

## Characterization of Alkaline Phosphatase (E.C 3.1.3.1) From The Seeds of *Dacryodes Edulis* (African Pear) A Likely Industrial Source For Enzyme Production.

<sup>1</sup>Fajana Olusegun .O., <sup>2</sup>Adu Oluwatosin .B., <sup>3</sup>Ogunrinola Oluwafunmilayo. O.,  
<sup>4</sup>Olaitain Samuel .N., <sup>5</sup>Raimi Olawale .G., <sup>6</sup>Ebenezer Olubukola .A., and <sup>7</sup>  
Elemo Babajide. O'

Cell and Tissue Culture Research Laboratory (Drug Discovery Unit) Department of Biochemistry, Lagos State University, Ojo, PMB 0001, LASU Post office, Lagos Badagry Expressway.

### -----Abstract-----

Alkaline phosphatase (EC 3.1.3.1) is an enzyme that catalyzes hydrolysis of phosphate ester bonds in extracellular organic molecules, enabling the availability of inorganic phosphate (Pi) for cellular metabolism. This study was therefore aimed at determining the activities of alkaline phosphatases (ALP) in producing and supplying inorganic phosphate for the various biochemical processes that require it during ripening in seeds of *D. edulis* (African Pear). The purification and characterization of these phosphohydrolytic enzymes in the seed was also carried out. The study revealed that the protein concentration ranged from  $11.18 \pm 0.053$  mg/ml to  $2.235 \pm 0.015$  mg/ml and ALP activity was highest on the seventh day when the fruit is fully ripe. Enzyme activity ranged from  $1577.80 \pm 108.10$  U/I to  $5480.440 \pm 10.61$  U/I. The activities of alkaline phosphatase was activated by  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ca^{2+}$  and inhibited by EDTA and fluoro oxalate in a concentration dependent manner. *Dacryodes edulis* seeds alkaline phosphatase had a  $K_m$  and  $V_{max}$  of 1.80 mM, and  $1.52 \mu\text{mol}/\text{min}/\text{ml}$  respectively, with an optimum pH of 10 and optimum temperature of  $40^\circ\text{C}$ . This seeds could serve as a source of alkaline Phosphate (ALP) enzyme production.

**Key Words:** Alkaline phosphatase, characterization, *Dacryodes edulis*, enzyme, extracellular

Date of Submission: 8 Dec 2012



Date of Publication: 25, April.2013

### I. INTRODUCTION

Alkaline phosphatases are enzymes that catalyze the hydrolysis of phosphate ester bonds in extracellular organic molecules, permitting the cellular uptake of inorganic phosphate (Pi) for metabolism. ALPs are the best characterized extracellular phosphatases. Their properties include low substrate specificity and an alkaline pH optimum. The active site of most ALPs contains a single Mg and two Zn atoms (Coleman 1992). Many phytoplanktons, such as diatoms, dinoflagellates, green algae, cyanobacteria, and coccolithophores express ALP under P-limitation (Riegman *et al.* 2000, Kruskopf and Du Plessis 2004). Alkaline phosphatases in plants play a major role in the supply and metabolism of inorganic phosphate for the maintenance of cellular metabolism (Tabaldi *et al.*, 2007; Mishra and Dubey, 2008).

Alkaline phosphatases are a group of enzymes found primarily in the liver (isoenzyme ALP-1) and bone (isoenzyme ALP-2) (Coleman 1992). There are also small amount produced by cells lining the intestines (isoenzyme ALP-3), the placenta and the kidney (in the proximal convoluted tubules) (Coleman 1992). What is measured in the blood is the total amount of alkaline phosphatases released from these tissues into the blood. Alkaline phosphatases act by splitting off phosphorus (an acidic mineral) creating an alkaline pH. The primary importance of measuring alkaline phosphatase is to check the possibility of bone disease or liver diseases (Coleman 1992).

