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Full Length Research Article

# Stability Profile of *Pistia stratiotes* Leaf Peroxidase

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## ABSTRACT

Peroxidases are widely distributed in nature and are used in multiple industrial applications. Sources of peroxidases with notable stability are therefore being explored for commercial purposes. *Pistia stratiotes* leaf peroxidase was purified and characterized. It exhibited maximum activity at pH 6.0 after 10 minutes of incubation, which shifted to pH 6.5 after 60 minutes of incubation. Peroxidase from *P. stratiotes* leaf showed moderate stability after heating at 50ºC for 60 minutes and at 60ºC for 30 minutes. This enzyme was strongly inhibited by EDTA while urea had only a slight denaturing effect with approximately 65% of the activity retained after 60 minutes exposure. There was reduction in *P. stratiotes* leaf peroxidase activity in the presence of $Pb^{2+}$ (67%), $Hg^{2+}$ (70%), $Ni^{2+}$ (75%), $Cu^{2+}$(12%), $Co^{2+}$ (60%), $Ca^{2+}$ (45%) and $Mn^{2+}$ (65%) at 6mM concentration. Acetone activated the enzyme in a concentration-dependent manner while the presence of methanol and ethanol in the reaction mixture led to 16% decrease in the activity of the enzyme at 20% concentration. Results obtained from this study revealed that *P.stratiotes* leaf peroxidase is moderately stable and thus has potential for some industrial applications.

**Keywords:** *Pistia stratiotes*, Urea, Enzyme Activity, Peroxidase, Stability

## INTRODUCTION

Plant peroxidases (EC.1.11.1.7) are very common group of enzymes with important physiological roles in plants (Passardi *et al.,* 2005; Hamid and Khalil-ur-Rehman, 2009). They are heme-containing enzymes which catalyse the single one electron oxidation of several substrates at the expense of $H_2O_2$ (Galati and O'Brien, 2004; Veitch, 2004). Due to their broad substrate specificity, peroxidases have importance in industrial applications such as decolorization of dyes, removal of phenolic contaminants, and production of analytic and diagnostic kits, ELISA, biosensors, and pharmaceuticals (Clarke *et al*., 1993; Ruzgas *et al.,* 1996; Agostini *et al.,* 2002; Carlos *et al.,* 2004; Aehle, 2007; Hamid and Khalil-ur-Rehman, 2009; Ng *et al.,* 2010; Arise *et al*., 2016).

Recently, Horseradish peroxidase (HRP) is being exploited for cancer treatment (Veitch *et al.,* 2004, Arise *et al*., 2016; Bonifert *et al*., 2016). HRP is the most extensively studied plant peroxidase due to its wide applicability (Regalado *et al.,* 2004). However, the cost of commercially available HRP has restricted its applications. This has led to efforts to identify viable forms of peroxidase from other sources (Ito *et al.,* 1991; Kvaratskhelia *et al.,* 1997; Yemenicioglu *et al.,* 1998; Castello *et al.,* 2002; Deepa and Arumughan, 2002). Efforts are still being made to identify peroxidases which have lower cost of purification and comparable or higher stability (Bansal *et al.,* 2012) as these will increase affordability of the purified enzymes for industrial and clinical applications. Studies have shown some peroxidases from various sources with potentials for different applications in bio-catalysis and bio-electrocatalysis (Adam *et al*., 1999; Torres *et al*., 2003; Carlos *et al*., 2004). Several of these peroxidases have been recognised as possessing some significant qualities such as thermal stability, pH stability, and resistance to chemical denaturation which makes them suitable for industrial applications (Deepa and Arumughan, 2002).

*Pistia stratiotes L*. (family Araceae), also known as water lettuce, water cabbage, Nile cabbage or shellflower, is a free-floating freshwater macrophyte and a perennial herb widely distributed in tropical and sub-tropical regions (Sajna *et al*., 2007; Tulika and Mala, 2015). It is an aquatic macrophyte with evasive characteristics and generally regarded as one of the world's worst weed and has several documented negative economic and ecological impacts (Suasa-Ard, 1976; Napompeth, 1990; Holms *et al.,* 1997). The objective of this study is to isolate, purify and characterize peroxidase from *Pistia stratiotes* leaf with the aim of understanding the conditions and factors which could identify its prospect for industrial applications.

