Purification and Effects of Some Divalent Metals ions on α and β –Amylases Produced from *Eleusine coracana* Malt

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Abstract-Amylases being industrial enzymes are in high demand for the hydrolysis of starch used for the production of various industrial products. Crude extracts of the enzymes were obtained from *Eleusine coracana* (Finger millet) and purified using Reverse Phase –Column Chromatography and gradient mobile phases. The enzymes were purified and various studies carried out to ascertain the extend of purification and activity of the enzymes. The enzymes were purified 1.82 fold and specific activity was obtained as 133µmol/mg. It was also observed that alpha amylase was most effective at temperatures 40-55^{0}C while beta amylase was between the temperatures of 40-45^{0}C. The crude enzyme extracts had an activity optimum temperature range of 35-55^{0}C . The optimum pH for the various enzymes were found to be: α_1 -7.0, α_2-6.0, β_1-6.0, β_2-7.5 while the crude enzyme extracts had pH optimum of 6.5-7.5. The effect of the metal ions on the activity of the enzymes showed that the crude enzyme extract and β_1-amylase were greatly influence while the other enzymes were not significantly affected by the metal ions. The results of the analysis showed that the enzymes obtained from the local grains *Eleusine coracana* are potential sources of enzyme for hydrolysis of cereal grains for use in the production of industrial products such as alcoholic and non-alcoholic drinks

Key Words – Alpha and beta amylases, *Eleusine coracana*, Enzyme activity, Finger millet, Metal ions, Purification and Starch hydrolysis

1. INTRODUCTION

Eleusine coracana (Finger millet) is an annual tufted grass and a tropical cereal crop with relatively wide range of adaptation, moderate temperature and grown extensively in various regions of India and Africa [15], [2]. Having such a tolerable weather conditions made it easier to be widely grown in the Northern and central part of Nigeria. Finger millet (FM) grains are used in these parts of the country for the production of alcoholic and non-alcoholic drinks and the by-products are used for animal feed. Eneje and colleagues [11] stated that the most important characteristics of good malt are high enzyme levels (measured by diastatic power to degrade starch and obtain high extract yield).

FM is eaten cooked, bread are made from its flour and popped products, as it is readily digestible [1]. FM is a staple food mostly consumed by low-income group [7] most especially the local alcoholic drink "Burukutu" that replace the 'High class lager beer.'

FM has been recognized because of its high content of calcium, dietary fiber, phenolic compounds, protein, vitamins, minerals and carbohydrate, which are comparable to other cereals [2], [7]. Germinated and non-fermented FM flour contain higher level of carbohydrate, protein and glycoprotein, however, when germinated and fermented, increased amino acid, phytochemical and free radical scavenging activity [19].

Starch-hydrolyzing amylases are interesting enzymes that split large polysaccharide chains into various smaller size segments. This digestive processes results in the products such as dextrin, maltose and glucose. The breakdown of large particles drastically reduces viscosity of gelatinized starch solution resulting in liquefaction. The final stage of de-polymerization is the formation of mono, di and tri-saccharides, which are observed in the partial purification, and characterization of alpha and beta amylases [10].

Generally, α- amylases are stable to heat compared to β-amylases [17] and the removal of one amylase from the preparation containing both α *and* β –amylases is a major problem when purifying enzymes from cereal sources. In addition, it was reported that separation of the amylases into two active components of each is not related to aggregation of dissolution of monomeric forms of the enzymes [13].

Amylase occupies a major share (around 25%) of total world enzyme market owing to its high demand eliminating chemical hydrolysis of starch in the starch liquefaction process. It has been utilized also in textile,

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food, brewing, and paper pulp industries [18].
The pH and temperature optimum for the crude enzymes extract of finger millet malt was recorded as 7 and between 45-30^{0}C respectively and hexose sugar was present on chromatographic analysis [8]. The advantage of enzymatic hydrolysis is that it is highly specific and its action can be easily controlled at milder condition of temperature and pH. El-non and others [10] concluded that the amylolytic activity initiated during germination of sorghum determined the Drastic Power of the malt to be no significant variation between the extraction procedure followed by either distilled water or water with 2% peptone. Finger Millet malt has much more of this saccharifying power than does sorghum or maize: only barley, the world's premier beer grain surpasses it.
This work report the purification steps of un-isolated and isolated α and β amylases, effect of temperature, pH and some divalent metal ions on the activities of enzyme from *Eleusine coracana* malt.