EDTA and Fluoro acetate are very good inhibitors of this enzyme, Mishra and Dubey (2008) also reported the inhibitory effect of Arsenite ( $\text{As}_2\text{O}_3$ ) on the activities of acid and alkaline phosphatases in rice (*Oryza sativa* L.) seedlings, which resulted in a decline in the level of the phosphate pool. LePS2 gene encodes acid phosphatase in tomato (*Lycopersicon esculentum*) and it was induced in tomato plant in the absence of inorganic phosphate (Baldwin *et al.*, 2001). The addition of a phosphate group may activate or de-activate an enzyme (e.g., kinase signalling pathways) (Seeger and Krebs, 1995), or enable a protein-protein interaction to occur (e.g., SH3 domains) (Ladbury, 2007); therefore phosphatases are integral to many signal transduction

pathways. A common phosphatase in many organisms is alkaline phosphatase, although, there is also an acid phosphatase.

African pear (*Dacryodes edulis*) also known as African plum or safou is an indigenous fruit tree in the humid lowlands and plateau regions of West, Central African and Gulf of Guinea countries. In south-east Nigeria, the trees are grown around homesteads and flowering takes place from January to April. The major fruiting season is between May and October (Emebiri and Nwufo, 1990; Kengue and Nyagatchou, 1990). The Fruits are ellipsoidal and their size varies approximately from 4 - 9 cm long and from 2 - 5 cm wide. They could be an important source of pulp oil, seed oil and even whole fruit oil (Awono *et al.*, 2002). It has been advocated that The Safou oil should take its place in the food industry, the pharmaceutical and the cosmetics industry (soap, perfume, creams) as well as in other branches of industry where fat raw materials are needed. The cake remaining after the production of pulp oil may be useful in human food industry (bakery, baby foods). Information on the consumption and composition of Safou not totally complete. As the fruit becomes more popular and is increasingly commercialized, such information is indispensable for proper valorization of the fruit. The seed of the African pear are usually not fully utilized and they seem to be the waste product in this plant since the skin and pulp are usually consumed. Hence, this study was carried out to investigate the utilization of the seed for the purpose of industrial production of enzyme.

## II. MATERIALS AND METHOD

25 pieces of unripe samples of African pear fruit were purchased from Iyana- Iba market, in Ojo area of Lagos state, Nigeria, RANDOX alkaline phosphatase kits produced by RANDOX Laboratories Ltd., Biuret reagent, Sodium hydroxide, Magnesium chloride ( $MgCl_2$ ), distilled water, Tris-HCl buffer, phosphate buffer. All other reagents were of high quality and purity.

### 2.1 ENZYME EXTRACTION

5.0g of seed sample were ground in a chilled mortar with acid - washed sand and 20 ml of chilled 0.05 M sodium carbonate buffer (PH 10). The homogenate was filtered through double layers of cheesecloth and centrifuged at 20, 000g for 20 min. The supernatant was used as the source of the crude enzyme (Agoreyo, 2010).

### 2.2 PROTEIN DETERMINATION

Protein concentration of crude enzyme was determined using Biuret method (Layne, 1957). 1.0 ml of the homogenate was mixed with 3.0 ml of Biuret reagent and incubated for 10 min at 37°C. Absorbance was taken at 540 nm.

### 2.3 DETERMINATION OF ALP ACTIVITY

Alkaline phosphatase activity was determined by a modified method of Bowers and McCom (Bowers and McComb, 1996) the method involves the incubation of 0.1 ml of seed homogenate with 80mM Tris-HCl buffer (pH 10.4), 0.5 ml p-nitrophenyl phosphate (PNPP, 10 mmol/L) at 37°C for 30 min. The reaction was terminated by adding 5.0 ml of 2 mM NaOH after which absorbance was taken at 405 nm against reagent blank. Then the activity of each tissue homogenate was calculated by the formulae:  $2760 \times \Delta A_{405 \text{ nm}}$

### 2.4 EFFECT OF METAL IONS

Mg, Mn and Ca were used for this assay. Five test tubes were used which contain the metal ion. A range of 0.1 to 1.5 mol/l of different metal concentration was added to 0.1 ml of seed homogenate in the test tubes and 0.1 ml 80 mM Tris-HCl buffer, (pH 10.4) and 2.0 ml of p-nitrophenylphosphate was added to it. The reaction mixtures were incubated for 30 mins at 37°C after which 5 ml of 0.02 M NaOH was added and the corresponding absorbance was read at 405 nm against blank.