## MATERIALS AND METHODS

### Materials

Ammonium sulphate and Sephadex G-150 were purchased from BDH Chemicals, England while guaiacol was obtained from Santa Cruz Biotech, USA. All other chemicals of analytical grade were ordered from Sigma-Aldrich (USA), Santa Crux Biotech (USA), and BDH Chemicals (England).

### Plant Material

The leaves of *Pistia stratiotes* were collected from Asa Dam area in Ilorin, Kwara State, Nigeria. The leaves were deposited at the herbarium of Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, Kwara state, Nigeria, for proper identification and authentication.

### Preparation of Crude Extract

Seventy five (75) grams of fresh healthy leaves were washed thoroughly with distilled water at room temperature. The leaves were homogenized with cold 100 mM Tris HCl buffer, pH 7.5 in a blender for 5 min. The homogenate was filtered using cheese cloth arranged into 4 folds. The clear filtrate was then centrifuged at speed of 10, 000 g for 10 min at 4°C and the supernatant obtained was used as crude extract enzyme source.

### Ammonium Sulphate Precipitation

This was carried out in ice bath, using carefully ground ammonium sulphate crystals. The appropriate amount of the powder (37.32g, 20.71 and 62.14g for 0-60%, 60-90% and 0-90% saturations, respectively) was weighed and gently added to the crude extract by constant stirring to ensure uniform and complete solubilisation of the salt. The solution was then allowed to stand overnight at 4ºC for complete precipitation. This was carried out in steps to achieve between 0-60%, 60-90% and 0-90% ammonium sulphate saturations of the crude enzyme extract.

### Dialysis and Gel filtration

Samples obtained after precipitation were subjected to dialysis. The dialysis process spanned a period of 24 hours with 100 mM Tris HCl buffer, pH 7.5. Stirring was done intermittently using magnetic stirrer with 3 buffer changes. The samples obtained after dialysis was subjected to gel filtration using Sephadex G-150.The dialysed fraction was loaded onto the column (1cm × 40 cm) previously equilibrated with 100 mM Tris HCl buffer, pH 7.5 and then washed with 50 ml of the eluting buffer (same as the equilibrating buffer). The eluates were collected as fractions of 5 ml each and then assayed for enzyme activity.

### Total Protein and Enzyme Activity Assay

Lowry method as described by Lowry *et al.* (1951) was used to determine the total protein in the different fractions using bovine serum albumin (BSA) as standard. Peroxidase activity was measured spectrophotometrically, using guaiacol as substrate according to the method of Tonami *et al*. (2004) with slight modifications. Briefly, 2.5 ml of 0.1 M phosphate buffer, pH 6.0 was added to a test tube followed by the addition of 0.1 ml of enzyme source after which 0.2 ml of 100 mM guaiacol was added to the reaction mixture. The reaction was initiated with 0.2 ml of freshly prepared 150 mM $H_2O_2$ solution and the change in absorbance of the mixture was read at 470 nm at 25℃ over a period of 3 minutes at 1 minute interval. One unit of activity (U) was

defined as the amount of enzyme that caused the oxidation of 1 µmole of substrate per min under standard conditions.

**Determination of Optimum pH and pH Stability**
Buffers at different pH were prepared with pH range from 2.5 to 10.0. The optimum pH was determined by incubating 0.1 ml of the enzyme in different pH solutions for 10 minutes. After this, the enzyme activity was determined. pH stability was determined by incubating the enzyme in different buffers pH range for a period of 60 minutes, as described above, after which the enzyme activity was determined.

**Optimum Temperature and Thermal Stability**
The optimum temperature was determined by incubating the enzyme sample at a particular temperature for 10 minutes and the enzyme activity immediately assayed at that temperature. The temperature ranges studied were between 20-80˚C. The thermal stability of the enzyme was determined at the range of 40-80˚C. Briefly, the enzyme sample was heated to a specific temperature over a period of 60 minutes using water-bath. Aliquots of the enzyme were withdrawn at 10 minutes intervals. The withdrawn samples were then cooled in ice-bath for 30 minutes and incubated for 10 minutes at 25 $^0$C. Percentage residual enzyme activity was then determined, as described above.