2. MATERIALS AND METHODS

2.1 Sample PREPARATION

Fresh dried grains of finger millet locally known as Tamba was collected from a farmland at Kadamo village, Jengre town in Bassa LGA of Plateau state, Nigeria. Some of the grains were grounded into powder, using mortar and pestle, stored in an airtight container for all the analysis.

2.2 Extraction of Crude Enzyme

Crude enzyme extracts from finger millet at optimum malting time was carried out using the method of MacGregoe and others [13]. While Isolation of α and β amylases from crude malt extract were carried out using Botes and colleagues [5] method.

2.3 Isolation of alpha-amylase

The prepared crude enzymes extract was centrifuged (7000g x 20mins) and filtered through glass wool, the supernatant was poured into a 250 ml beaker while the residue was re-extracted with 100ml acetate buffer of pH 7 and pooled together with the former. About 150ml of the supernatant was heated in a controlled water bath at 70^{0}C for 24 hrs with frequent stirring, in order to deactivate the β-amylase, which is heat labile. The extract was cooled immediately to room temperature and the centrifuge to remove the precipitate. The supernatant was concentrated using a freeze dryer and prepared for further purification on column chromatography.

2.4 Isolation of beta-amylase

The prepared crude enzymes extract was also subjected to centrifugation as above, 150ml of the pooled supernatant was maintained at pH of 3.7 with acetic acid at a temperature of 10-15^{0}C for 2 hrs to deactivate the α-amylase since is acid labile. The solution was then centrifuge again to remove the precipitate and **concentrate** the supernatant.

2.5 Protein Determination

The percentage protein of the crude enzyme extracts was quantified using Kjeldahl method for determination of organic nitrogen [8].

2.6 Purification of the Enzyme Extracts

The isolated α and β amylases and the crude enzyme extracts (unisolated enzyme extract 1&2), were subjected to column chromatography using reverse phase technique (C-18 silica gel as adsorbent). The gradient mobile phase in 0.1% Trifluoroacetic acid (TFA) solutions of; 100% distilled water, acetonitrile/H_2O solution(3:7), methanol/H_2O solution(7:3), added to the columns successively in a stepwise manner.

2.7 Amylase Assay

The activity of the α and β amylases was determined by Dinitrosalicylic (DNS) acid, measuring the maltose liberated in μM/ml when treated with 0.1ml of enzyme and 0.05g/L starch solution using 3,5 DNS reagent [3]. The amount of the reducing sugars liberated during assay was estimated by measuring color development at 540nm by UV-VIS spectrophotometer. One unit of amylase activity is defined as the amount of enzyme producing 1μmol of reducing sugar in maltose per minute under standard assay condition.

2.8 Determination of optimum pH

The optimum pH was determined by incubating the unisolated, α and β enzymes extracts in 0.05M acetate buffer at different pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The hydrolysis reaction was performed at 37^{0}C for 10 minutes.

2.9 Determination of optimum temperature

The optimum temperature for maximum enzyme activity was determined at temperatures of 35, 40, 45, 50, 55, 60, 65 and 70^{0}C at pH of 5.5 for unisolated amylases extract and pH 6.5 for α and β amylase extracts.

2.10 Determination of Metal ions Activity:

The 0.3ml of 100ppm Ca^{2+}, Mg^{2+}, Pb^{2+} and Cd^{2+} ions were pre-incubated with 0.7ml of amylase extracts at 37^{0}C for 20mins then assayed for amylase activity; and the activity in the absence of metal ion was taken as 100%.

3. RESULT AND DISCUSSION

3.1 Purification of Enzyme Extracts

Purification of α and β amylases and un-isolated enzyme extracts-1&2 produced by *Eleusine corcana* using different mobile phases were summarized in Table 1. The two pairs of enzyme isomers α_1, α_2 and β_1, β_2 $_{amylases}$ were preferentially isolated through heat treatment of 70^{0}C, and pH in conformity with the work by MacGregoe and

colleagues [13].

