

Original Article

Characterization of Alkaline phosphatase (E.C.3.1.3.1) from freshwater Fish Species (*Coptodon zillii*, *Heterobranchus bidorsalis*, *Clarias gariepinus* and *Chrysichthys nigrodigitatus*)



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ABSTRACT

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Alkaline phosphatases (APs) are homodimeric enzymes that catalyze the hydrolysis and transphosphorylation of phosphate monoesters. These enzymes help to modulate and remove the phosphate group from a protein. In this study, **alkaline phosphatase** activity was determined in different tissue homogenates (flesh and bones) of species four fish species - *Coptodon zillii*, *Heterobranchus bidorsalis*, *Chrysichthys nigrodigitatus* and *Clarias gariepinus*. The bone was found to have the highest activity of alkaline phosphatase. The K_m and V_{max} values for the fishbone alkaline phosphatase (FBAP) were estimated to be 0.0106mM and 2.1730 $\mu\text{mol}/\text{min}/\text{mol}$ for *C. zillii*, 0.0074mM and 3.1868 $\mu\text{mol}/\text{min}/\text{mol}$ for *H. bidorsalis*, 0.0226mM and 8.9928 $\mu\text{mol}/\text{min}/\text{mol}$ for *C. gariepinus* and 0.0104mM and 6.0753 $\mu\text{mol}/\text{min}/\text{mol}$ for *C. nigrodigitatus* respectively. The pH optimum of the enzyme for the categories of fish was estimated to be 9.0. Divalent metals such as Mg^{2+} and Ca^{2+} enhanced enzyme activity but have an inhibitory effect at concentrations above 1.5 mM. The highest activity of the enzyme in the bone of the four species of fishes in this study suggests that most of the organic phosphates in the species are hydrolysed principally in this tissue.

INTRODUCTION

Aquatic fauna have a complex physiological and metabolic role in essential nutrients in active tissues (Cho and Bureau, 2001). Alkaline phosphate is a transmembrane glycoprotein of the plasma membrane (Hua and Bureau, 2006, Sugiura, 2018). Alkaline phosphatases [ALP; orthophosphoric monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1] are

plasma membrane-bound glycoproteins, situated on the cell membrane's outermost layer (Lowe *et al.*, 2023). The efficient absorption of dietary phosphorus is important for fish to support numerous processes (Sagiura *et al.*, 2006). High phosphate poses a serious threat to aquatic environment (Sagiura, 2000, 2018), such as algae bloom and eutrophication of water bodies (Sagiura and Hardy,



2000). Reducing the phosphate content in fish environments is important to production and Aquaculture (Luckstadt, 2008).

Alkaline phosphatase (ALP, ALKP) Orthophosphate monoester phosphohydrolases; (EC. 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotide, protein and alkaloids to give inorganic phosphate and the corresponding alcohol, phenol and sugar (Lallès, 2019). These enzymes are widely distributed in nature, including prokaryotes and higher eukaryotes, apart from some higher plants. Despite being found in many bodily tissues, the exact physiological role of ALPs is yet unknown (Vilmaraj, 2020). Alkaline phosphatase forms a large family of dimeric enzymes, usually confined to the cell surface hydrolyzes various monophosphate esters at a high pH optimum with release of inorganic phosphate (Prasad et al., 2014). It is a family of non-specific ecto-phosphomonoesterases. The isolation and characterization of alkaline phosphatase have been carried out from several sources (van Hoof et al., 1994, Larsen et al. 2002, Miandare et al. 2016, Shakoori et al., 2019). The process of removing the phosphate group is called dephosphorylation. As the name suggests, alkaline

phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphatase (Tamas et al., 2002). Alkaline Phosphatase is a non-specific metalloenzymes that hydrolyzes many types of phosphate esters at an alkaline pH in the presence of zinc and magnesium ions. What is measured in the blood is the total amount of alkaline phosphatases released from these tissues into the blood. As the name implies, this enzyme works best at an alkaline pH and thus the enzyme itself is inactive in the blood. Alkaline phosphatases act by splitting off phosphorus (an acidic mineral) creating an alkaline pH.