**Effect of EhylenediamineTetraacetate (EDTA) and Urea**
*P. sratiotes* leaf peroxidase activity was studied in the presence of 20, 15 and 10 mM EDTA concentrations. The enzyme and EDTA were incubated at optimum temperature of 40˚C for a period of 10 min after which the enzyme activity was assayed. Urea solution of 10 mM was incubated with the enzyme for the period of 3, 10, 30 and 60 minutes. The extent of denaturing was determined by assaying for the residual activity of the enzyme.

**Effect of Metal Ions and Some Common Organic Solvents**
The effect of manganese ion ($Mn^{2+}$),lead ion ($Pb^{2+}$),mercury ion ($Hg^{2+}$),Nickel ion ($Ni^{2+}$),copper ion ($Cu^{2+}$), calcium ion ($Ca^{2+}$) and cobalt ion ($Co^{2+}$) were studied using different concentrations of 20, 15, 10 and 5 mM. The mixture containing the enzyme and the metal ion was incubated at 40˚C for 60 minutes after which the enzyme activity was assayed and expressed as residual enzyme activity. The effect of acetone, methanol and ethanol were as well studied. This was done at 2, 5, 15, 20, and 25%solvents concentrations. The reaction mixture was incubated with the various solvent concentrations at 40˚C for 10 minutes and the residual enzyme activity was determined.

## RESULTS

**Isolation and Purification of *Pistia stratiotes* Leaf Peroxidase**
The summary of the purification process from crude extract to gel filtration is shown in Table 1. The percentage yield and purification fold obtained after the purification procedure were 8.9% and 10.82 respectively. The elution profile of the gel filtration purification step revealed that fractions F6, F8 and F10 exhibited the highest activity (Figure 1) and were thus pooled together for further characterization.

**Optimum pH and pH Stability**
Different pH values influenced the activity of *P. stratiotes* leaf peroxidase as presented in (Figure 2). which shows 6.0 as the optimum pH for peroxidase with steep decrease in enzyme activity on both sides of the optimum pH.

Figure 3 shows the pattern of *P. stratiotes* leaf peroxidase activity and stability after 60 min incubation at different pH range in comparison to 10 min short period of incubation. The optimum pH was observed at pH 6.0 after 10 minutes of incubation, which shifted to pH 6.5 after 60 minutes of incubation.

**Table 1:** Summary of *Pistia stratiotes* leaf peroxidase purification

| Purification step | Total Enzyme Activity (µM $min^{-1}$) | Total Protein (mg) | Specific Activity (µM/min/mg) | Purification Fold | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 672.180 | 11.270 | 59.64 | 1.00 | 100.0 |
| $(NH_4)_2SO_4$ precipitation | 191.730 | 2.250 | 85.21 | 1.43 | 28.5 |
| Gel filtration | 60.098 | 0.092 | 645.33 | 10.82 | 8.9 |

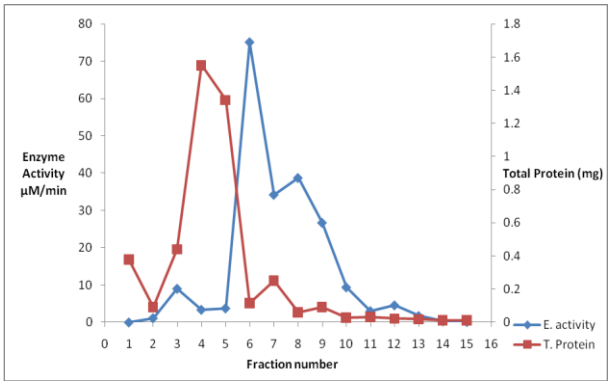


**Figure 1**: Purification profile of *Pistiastratiotes*leaf peroxidase by sephadex G-150 gel filtration

**Optimum pH and pH Stability**

Different pH values influenced the activity of *P. stratiotes* leaf peroxidase as presented in (Figure 2). which shows 6.0 as the optimum pH for peroxidase with steep decrease in enzyme activity on both sides of the optimum pH.