### 2.5 EFFECT OF EDTA AND FLUORO OXALATE

The effect of EDTA and Fluoro-oxalate was investigated. EDTA was used for this assay. A range of 5.0 to 30.0 mmol/L of EDTA concentration was added to 0.1ml of seed homogenate in the test tubes and 0.1 ml 80mM Tris-HCl buffer, (pH 10.4) and 2.0 ml of p-nitrophenylphosphate was added to it. The reaction mixtures were incubated for 30 mins at 37°C after which 5 ml of 0.02 M NaOH was added and the corresponding absorbance was read at 405 nm. Same approach was done for fluoro oxalate, a range of 5.0 to 30.0 mmol/L of Fluoro oxalate concentration was added to 0.1ml of seed homogenate in the test tubes and 0.1 ml 80mM Tris-HCl buffer, (pH 10.4) and 2.0 ml of p-nitrophenylphosphate was added to it.

The reaction mixtures were incubated for 30 mins at 37°C after which 5 ml of 0.02 M NaOH was added and the corresponding absorbance was read at 405 nm EFFECT OF pH The reaction tubes contain 2.0 ml of p-

nitrophenylphosphate and 0.1 ml of seed homogenate in phosphate buffer of pH range of between 7.0 and 11.0. The reaction mixtures were incubated for 30 min at 37°C after which 5 ml of 0.02 M NaOH was added and the corresponding absorbance was read at 405 nm.

### 2.6 EFFECT OF TEMPERATURE

The reaction tubes contain 2.0 ml of p-nitrophenylphosphate and 0.1 ml of seed homogenate and 0.1 ml 80 mM Tris-HCl buffer, (pH 10.4). The reaction tubes were incubated at a temperature range of between 20°C and 50°C for 30min after which 5 ml of 0.02 M NaOH was added and the corresponding absorbance was read at 405 nm. DETERMINATION OF  $K_M$  and  $V_{Max}$  A range of 0.5 - 3.0 ml of p-nitrophenylphosphate, PNPP was used. 0.5 ml, 1.0 ml, 1.5 2.0ml, 2.5ml and 3.0 ml of 80 mM Tris-HCl buffer, (pH 10.4) was added and then 2.5 ml, 2.0 ml, 1.5 ml, 1.0 ml, 0.5ml and 0.0 ml of p-nitrophenylphosphate (10 mmol/L), was added to start the reaction. The reaction mixtures were incubated for 10 min at 37°C after which 5.0 ml of 0.02 M NaOH was added and the corresponding absorbance was taken at 405 nm. The concentration of the p-nitrophenylphosphate was calculated and a graph of reciprocal of absorbance against concentration of the substrate was plotted to obtain the  $K_M$  and  $V_{max}$ .

### III. RESULTS

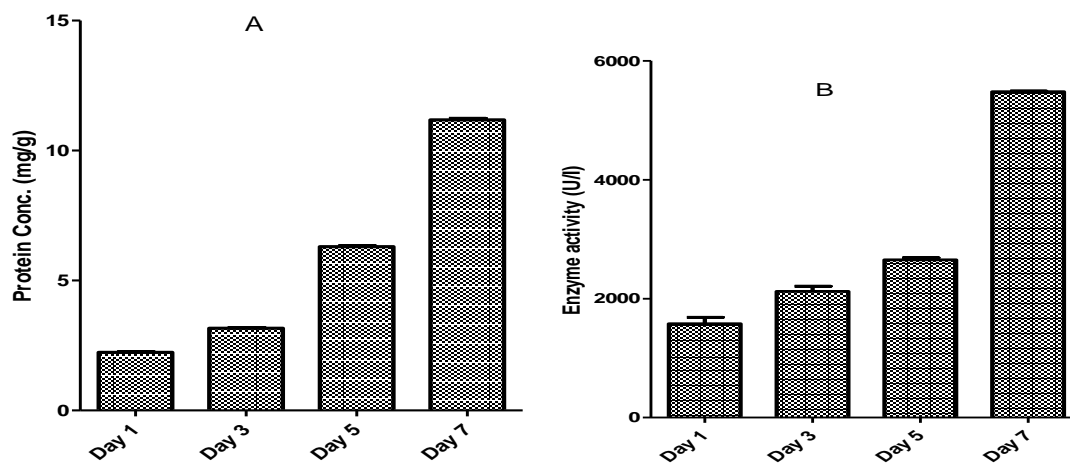


Figure 1 (A) Protein concentration in African pear seed during ripening (B) Alkaline phosphatases activity in African pear seed during ripening.