Phosphatases are enzymes that catalyze reactions which remove the phosphate groups from various molecules. This action of these enzymes is called dephosphorylation. The enzyme removes a phosphate group from the substrate which is monoesters and releases phosphate ions as products. These reactions take place in various types of cells in the body. Its name indicates it is active in alkaline or basic conditions (Tamas et al., 2002). This work is aimed at comparing the distribution and characteristics of ALP levels in four species of freshwater fishes, *C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus*

Sampling site

Ojo local government is located between Badagry and Amuwo-Odofin Local government Areas in Lagos, Nigeria. The creek is an extension of Badagry Creek and is situated on the Eastern section of the Trans-West African Coastal Highway, about 37 kilometers west of Lagos. It is coordinated at 6°28'N30°11'E, showing latitude 4°55'N, latitude 4°17'N, and longitudes

MATERIALS AND METHODS

12°55'E and 13°0'E. The purpose of choosing this site was due to the large waste disposal it receives from the growing population of the town, estimated at about 2 million inhabitants. Another reason was attributed to the evolution of industries such as Doyin groups of companies that is about located about 28km to the sampling site which contributes to the waste discharged into the Lagoon.

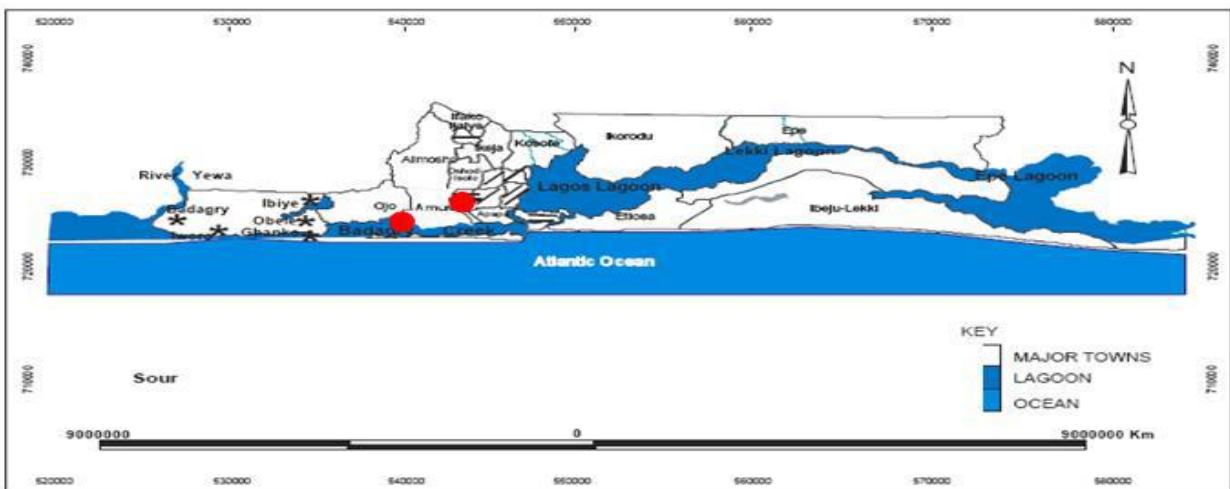


Fig. 1: Ojo coaster water and sample collection site. Source: Hammed et al., (2020)

Sample collection

Ten specimens each of four different fish species namely *C. gariepinus*, *C. zillii*, *H. bidorsalis*, and *C. nigrodigitatus* were procured from the landing site (Ojo market) early in the morning between 6:00hrs and

6:30hrs, and transported on ice to the laboratory for the extraction of the fish muscle and bone for analyses. The laboratory procedure was done at the Biochemistry Department of the Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos, Nigeria. These specimens of

muscles and bone were removed, tagged and kept in a small container for further studies.

Sample preparation: The purchased fishes were transported in a vacuum cooler box filled with ice cubes to the laboratory for morphometric examination. Each sample was degutted with the use of a knife; a sample of the flesh and bones of each fish species was removed into standard crucible and were properly labelled. The bone samples mixed with 5ml of saline solution were pounded in a mortar with a pestle and homogenization was performed to get the substance out of the bone and saline solution. After grinding, the homogenate was obtained. The homogenate plus the solution was poured into sample bottles and then put in the centrifuge machine for 15mins at the speed of 5000 resolution/min. After 15 minutes, the supernatants of each centrifuged homogenates were collected.