Figure 3 shows the pattern of *P. stratiotes* leaf peroxidase activity and stability after 60 min incubation at different pH range in comparison to 10 min short period of incubation. The optimum pH was observed at pH 6.0 after 10 minutes of incubation, which shifted to pH 6.5 after 60 minutes of incubation.

**Optimum Temperature and Thermal Stability**

Figure 4 shows the effect of temperature on the activity of *P. stratiotes* leaf peroxidase. The highest activity was demonstrated at temperature of 40ºC and a steep decrease was observed in enzyme activity as temperature increases.

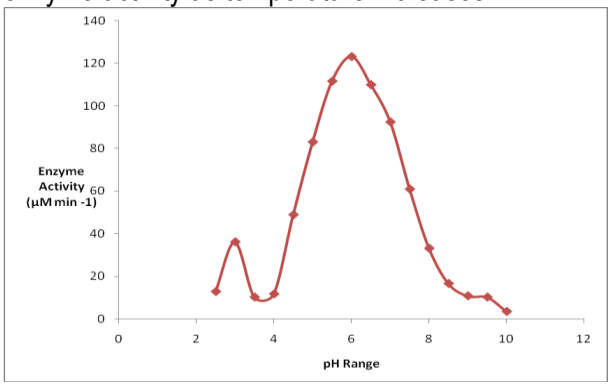


**Figure 2**: Effect of pH on *Pistia stratiotes* leaf peroxidase activity using guaiacol as substrate

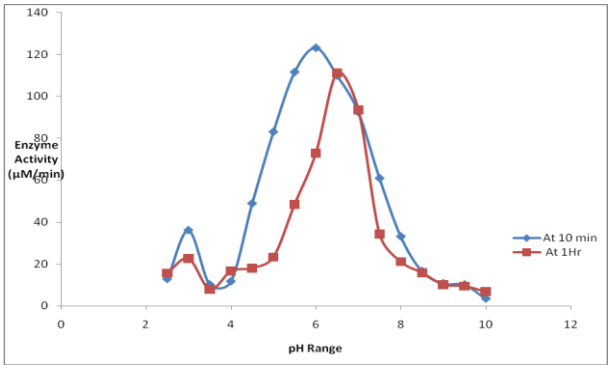


**Figure 3**: Time-dependent effect of pH on *Pistia stratiotes* leaf peroxidase activity

Figure 5 represents the time-dependent thermal stability profile of *P. stratiotes* leaf peroxidase. *P. stratiotes* leaf peroxidase residual activities at 30 and 60 min thermal exposure were compared. Reduction in enzyme activity as result of

increment in temperature was pronounced between temperatures of 40 and 50˚C after which a relative stability was maintained in spite of difference in exposure time.