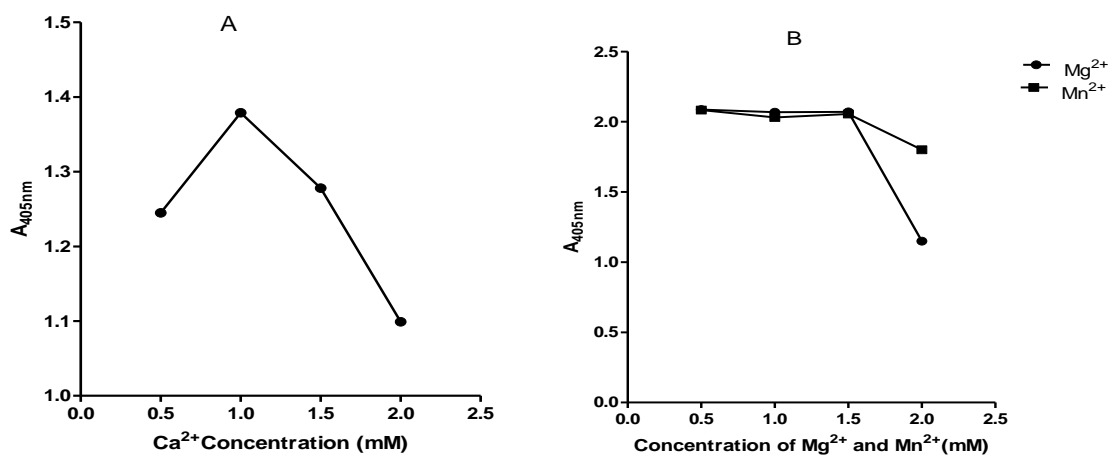


Figure 2: (A) Effect of Calcium, (B) Magnesium and Manganese ion on alkaline phosphatase activity in African pear seed

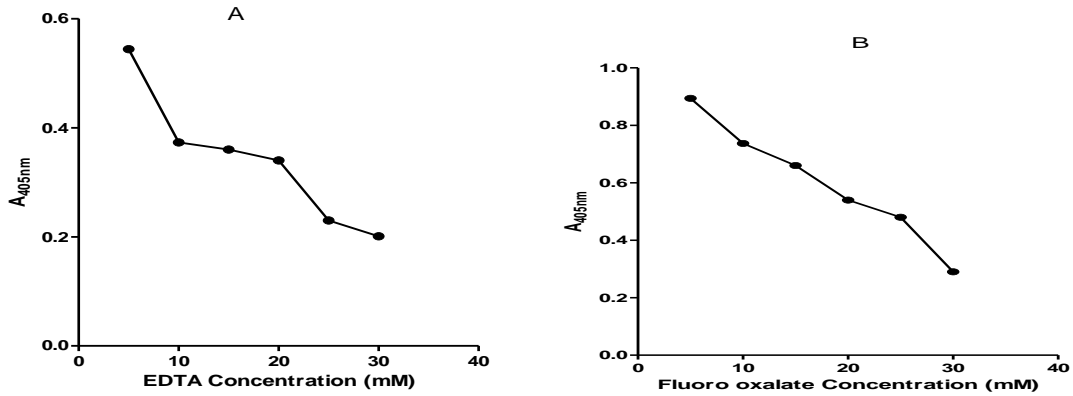


Figure 3: (A) Effect of EDTA and (B) Fluoro oxalate on alkaline phosphatases activity in African pear seed