Purification of un-isolated enzyme extract1 on Acetonitrile (Acn)/water (3:7) gave a specific activity of 110μmol/mg with fold purification of 1.5 and 8% recovery yield while un-isolated enzyme extract2 on methanol (MeOH)/water (3:7) treatment lowered the specific activity by 34μmol/mg, fold purification by 0.4 and increased in recovery yield by 81%.

Purification of α_1 on Acn/water (3:7) gave 131 μmol/mg, 1.82 and 6%. Nevertheless, it was pooled for further purification on methanol (MeOH)/water (3:7) as α_2, recorded an increase by 2 μmol/mg, lowered fold purification by 0.02 and an increased 10% recovery yield. Low yield of the purified enzyme is attributed to the loss during Acn/water pool as earlier stated by Massade, and others [14], while Raul and colleagues [18] reported less activity.

Furthermore, purification on water (100%) of β_1 gave 96 μmol/mg, 1.3 and 70%, treatment on Acn/water (3:7) as β_2, caused an increase in the specific activity by 14 μmol/mg, fold purification of 0.2 and lowered recovery yield by 7%.

The high percentage yield of beta amylase compared to alpha amylase in this work affirmed the result of Egwin, and Oloyede, [9], who suggested it could be the presence of the other complementing hydrolyzing enzymes such as glucoamylase. Similarly, Nirmala, and Murallikrishma [17], reported that this disparity could be due to the technique used for the isolation of the enzyme, which if developed further might produced a more purified amylase even though Bertoft and colleagues [4] attributed it to limited capacity of the column used.

Table 1: Enzymes Purification at pH 6.5 and Temperature of 45^0C

S/No	Sample	Total activity (µMol)	%Protein	Specific activity (µMol/mg)	Fold Purification	Total Recovery (%)
1.	Crude extract	1120	15.53	72	1	100
2.	Unisolated enzyme in Acn/H_2O(3:7)	107	0.94	110	1.5	8
3.	Unisolated enzyme MeOH/H_2O (3:7)	1090	14.26	76	1.1	97
4.	β_1-extracts in 100%H_20	781	8.10	96	1.3	70
5.	β_2 extracts in Acn/H_2O (3:7)	708	6.45	110	1.5	63
6.	α_1 extracts in Acn/H_20 (3:7)	62	0.47	131	1.82	6
7.	α_2-extract in MeOH/H_2O (3:7)	177	1.33	133	1.8	16

3.2 The effect of Temperature

The effect of temperatures on partially purified enzyme extracts is as stated in figure 1. The optimum temperatures for the enzymes are as follow: α_1 55^0C , α_2 40^0C, β_1 45^0C , β_2 40^0C , unisolated enzyme extracts 1&2 are 45^0C and 55^0C respectively. The enzymatic activity increased progressively with increase in temperature from 40^0C- 55^0C. However, this begins to decline at higher temperature than 55^0C. This trend agrees with the earlier work by Bertoft and others [4] who stated that at higher temperatures, the enzymes are inactive and depend on the enzymes concentration and incubation time. He further established that under the same condition treatment, α-amylase has high temperature optimum than β amylase despite the fact that α amylase is much more sensitive to high temperature, which correlates with our findings.

3.3 The effect of pH

The enzyme activity was assayed at pH 4.5-8.0. There was increase in activity from 4.5 to maximum of 7.0 for α_1 amylase and 6.5 for α_2 amylase, 6.0 and 7.5 for β_1 and β_2 amylases respectively, as depicted in figure 2. These results obtain are similar to earlier work reported by some researchers [6], [8], [9].