Protein determination: Using Lowry's method, 0.2 ml of each supernatant was obtained and 2ml of the protein test reagent was added to each supernatant in new sample bottles. The absorbance of the samples were measured with the use of a spectrophotometer to give 0.170nm, 0.167nm, 0.254nm and 0.230nm respectively.

Enzyme activity: Alkaline phosphatase activity in tissue homogenates was determined by a modified method of Bowers and McComb (1996). The method involves the incubation of 0.1ml of the tissue homogenate with 80mM Tris-HCl buffer (pH 10.5, 0.5mM P-nitrophenylphosphate (PNPP, 10mMol/l) at 37°C for 30 minutes. The reaction was terminated by adding 5.0ml of 2 mM NaOH after which absorbance was taken against the reagent blank.

Determination of K_M and V_{max} : The reaction mixtures contain 1.0ml of the tissue homogenates which has the

highest alkaline phosphatase enzyme activity (bone) was added, 2.0ml, 1.5ml, 1.0ml, 0.5ml of 80mM Tris-HCl buffer, (pH=10.4), differently was added and then 1.0ml, 1.5ml, 2.0ml, 2.5ml and 3.0ml of p-nitrophenylphosphate (10mMol/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 taken. The concentration of the PNPP was calculated and a graph of reciprocal of absorbance against the concentration of the substrate was plotted to obtain K_m and V_{max} .

Effect of divalent cations: The effect of magnesium and calcium ions on phosphatase activity was investigated using $MgCl_2$ and $CaCl_2$. A concentration range of 0 - 1.5 mM of the two metals was used. The reaction was incubated for 10 min and terminated with 1.0 mL of 0.1M NaOH. Absorbance was read.

Effect of pH: The reaction tubes contained 2.0 ml of PNPP and 0.1 ml of tissue homogenate in a Tris-HCl buffer of a pH range of between 7 and 11. It was incubated for 30min at 37°C after which the reaction was terminated by adding 5ml of 0.02M NaOH.

RESULTS Alkaline phosphatase was isolated and characterized from different tissues (flesh and bone) of fishes, (*C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus*). Of all the tissues examined, the flesh contains the highest concentration of protein. 435.20 ± 0.02 g/l, 386.45 ± 0.02 g/l, 248.66 ± 0.01 g/l and 365.80 ± 0.01 g/l were obtained for *C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus* respectively (Table 1). The highest activities of alkaline phosphatase in the bones are 701.04 ± 0.02 u/l, 760.3 ± 0.02 u/l, 801.04 ± 0.02 u/l and 786.07 ± 0.02 u/l for *C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus* respectively. The bone alkaline phosphatase has an optimum pH of 9°C.

Table 1: Protein concentration of tissue homogenates.

Sample	Fish species			
	<i>C. zillii</i>	<i>H. bidorsalis</i>	<i>C. gariepinus</i>	<i>C. nigrodigitatus</i>
Flesh (u/l)	435.20 ± 0.02^a	386.45 ± 0.02^b	248.66 ± 0.01^c	365.80 ± 0.01^d
Bone (u/l)	273.59 ± 0.01^a	293.70 ± 0.01^b	352.94 ± 0.00^c	372.37 ± 0.02^d

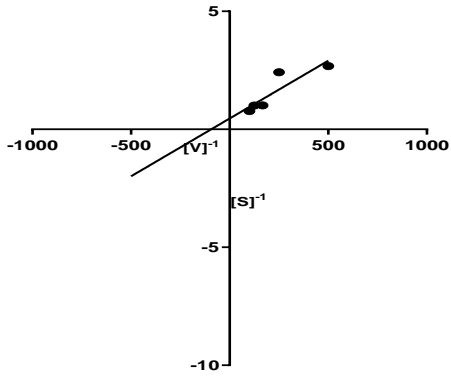
Data represent mean \pm SD (n = 3) concentration expresses total protein in grams per liter (g/l).