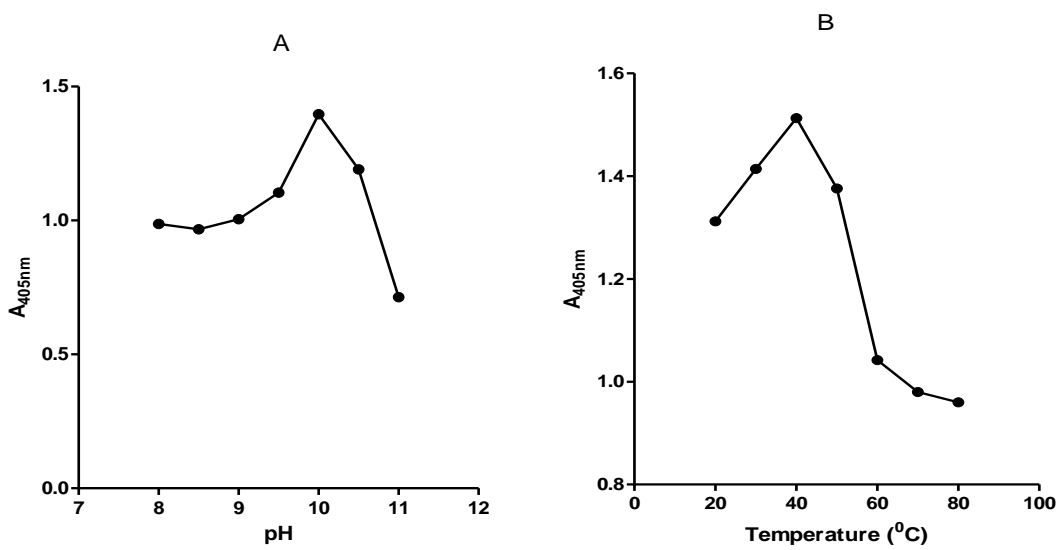


Figure 4: (A) Effect of pH and (B) Temperature on alkaline phosphatases activity in African pear seed

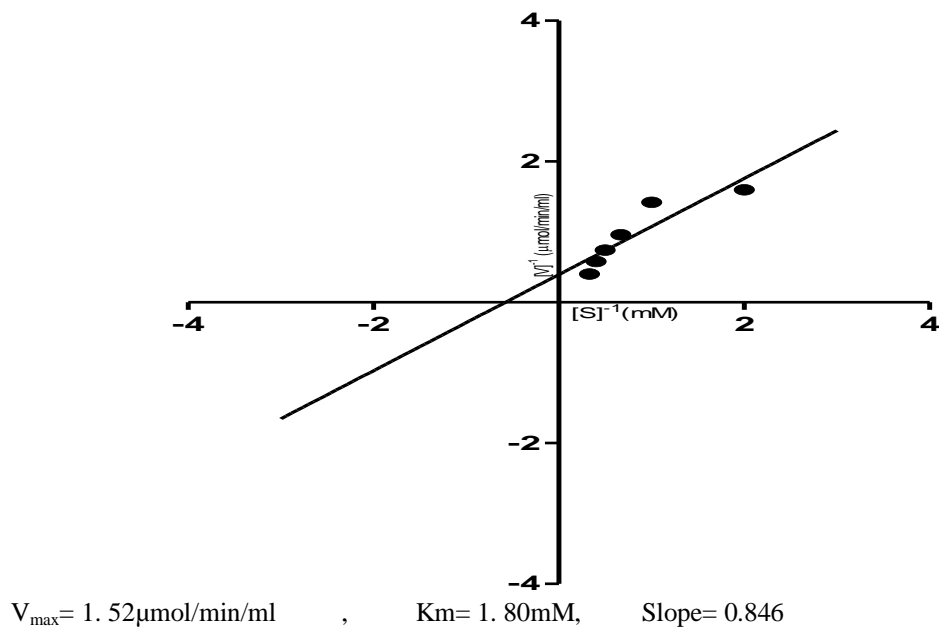


Figure 5: Double reciprocal plot of absorbance against substrate concentration of African Pear

#### IV. DISCUSSION

Alkaline phosphatase was isolated, purified and characterized from seeds of African pear (*Dacryodes edulis*). It was observed that there is a progressive increase in the total protein level as the fruit ripens. The highest concentration of protein was observed at day seven when the fruit was fully ripped and softens which gave the highest concentration which was  $11.181 \pm 0.053$ mg/g. According to Agoreyo, 2010, during fruit ripening, peel colour has been reported to be an important characteristic which often serves as one of the major criteria used to determine whether a fruit is ripe or unripe (Williams, 2002).