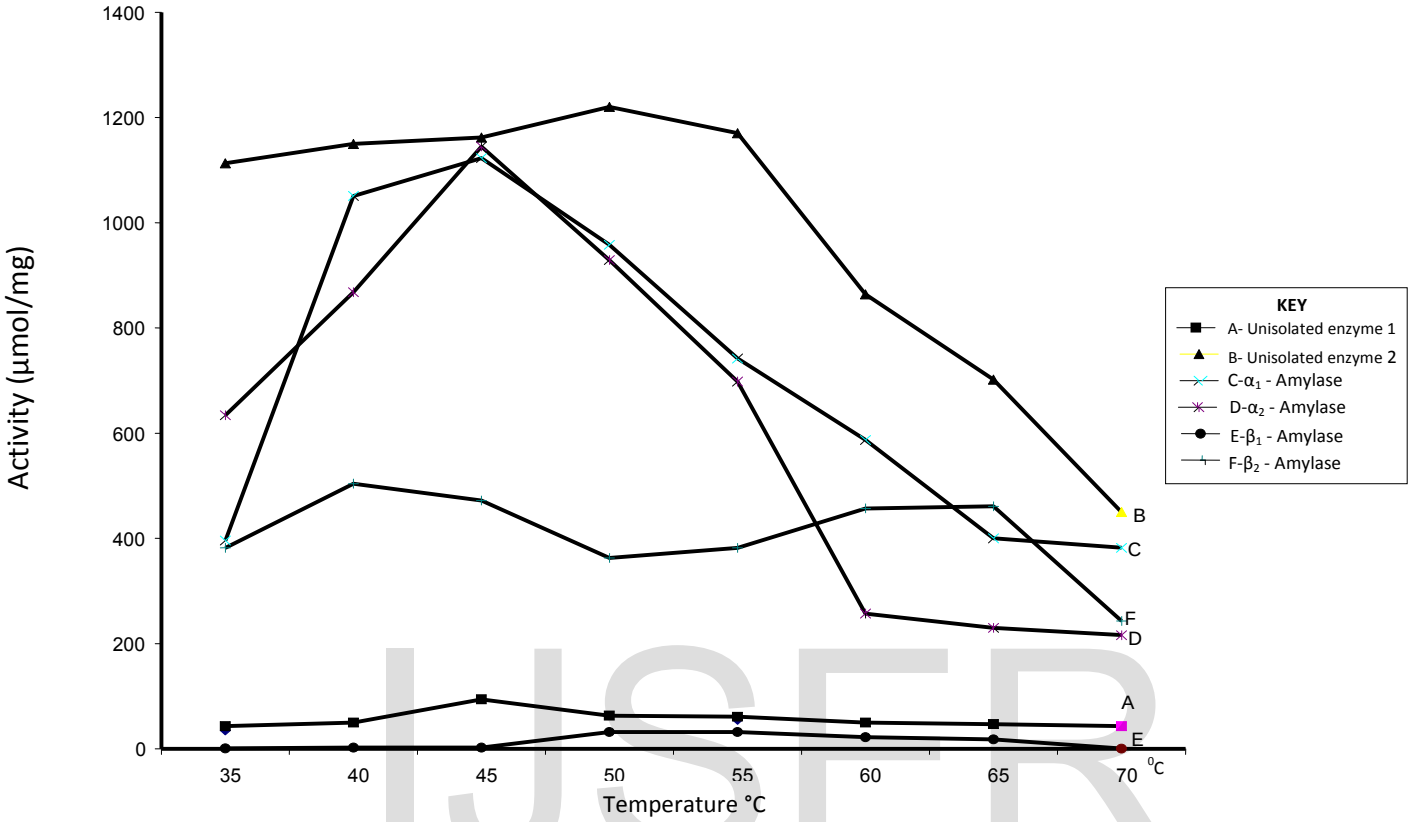

Figure 1: Activity of Enzymes in the Elution Profile against Temperature

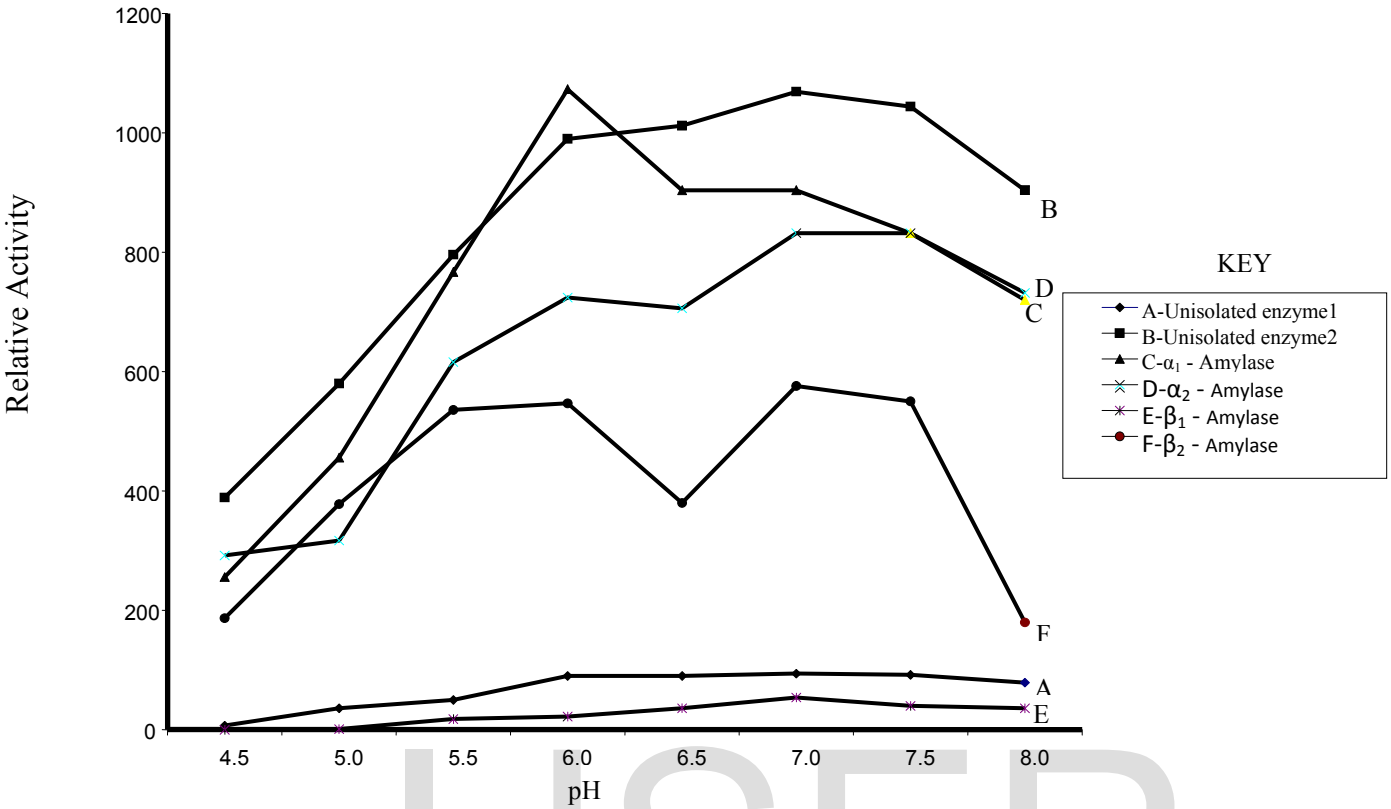

Figure 2: Activity of Enzymes in the Elution Profile against pH

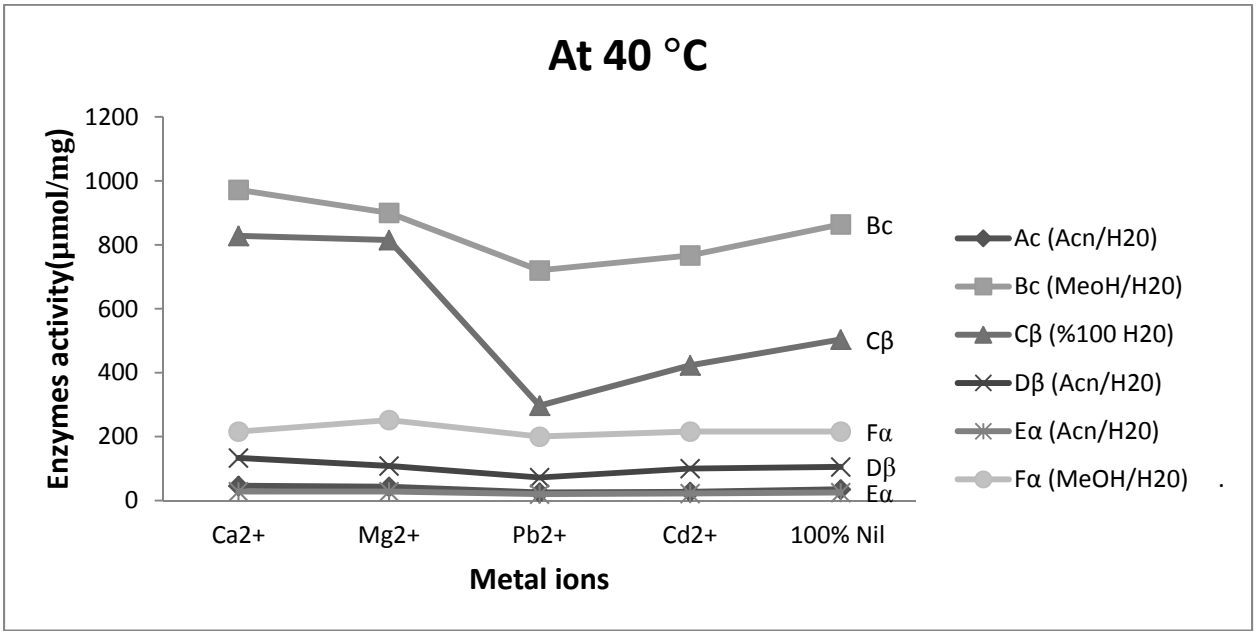

Figure 3: Effect of metal ions on enzymes activity using starch as substrate
AC= Eluted unisolated enzyme extract1, BC=Eluted unisolated enzyme extract 2
Cβ=Eluted β-amylase enzyme extract 1, Dβ=Eluted β-amylaseenzyme extract 2
Eα= Eluted α-amylase enzyme *extract 1, Fα=Eluted α-amylaseenzyme extract 2*