Table 2: Alkaline phosphatase activity in tissue homogenates.

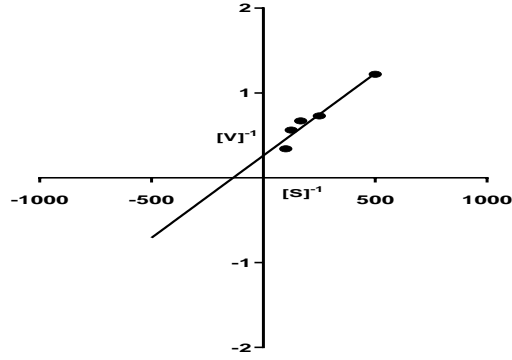
Sample	Fish species			
	<i>C. zillii</i>	<i>H. bidorsalis</i>	<i>C. gariepinus</i>	<i>C. nigrodigitatus</i>
Flesh (u/l)	634.80 ± 0.01^a	604.60 ± 0.00^b	687.60 ± 0.00^c	664.34 ± 0.00^d
Bone (u/l)	701.04 ± 0.02^a	760.3 ± 0.02^b	801.04 ± 0.02^c	786.07 ± 0.02^d

Data represent mean \pm SD (n= 3). Activity is expressed in units per liter (u/l).

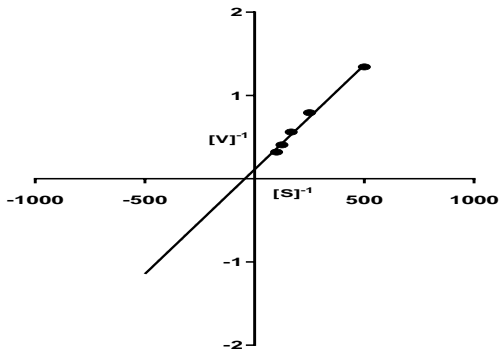




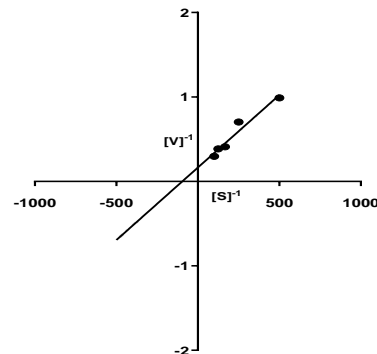
C. zillii- $K_m = 0.0106\text{mM}$; $V_{max} = 2.1730 \mu\text{mol}/\text{min}/\text{mol}$



H. bidorsalis - $K_m = 0.0074\text{mM}$; $V_{max} = 3.1868 \mu\text{mol}/\text{min}/\text{mol}$



C. gariepinus - $K_m = 0.0226\text{mM}$; $V_{max} = 8.9928 \mu\text{mol}/\text{min}/\text{mol}$



C. nigrodigitatus- $K_m = 0.0104\text{mM}$; $V_{max} = 6.0753 \mu\text{mol}/\text{min}/\text{mol}$

Fig. 1: Reciprocal plot of absorbance against substrate concentration of the fish species