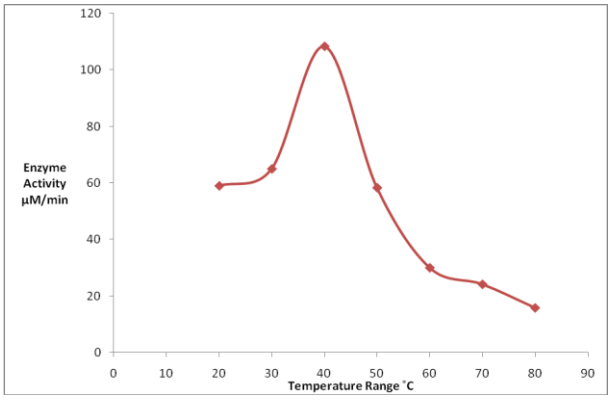


**Figure 4**: Effect of temperature on the activity of *Pistia stratiotes* leaf peroxidase

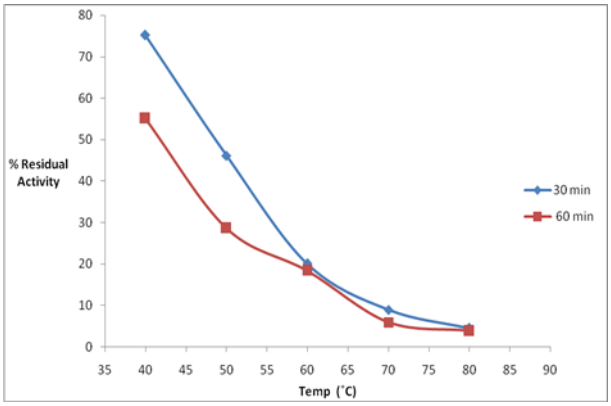


**Figure 5**: Time-dependent thermal stability of purified peroxidase from *Pistia stratiotes* leaf

### Effect of EDTA and Urea on *Pistiastratiotes* Leaf Peroxidase

Figure 6 presents the effect of different concentrations of EDTA on *P. stratiotes* leaf peroxidase. The result obtained shows that EDTA is a concentration-dependent inhibitor of peroxidase from *P. stratiotes* leaf. Residual activities obtained were 16.67%, 17.43% and 29.74% at 20, 15, and 10 mM EDTA concentrations respectively. The residual activity of the enzyme decreased continually with increasing EDTA concentration. However, the residual activity was slightly stable as the concentration of EDTA increased from 15-20 mM. Figure 7 shows the time-dependent effect of urea (at a fixed concentration of 10 mM) on the activity of *P. stratiotes* leaf peroxidase. The result was expressed as percentage residual activity and it was observed that the presence of urea resulted in the loss of about 25% of the enzyme activity, after 30 minutes of incubation. As the time of incubation with urea increases, the loss of enzyme activity became more pronounced. However, *P. stratiotes* leaf peroxidase retained 65% of its activity after 60 minutes of incubation with 10mM urea.

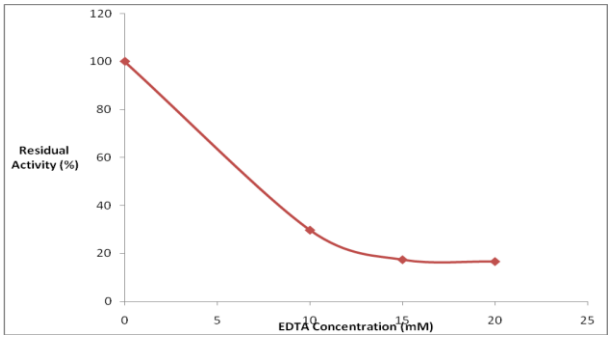


**Figure 6**: Concentration dependent effect of EDTA on *Pistia stratiotes leaf* peroxidase

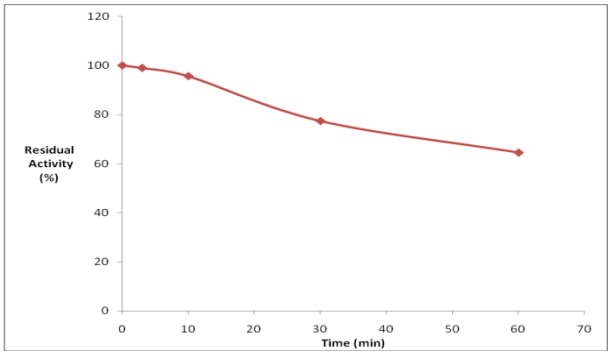


**Figure 7**: Time-dependent effect of urea on *Pistia stratiotes* leaf peroxidase activity

### Effect of Metal Ions on *Pistia stratiotes* Leaf Peroxidase Activity

The effects of $Mn^{2+}$, $Pb^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Ca^{2+}$ and $Co^{2+}$ were examined at different concentrations of 20, 15, 10 and 5 mM (Figure 8).