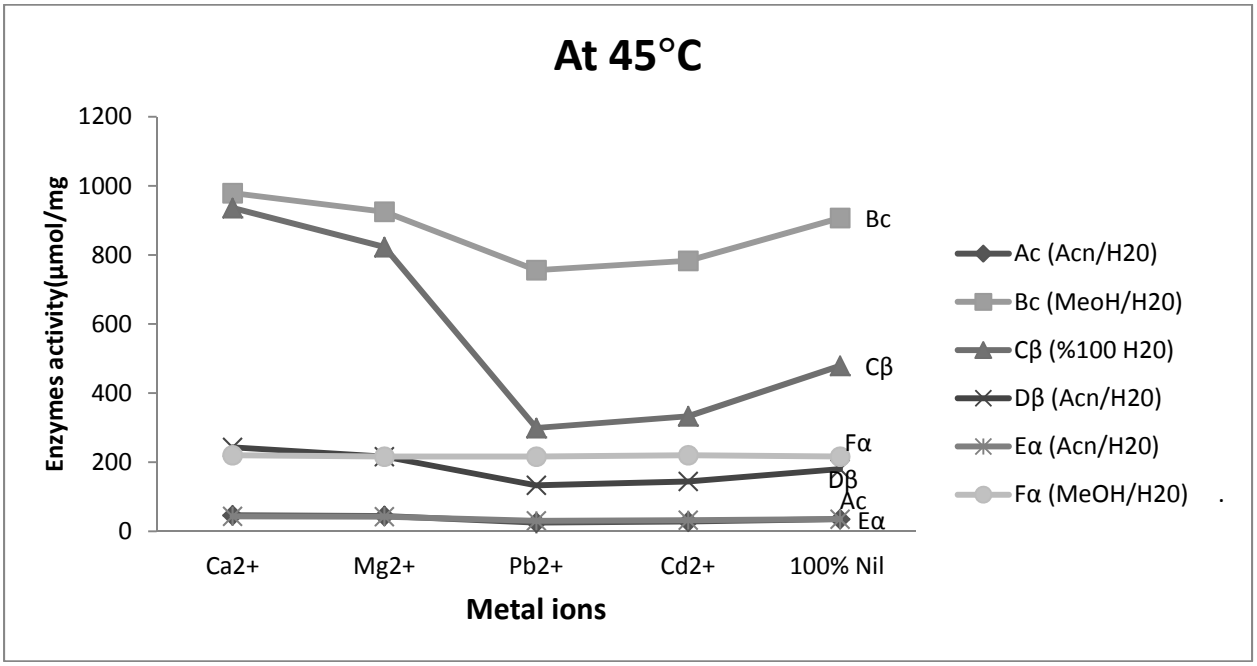

Figure 4: Effect of Metal ions on Enzymes Activity Using Starch as Substrate