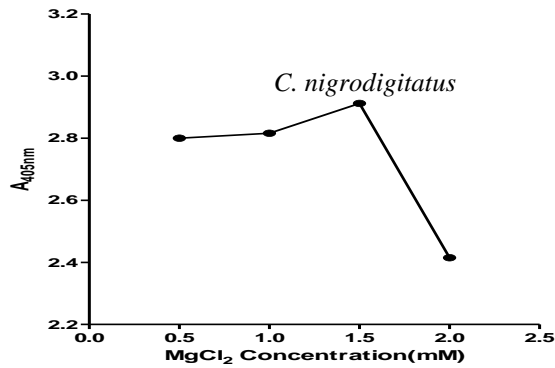
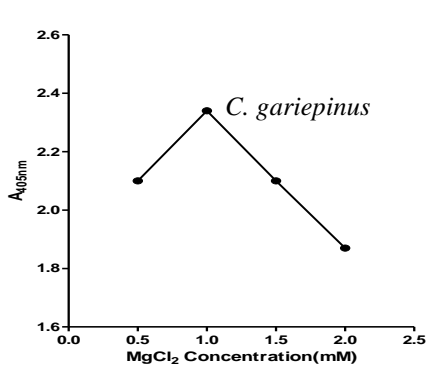
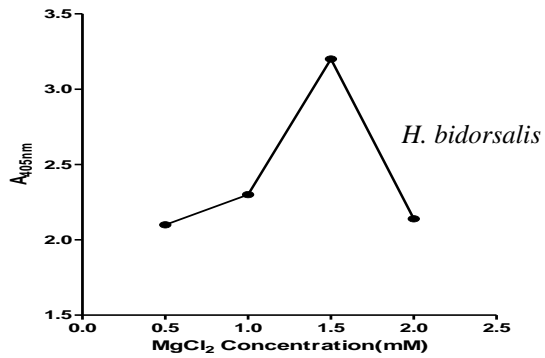
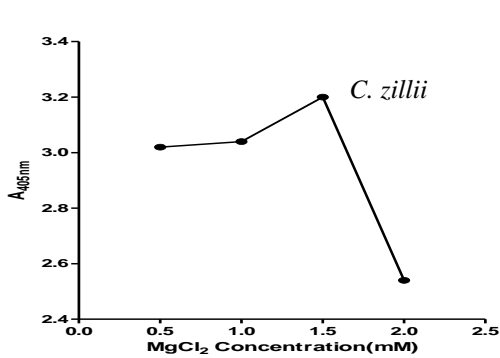


