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Studies on the Physicochemical Properties of Amylases Obtained from *Digitaria Exilis* (White Fonio, Acha) and *Digitaria Iburua* (Black Fonio, Iburu) Under Optimized Conditions

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Abstract

The need for indigenous cereals as a substitute to popular ones such as barley and wheat has necessitated the search for unpopular cereals like fonio. This study investigated the most appropriate conditions for the induction of amylases from acha and iburu using response surface methodology in water which is of traditional practice and water containing phosphate salts (10 mM sodium phosphate buffer, pH6.5). Malted grains as a function of days of germination produced the highest amylolytic activities on second day of germination with 24337 U/mgprotein when steeped in water and 15794 U/mgprotein for iburu steeped in water containing phosphate salt. However, white fonio produced lesser amount of amylases when steeped in water with 17948 U/mgprotein and 12046 U/mgprotein in water containing salt. These were purified using Concanavalin A Sepharose and were further purified on Bio-gel P100 gel filtration technique for a pure homogenate. The physicochemical properties of these homogenates were studied and white fonio was optimum at pH 6.0 while black fonio was pH 6.5. Similarly, both were stable to temperature at above 65ºC. $CaCl_2.2H_2O$ and $MnCl_2.4H_2O$ activated activities of amylases from two grains but NaCl and KCl led to a reduction in activities while $CuSO_4$ and $MnCl_2$ absolutely inactivated activities of these enzymes. Physicochemical properties of these amylases are similar to that obtained from other grains, hence fonio can be used to totally replace amylases from other grains and as such promote the utilization of these local grains and as such improve the economy of our nation Nigeria.

Keywords: Physicochemical properties; Amylases; *Digitaria Exilis*; *Digitaria Iburua*; Optimized Conditions

1.0 Introduction

Amylolytic enzymes (E.C 3.2.1.0), are collectively referred to as α-amylase, β-amylase and γ-amylase which are among the most important enzymes and of great significance due to their wide areas of application and also represents second largest group of enzymes in the market after proteases. They are important industrial enzymes covering around 30% of total enzyme market in the world (Aiyer, 2015; Sivaramakrishnan *et al*., 2016). Amylolytic enzymes simply referred to as amylases catalyze the hydrolysis of the 1,4-glycosidic linkages found in polysaccharides such as amylose, amylopectin, glycogen, or their degradation products. Since the breakdown products of starch are the usual sources of dietary carbon, amylases are universally distributed throughout the animal, plant and microbial kingdoms (Dehkordi-Mobini

and Javan, 2012; El-Fallal *et al*., 2012; Hashemi *et al*., 2014; Shah *et al*., 2014).

Both α- and β-amylases are present in seeds, β-amylase is present in an inactive form prior to germination whereas α-amylase appears once germination has begun**.** This unique enzyme has been isolated from barley and rice plants. Adewale *et al*. (2006) and Egwim and Oloyede (2006) extracted α-amylase (which is the most abundant of amylolytic enzymes) from common cereals like maize, millet, rice, sorghum and very good yield of this enzyme was obtained. The search for an alternative source of amylases as a good substitute to those of microbial sources has led to increased studies in cereal malting which involves development of hydrolytic enzymes via a three-step process namely: steeping, germination and drying (Saxena *et al*., 2014). α-Amylases (E.C.3.2.1.1) are a liquefying enzyme because they hydrolyze bonds located in the region of the substrate. Endoamylases are termed α-amylases because they cleave α-1,4-glycosidic linkages in amylose, amylopectin and related polysaccharides such as glycogen randomly resulting in rapid decrease in viscosity of starch solution (Gupta *et al*., 2003; Drauz *et al*., 2010).

Starch is the commonest storage carbohydrate in plants. It is used by the plants themselves by microorganisms and by higher organisms. So, there is a great diversity of enzymes being able to catalyze its hydrolysis. Starch from all plant sources occurs in the form of granules which differ markedly in size and physical characteristics from species to species. Chemical differences are less marked. The major difference is the ratio of amylose to amylopectin e.g corn starch from waxy maize contains only 80% amylose. The main location of starch synthesis and storage in cereals is the endosperm. Major starch sources are cereals (40 to 90%), roots (30 to 70%), tubers (65 to 85%), legumes (25 to 50%) and some immature fruits like bananas or mangos, which contain approximately 70% of starch by dry weight (Santana and Meireles, 2014). Of the two components of starch, amylopectin presents the great challenge to hydrolytic enzyme systems. This is due to the residue involved in α-1,6-glycosidic branched points which constitute about 4-6% of the glucose present. Most hydrolytic enzymes are action specific for α-1,4-glycosidic link yet the α-1,6-glycosidic links must also be cleaved for complete hydrolysis of amylopectin in order to give some of the most impressive recent exercises in the development of new enzymes. Amylases have a great commercial value in biotechnological applications ranging from bakery, alcohol, textile to paper industries (Parmar and Pandya, 2012). They are capable of digesting glycosidic linkages in starch components and glycogen molecules and are widely utilized in brewing and detergent industries (Khan and Priya, 2011). However, amylolytic enzymes are widely distributed in plant, microbial and animal kingdoms, with varying action patterns depending on the source (Padhiar and Konmu, 2016).