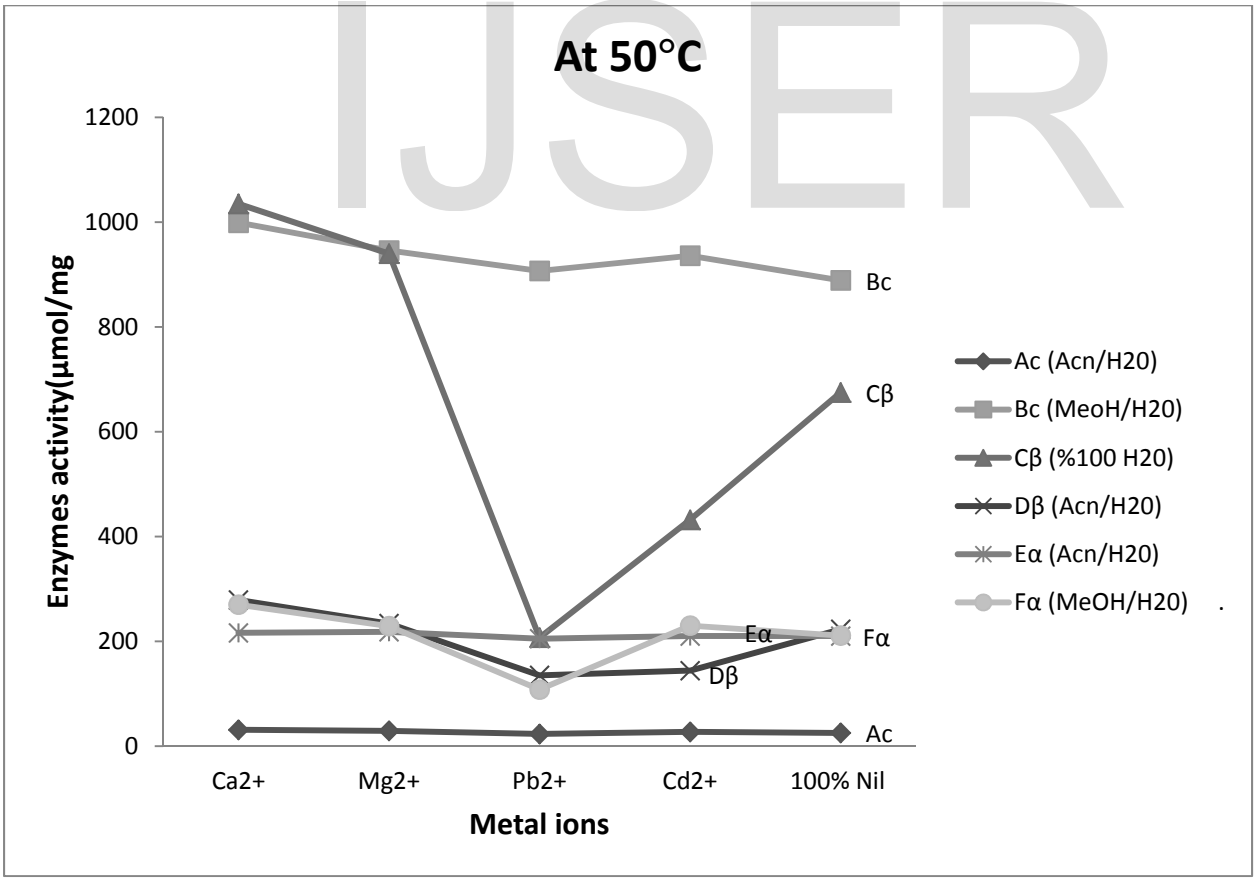

Figure 5: Effect of metal ions on enzymes activity using starch as substrate.