There is a significant increase in the enzyme activity as the ripening progresses, with the highest activity of  $1577.440 \pm 10.610$  U/I gotten on day 7 and the lowest activity of  $1577.800 \pm 108.103$  U/I on day 1. Alkaline phosphatase can hydrolyze phosphate bound to cell surface phosphoproteins and in this way, it may modulate their biological activities (Scheibe *et al.*, 2000). The increase observed in this study may be an indication that the organic phosphate in the seeds is hydrolyzed (Agoreyo, 2010). Alkaline phosphatase is a divalent metalloenzymes which requires  $Mg^{2+}$  for its activity, absence of this divalent metal has been seen to lower the alkaline phosphatase activity in serum and several cellular tissues from experimental animals (Loveless *et al.*, 1989). The effect of magnesium ion on alkaline phosphatase activity in African pear seed in figure 2b shows that there is a strong activation of the enzyme at a concentration of 0.5 to about 1.5mM but a sharp decrease in the enzyme action at 2.0mM, which show that a divalent metal like magnesium activates the enzyme. This result is similar to that of Raimi *et al.*, 2011 in which the activity of ALP is inhibited at a high concentration that is higher than 0.8mM of metal such as magnesium Metals such as  $Mn^{2+}$ ,  $Cd^{2+}$  has unstable inhibitory effect on ALP (Raimi *et al.*, 2011), this trend was observed in the activities of  $Mn^{2+}$  and  $Ca^{2+}$  used in this study as seen in figure 2a and b, there is a strong activation of the enzyme at a concentration of 0.5 to about 1.5mM but a sharp increase in the enzyme inhibition at 2.0mM.

There is a strong activation of the enzyme at a concentration of 0.5 to about 1.5mM and a peak activity at concentration at 1.0mM with a decrease in the enzyme and inhibitory activity at 2.0mM, this corroborate the work of previous researchers like Moss *et al.*, 1985; and Raimi *et al.*, 2011. There is a concentration dependent inhibition of the enzyme activity, as depicted in figure 3a and b, the higher the concentration of EDTA or fluoro oxalate the higher the inhibitory action of the enzyme, the concentration of 30mM has the highest inhibition on the enzyme activity. In figure 4a and b, The optimal pH of ALP from African pear seed was observed to be pH 10, which is an alkaline pH and its optimal temperature was 40°C, but the enzyme is optimally stable at temperature of 20-40°C after which the enzyme starts losing its activities. At a constant enzyme concentration, the kinetics of the enzyme was defined by Michaelis- Menten; the  $K_m$  and  $V_{max}$  of African pear seeds alkaline phosphatase was estimated to be 1. 80mM and 1. 52 $\mu$ mol/min/ml respectively (figure 5).

#### V. CONCLUSION

The results of this study suggest that alkaline phosphatase activity is highest on the seventh day when the fruit is fully ripped. The activities of alkaline phosphatase is activated by  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ca^{2+}$  and inhibited by EDTA and fluoro oxalate. The alkaline phosphatase has a  $K_m$  and  $V_{max}$  of 1. 80mM and 1. 52 $\mu$ mol/min/mL respectively. The ALP from seeds of African pear has an optimum pH of 10 and optimum temperature of 40°C as the above results depicts. Hence the potential of the seed as an industrial source of enzyme should be fully exploited.