Fig. 2: Effect of magnesium ion on ALP activity of the fish species



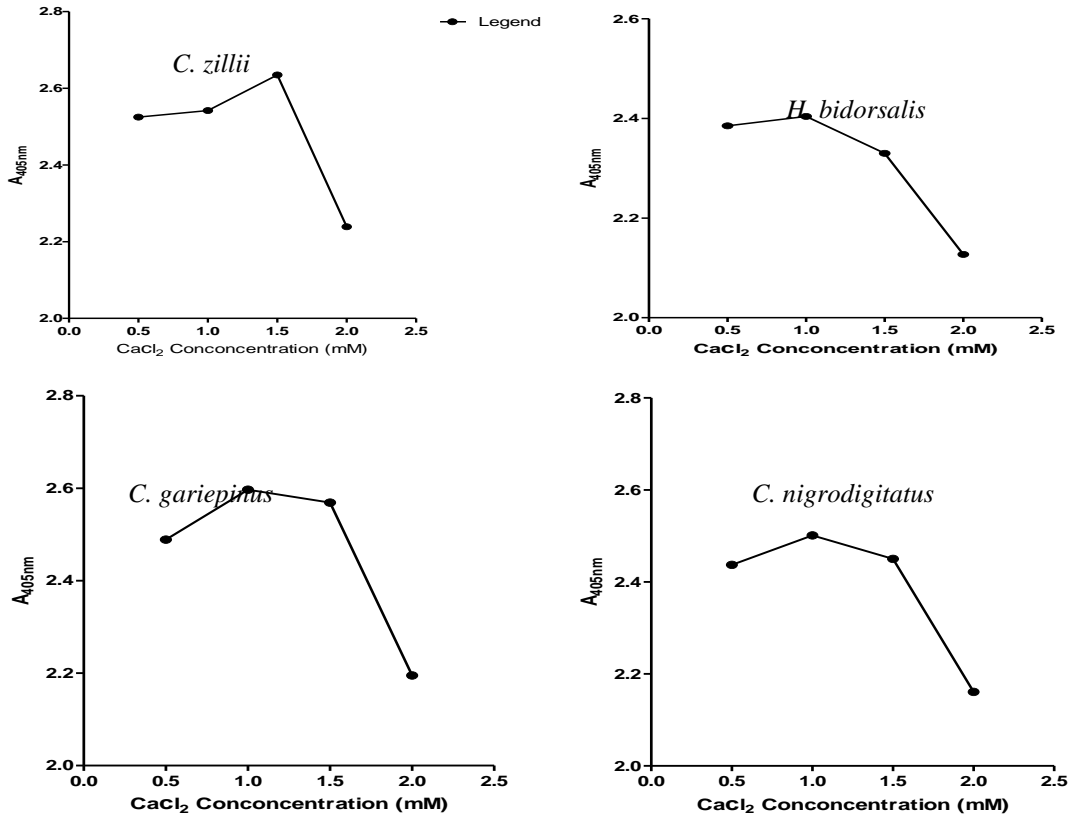


Fig. 3: Effect of calcium ion on ALP activity on the fish species

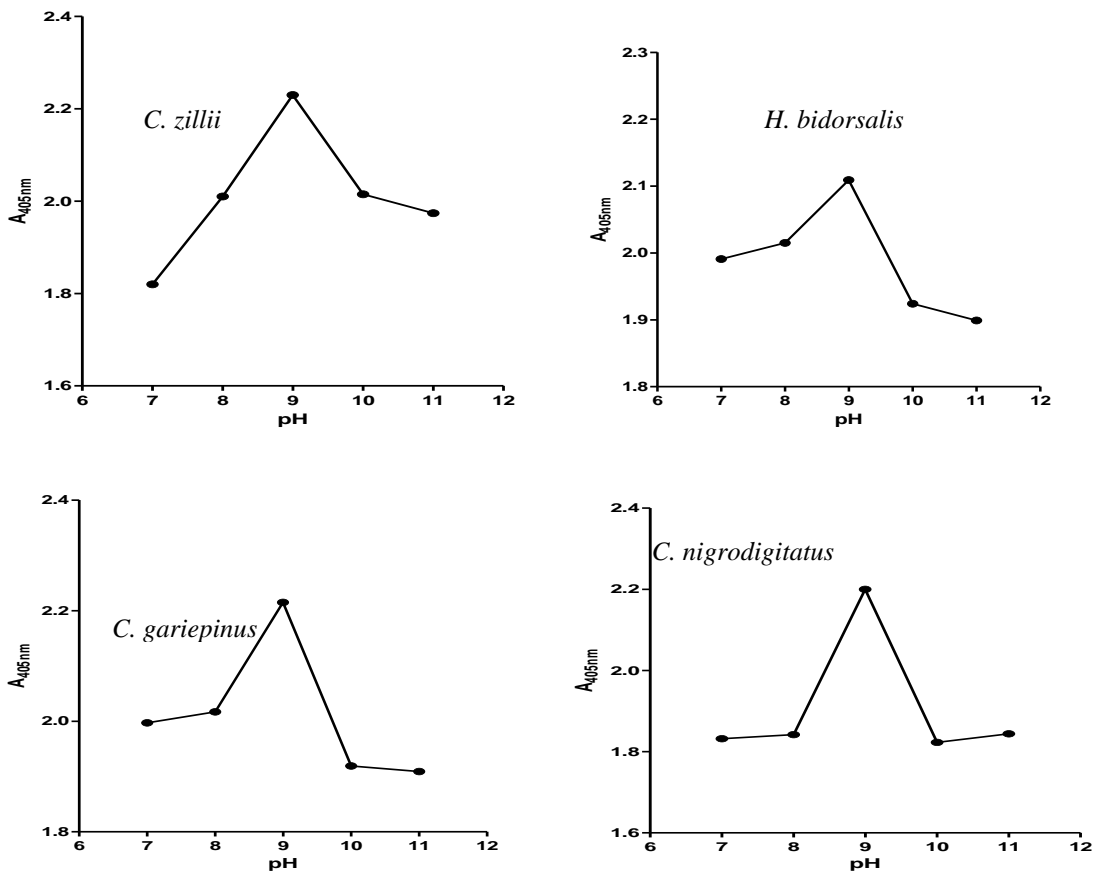


Fig. 4: Effect of pH on ALP activity on the fish species



DISCUSSION

Alkaline phosphatase was isolated from different tissues of *C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus* respectively. Of the tissues examined from these fishes, the flesh contained the highest concentration of protein in *C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus*. The values were 248.66 ± 0.014 g/l for *C. gariepinus* which is the lowest, followed by *C. nigrodigitatus* (365.80 ± 0.010 g/l), *H. bidorsalis* (386.45 ± 0.020 g/l) and the highest which is *C. zillii* (435.20 ± 0.020 g/l). The difference in the protein concentration of these fish species was likely due to the nature of the diet (nutrient) intake and the bone being the tissue for the storage (i.e. concentration of protein was accumulated in the bone) Hammed *et al.*, 2024. This implies that *C. zillii* meets the nutritional requirements of humans more than the rest of the fish species.