All the metal ions demonstrated inhibitory effects against *P. stratiotes* leaf peroxidase. The inhibitory activity of each of these metal ions except $Cu^{2+}$ and $Ca^{2+}$ was more pronounced at lower concentrations of ≤ 5 mM after which there was no appreciable reduction in the residual enzyme activity. However, as the concentration of $Cu^{2+}$ ion was increased, the residual enzyme activity continued to reduce.$Ni^{2+}$ showed the strongest inhibitory activity against *P. stratiotes* leaf peroxidase as it resulted in < 25% residual enzyme activity at concentration of 6 mM.

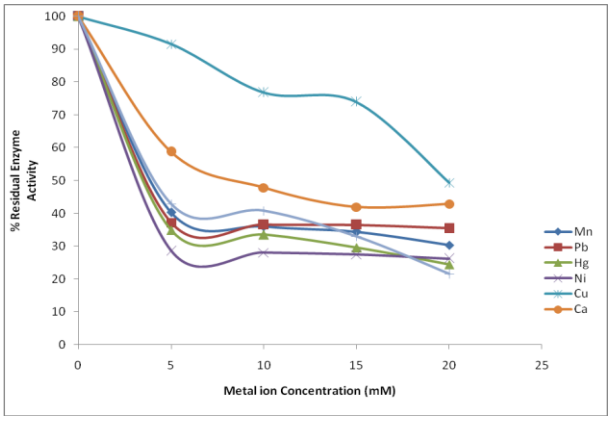


**Figure 8**: The effect of metal ions at various concentrations on *Pistia stratiotes* leaf peroxidase activity

### Effects of Acetone, Ethanol and Methanol on *Pistia stratiotes* Leaf Peroxidase

The effects of some common organic solvents are presented in Figure 9. Unlike acetone which exhibited concentration-dependent activating effect on *P. stratiotes* leaf peroxidase, ethanol and methanol demonstrated inhibitory effect against the enzyme. Ethanol however showed higher inhibitory activity than methanol as the former caused > 20% reduction in the activity of *P. stratiotes* leaf peroxidase at 25% solvent concentration.

## DISCUSSION

Peroxidases from plant sources are being studied extensively due to their broad substrate specificity, distributions and potential industrial applications (Carlos *et al.,* 2004; Aehle, 2007; Hamid and Khalil-ur-Rehman, 2009; Arise *et al*., 2016). In this study, purification of *Pistia stratiotes* leaf peroxidase was achieved through a three-step procedure that included crude extraction, ammonium sulphate precipitation and gel filtration. This procedure gave a purification fold and percentage yield of 10.82 and 8.9%, respectively. The specific activity of *Pistia stratiotes* leaf peroxidase in this study, increased considerably after the last step of the purification process (from 59.64 to 645.33µMmin$^{-1}$mg$^{-1}$).

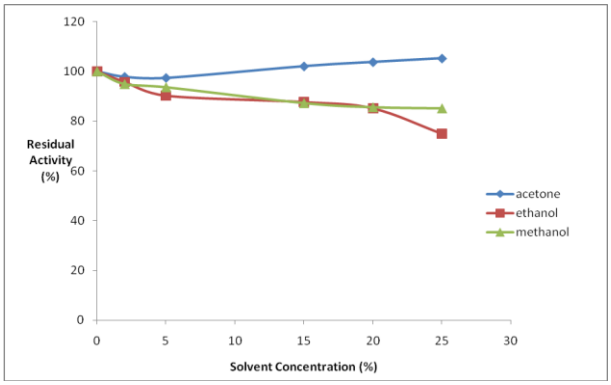


**Figure 9**: Effect of acetone, ethanol and methanol on *Pistia stratiotes* leaf peroxidase

The optimum pH for *P. stratiotes* leaf peroxidase was 6.0 with the optimum range between 5.0 and 7.0. This is in agreement with previous studies where peroxidases from different sources have been reported to have pH optima often within the range 4.5-6.5 (Tomas-Barberan *et al*., 1997; Bestwick *et al.,* 1998; Thongsook and Barrett, 2005; Onsa *et al.,* 2006; Hu *et al.,* 2012; Khatun *et al.,* 2012; Arise *et al*., 2016). The shift in optimum pH from 6.0 to 6.5, however, indicate that *Pistia sratiotes* leaf peroxidase largely retains its enzymatic activity over a narrow range of pH after 60 minutes of incubation.