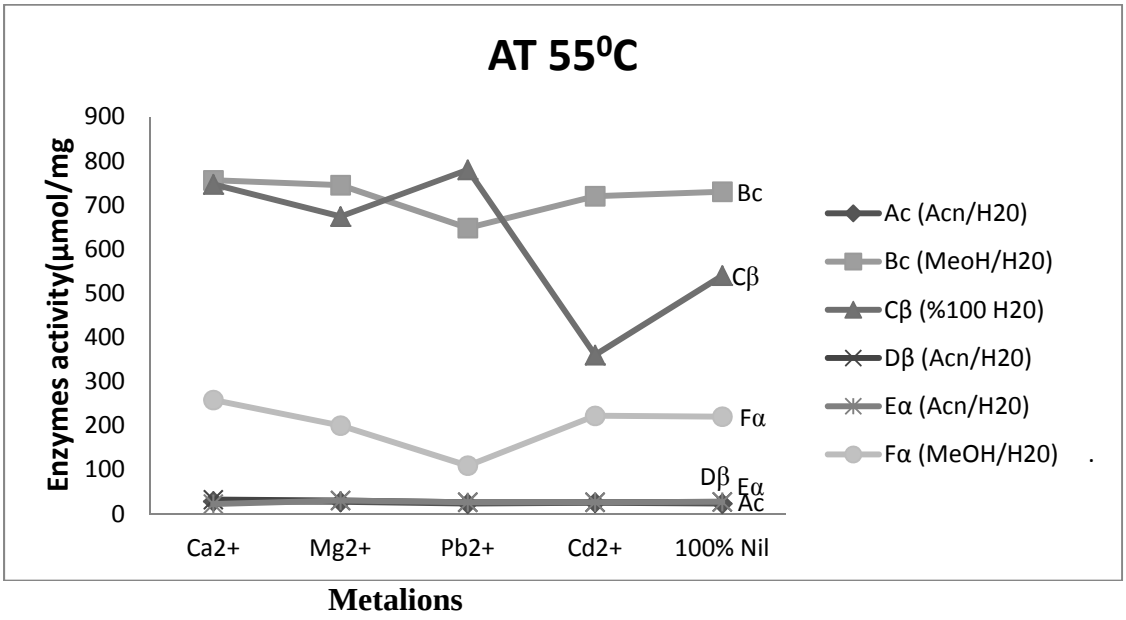

Figure 6: Effect of metal ions on enzymes activity using starch as substrate.

3.4 Effect of Metal ions on the Activity of the Enzymes

The activity of the enzymes on addition of metal ions was evaluated at different temperature ranges, 40^0C-55^0C (Bc) and β_1-amylase enzyme extract (Cβ) at temperatures 40-55^0C. Ca^{2+} and Mg^{2+} significantly increase the activity of Bc and Cβ, while the other enzyme extracts were not significantly influenced. Earlier authors [4], [20] had reported in their work that Ca^{2+} has a binding capacity and is independent on the configuration of the enzymes, further, Zanna and others [20] confirmed other divalent metal ions to have such characteristic. On other hand, Pb^{2+} and Cd^{2+} ions produced decrease activity at temperature range between 40^0C-50^0C on Bc, β_2-amylase enzyme extract (Dβ) and α-$_{1\&2}$ amylases enzyme extracts (Eα & Fα) respectively, but at 55^0C and above, the inhibition effects begin to increase on the enzymes. This decrease in activity of Pb^{2+} and Cd^{2+} ions proves that the metal ions are deleterious to the structure of the amylases, making it a competitive inhibitor as reported by the work of Nam [16]. Also Zana and others [20] reported that the inhibition cause by divalent metal ions suggested that the enzyme is a SH-enzyme, and concluded that divalent metal ions can be involved in enzyme catalysis.

1. 4. CONCLUSION

The malt of *Eleusine coracana* has demonstrated high potential of producing amylase enzymes during malting, making it very good source for producing high quality malt for beverage production. Nigeria is blessed with a vast land to produced large quantities of finger millet that can offer the beverage industries, opportunity to explore it usage. Instead of the conventional means of producing amylases from microbes, which are often difficult to handle especially during culturing, due to unstable source of power supply. In addition, the cost of building large tanks for cultivation of microbes is very high; therefore, producing amylase from cereals' malt would be a welcoming development.

under assay conditions and the amount was determined as shown in figures 3 – 6. All the metal ions used except Pb^{2+} ion increased the activity of unisolated enzyme extract-2

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