According to Raimi *et al.*, 2014, the difference in the protein concentration of '*Wesafu*, *Oreochromis niloticus* and *Sarotherodon melanotheron* was likely due to the nature of the nutrient intake and the muscle being the tissue for storage of the species. Raimi *et al.*, 2012 also observed that the stomach of *C. gariepinus* contained the highest concentration of protein in the cultured and wild catfishes. The highest activity in the stomach of the species implied that most of the organic phosphates in the species were principally hydrolysed in the stomach, followed by the liver. Furthermore, Raimi *et al.*, 2013 discovered that *Macrobrachium vollehovienii* and *Penaeus notialis* contained the highest concentration of protein followed by the head. The highest activity of alkaline phosphatase was observed in the head of both species. This might be as a result of the fact that the digestive system of this invertebrate is contained in the head. All the above mentioned corroborates our new findings.

Alkaline phosphatase activity was determined using *p*-nitrophenylphosphate as substrate, and its highest activity was observed in the bone homogenate (Table 2). The enzyme has been described as responsible for bone matrix formation (Kaup *et al.*, 1994, Vilmaraj, 2020). Alkaline phosphatase can hydrolyze phosphate bound to cell surface phosphoproteins and in this way, it may

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modulate their biological activities (Scheibe *et al.*, 2000; Burch *et al.*, 1985).

At a constant enzyme concentration, the kinetic of the enzyme was defined by Michalis-Menten, the K_m and V_{max} values of the bone alkaline phosphatase were estimated to be 0.0106mM and 2.1730 $\mu\text{mol}/\text{min}/\text{mol}$ for *C. zillii*, 0.0074mM and 3.1868 $\mu\text{mol}/\text{min}/\text{mol}$ for *H. bidorsalis*, 0.0226mM and 8.9928 $\mu\text{mol}/\text{min}/\text{mol}$ for *C. gariepinus* and 0.0104mM and 6.0753 $\mu\text{mol}/\text{min}/\text{mol}$ for *C. nigrodigitatus* respectively (Fig. 1). Alkaline phosphatase is a divalent metalloenzyme which requires Zn^{2+} , Mg^{2+} and Ca^{2+} for its activity; absence of these divalent metals has been seen to lower the alkaline phosphatase activity in serum and several cellular tissues from experimental animals (Hui-Chili *et al.*, 2001). In this experiment however, Mg^{2+} has an unstable activation on fish bone alkaline phosphatase (FBAP) but inhibited the alkaline phosphatase activity at a concentration above 1.5 mM (Fig. 2). A similar effect on FBAP activity was observed for calcium ion (Fig. 3). This confirms the preference of AP for a wide range of divalent cations for its activity, provided it is available at physiological concentrations.

The optimum pH was investigated by incubating the reaction mixture in buffers of different pH. Alkaline phosphatase from the four species of fish has an optimum pH of around 9.0 after which the enzyme begins to lose activity (Fig. 4).

CONCLUSION

The results of this study shows that alkaline phosphatase activity was high in the bone of the fishes (*C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus*). The activities of alkaline phosphatase in the four species of fish are activated by MgCl_2 , CaCl_2 and have the pH optimum of 9.0 in the results. Overall, the ALP activities are helpful in numerous activities in fish tissues including bone formation, membrane transport, osmoregulation, etc. Thus, the distribution of ALP in the fish tissues indicate that *C. zillii*, *H. bidorsalis*, *C. gariepinus* and *C. nigrodigitatus* can be incorporated together into the integrated fish farming system for mass-scale production. However, same study can be conducted using different aquatic species from marine environment.

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