The optimum temperature of 40ºC obtained was similar to that reported by other authors from different sources (Altunkaya and Gokmen, 2011; Hu *et al.,* 2012; Arise *et al*., 2016). Bhatti *et al.* (2006) reported that lettuce stem peroxidase has

an optimum temperature of 45˚C, and peroxidase from cauliflower had optimum temperature of 30ºC (Koksal and Gulcin (2008). When subjected to thermal treatments peroxidase retained at least 28% of its activity after thermal treatment for 60 min at 50˚C and 18.38% when heated for 60 min at 60˚C.*P. stratiotes* leaf peroxidase maintained over 60% of its activity over a temperature range of 30ºC to 50ºC. Although, other studies have reported higher stability, comparison is however quite difficult to make since most studies were carried out with enzyme sample with inconsistent level of purity (Marzouki *et al.,* 2005; Aiman and Quayyum, 2008; Wang *et al*., 2009; Hu *et al*., 2012). The difference observed in activities of *P. stratiotes* leaf peroxidase incubated for 30 and 60 minutes at temperatures below 60˚C (Figure 5) is related to the rate of denaturation, since denaturation is both time and temperature dependent. At temperatures beyond 60ºC, similar stabilities were observed for *P. stratiotes* leaf peroxidase incubated for 30 and 60 minutes. This observation might be due to relative increase in the conversion of active forms of the enzyme to inactive/denatured forms, with corresponding reduction in activity (Daniel *et al*., 2001).

EDTA usually results in inactivation of many metalloenzymes (Stoecker *et al*., 1988; Peeters, 2014). The deactivation of *P. stratiotes* leaf peroxidase was pronounced in this study and may be due to the ability of EDTA to sequester $Fe^{3+}$ and $Ca^{2+}$ which are required for structural and functional stability of the enzyme and thus its catalytic ability.

Urea acts as a protein denaturant by disrupting the native protein conformation. In this study, the concentration of urea used revealed that the enzyme was quite stable to chemical denaturation. However, the concentration of urea used was low, and thus it cannot be conclusively deduced if higher concentration could have resulted in stronger disruption of the protein conformation. $Mn^{2+}$, $Pb^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Ca^{2+}$ and $Co^{2+}$ all resulted in inhibition of the enzyme activity just as reported in other studies (Marzouki *et al.,* 2005; Bhatti *et al.,* 2006; Einollahi *et al.,* 2006; Hu *et al.,* 2012; Khatun *et al.,* 2012; Arise *et al*., 2016). The inhibitory effect of these metal ions could have resulted from their interaction with the side chains of some amino acid residues of the enzyme molecule such as seen in the interaction of $Hg^{2+}$ with the cysteine sulphurhydryl group (Khatun *et al.,* 2012).

Organic solvents can inactivate enzymes through several mechanisms: the organic solvent molecules can interact with the enzyme, disrupting the native secondary structure; they can strip hydration shell of essential water molecules altering the enzyme structure; or they can alter with the enzyme active site, causing inactivation (Sahare *et al*., 2014). The results obtained for the effects of acetone, ethanol and methanol on *P. stratiotes* leaf peroxidase activity indicated that the presence of acetone induced the activation of the enzyme in a concentration-dependent manner. The observed activation of *P. stratiotes* leaf peroxidise by acetone, may be due to change in conformation of the enzyme to a more active form, as a result of acetone binding. This is in contrast to what was reported by Hu *et al*. (2012) and Marzouki *et al*. (2005) where the introduction of acetone was reported to result in the loss of peroxidase activity. Methanol and ethanol however on the other hand resulted in the loss of *P. stratiotes* leaf peroxidase activity as similarly reported in other studies (Marzouki *et al.,* 2005; Hu *et al.,* 2012).

## CONCLUSION

The partially purified *P. stratiotes* leaf peroxidase showed stability under some of the conditions observed in this study. Thus, *P. Stratiotes* leaf peroxidase has prospects for industrial applications.

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