#### REFERENCES

- [1] B.O. Agoreyo, (2010) Acid phosphatase and alkaline phosphatase activities in ripening fruit of *Musa Paradisiaca* L. POJ 3(3):66-69 ISSN:1836-3644
- [2] Awono A., Ndoye O., Schreckenber K., Tabuna H., Isseri F. and Temple L. (2002). Production and marketing of Safou (*Dacryodes edulis*) in Cameroon and internationally: market development issues. For. Trees Livelihoods 12: 125–148.
- [3] J.C.Baldwin, Karthikeyan AS, Raghobama KG (2001) LEPS2, a phosphorus starvation – induced novel acid phosphatase from tomato. Plant Physiol 125: 728 – 737
- [4] J. E. Coleman, (1992). Structure and mechanism of alkaline phosphatase. Annu. Rev. Biophys. Biomol. Struct. 21:441–83.
- [5] L.C Emebiri, M.I Nwufu (1990). Effects of fruits types and storage treatment on the biodeterioration of African pear (*Dacryodes edulis*) (G. Don) H.J. Lam). Int. Biodeterior. 26(1): 43-50
- [6] J.C Kengue, and J.Nyangatou (1990). Problem of preserving the germination power of the seeds African pear (*Dacryodes edulis*).Fruits 45(4): 409-412.
- [7] G.A Kormanik, and R.R Harris, (1981). Salt and water balance and antennal gland function in three Pacific species of terrestrial crab (*Gecarcinus lateralis*, *Cardisoma carnifex*, *Birgus latro*). I. Urine production and salt exchanges in hydrated crabs. Journal of Experimental Zoology 218: 97-105.
- [8] M. M. Kruskopf, and S. Du Plessis, (2004). Induction of both acid and alkaline phosphatase activity in two green algae (chlorophyceae) in low N and P concentrations. Hydrobiologia 513:59–70.
- [9] J.E Ladbury (2007). Measurement of the formation of complexes in tyrosine kinase-mediated signal transduction. *Acta Crystallogr. D Biol. Crystallogr.* 63 (Pt 1): 26–31.

- [10] R W. Loveless, T. Feizi, R. A. Childs, T. Mizuochi, M. S. Stoll, R. G. Oldroydt and P. J. Lachmann (1989) Bovine serum conglutinin is a lectin which binds non-reducing terminal N-acetylglucosamine, mannose and fucose residues. *Biochem. J.* 258, 109-113
- [11] R..B McComb, G.N. Bowers, S.Posen, (1979). Alkaline phosphatase. Plenum Press, New York.
- [12] S. Mishra, and R.S.Dubey, (2008) Changes in phosphate content and phosphatase activities in rice seedlings exposed to arsenite. *Braz J Plant Physiol* 20:19 -28
- [13] D. W. Moss, and K.B. Whitaker, (1985). *Enzymes*; 34: 212-216
- [14] O. G.Raimi, A.A. Fatai, H.A. Bankole, S. N. Olaitan, O. O. Fajana, M. I. Kazeem and K. A. Akobada (2011) Characterization of Alkaline Phosphatase (E.C.3.1.3.1) from Giant African Snail (*Archachatina Marginata*). *Journal of Cell and Tissue Research* Vol. 11(1) 2485 – 2489
- [15] R. Riegman, W.Stolte, A. A. M. Noordeloos,. and, D. Slezak (2000). Nutrient uptake and alkaline phosphatase (EC 3:1:3:1) activity of *Emiliana huxleyi* (Prymnesiophyceae) during growth under N and P limitation in continuous cultures. *J. Phycol.* 36:87–96.
- [16] F .Schiele, M. Vincent-Viry, B. Fournier, M .Starck, and G .Siest (1998). Biological effects of eleven combined oral contraceptives on serum triglycerides, gamma-glutamyltransferase, alkaline phosphatase, bilirubin and other biochemical variables. *Clin. Chem. Lab. Med.* 36 (11): 871–8.
- [17] R .Seeger, and E.G Krebs (1995). The MAPK signaling cascade. *FASEB J.* 9 (9): 726–35. PMID 7601337.
- [18] L.A. Tabaldi, R .Ruppenthal, D. Cargnelutti, V.M. Morsch, L.B. Pereira, M.R.C. Schetinger (2007) Effects of metal elements on acid phosphatase activity in cucumber (*Cucumis sativus* L.) seedlings.*Journal Environmental and Experimental Botany* 59 (1) 43-48 ISSN: 0098-8472
- [19] O.J. Williams, (2002) Enzyme studies and postharvest technology of tropical fruits. PhD Thesis, the University of the West Indies. Mona, Jamaica.