The search for an alternative source of enzymes has led to increased studies in cereal malting (Hammond and Ayernor, 2000). With the advent of new frontiers in biotechnology, the spectrum of amylolytic enzymes application has widened. There is need for the possibility of other cereals that can tolerate wider range of soil and climatic conditions (Doss and Anand, 2012) and which can possibly replace the importation of wheat and barley. Sorghum according to Adefila *et al.* (2012) would have been a very good substitute but research had shown, it has very small quantity of β-amylase and a good mixture of α and β-amylases however are required for complete saccharification of starch, hence the need to shift studies to *D. exilis* and *D. ibura* which are African unpopular grains and could possibly serve as a good substitute.

2. Materials and Methods

2.1 Materials

Digitaria exilis and *Digitaria iburua* grains were purchased from Sabon Gari market in Zaria, Kaduna State, Nigeria. These grains were authenticated at the Herbarium of Department of Biological Science, Kaduna State University, Kaduna, Nigeria. The Voucher number is 6/6.

2.1.1 Chemicals

3,5,-Dinitrosalicylic acid (DNSA), Sodium hydroxide pellet, Sodium potassium tartrate (Rochelle Salt), blue dextran, glucose, bovine serum albumin (BSA), 2-mercaptoethanol, acetic acid, sodium phosphate dibasic (Na_2HPO_4), anhydrous sodium phosphate monobasic ($NaHPO_4$), Soluble Starch, , ammonium persulphate, Coomassie brilliant blue G-250, phosphoric acid and calcium chloride dihydrate were obtained from Sigma Chemical Company, St Louis, Mo, U.S.A. Glycerol, sodium chloride, methanol were obtained from British Drug House Chemicals Limited, Poole, England. All other reagents were of analytical grade.

2.2 Methods

2.2.1 Induction of Amylolytic Enzymes

Amylolytic enzymes were induced by steeping 100 g of screened *D. exilis* and *D. iburua* grains in water and water containing phosphate salts (10 mM sodium phosphate adjusted to pH 6.5) for 24 h at room temperature. Steeped grains of *D. exilis* and *D. iburua* were blotted to remove excess water after 24 h and were spread out in a locally constructed malting chamber at room temperature. Grains were sprinkled with water and water containing phosphate salts at 12 h intervals (twice a day) for optimization of the germination condition.

Optimum day of germination was determined for each grain by harvesting malts on each day of germination, homogenizing to obtain a crude extract and assaying for amylolytic enzymes until a decline in induced amylolytic activity was obtained.

2.2.2 Extraction of Amylolytic Activity

Induced amylolytic enzyme from *D. exilis* and *D. iburua* malts were extracted by preparing 30% homogenate of the malted grains using water and water containing phosphate sulphate (10 mM sodium phosphate buffer pH 6.5 containing 1 mM $CaCl_2$), following the method of Adefila *et al*. (2012). The 30% homogenate was prepared by homogenizing 100 g of malted *D. exilis* and 170 g of malted *D. iburua* in cold 10 mM sodium phosphate buffer pH 6.5 containing 1 mM $CaCl_2$. The crude homogenates were centrifuged at 13,000x*g* for 30 min at 4 ˚C using Hitachi High speed Refrigerated Centrifuge Himac CR21G H. The pellets were discarded while the supernatants were collected. Amylolytic activities and protein concentration in each supernatant were determined and the supernatants were stored at -20 ˚C until further use.

2.2.3 Standard Procedures for Amylase Activity Assay

The amount of reducing ends released upon starch hydrolysis by amylolytic enzymes was estimated using the modified method of Bernfeld (1951). A unit of amylolytic activity was defined as the amount of enzyme which liberated reducing sugar equivalent to 1 µg of D-glucose per minute at 25 ˚C under the standard assay condition.

An assay mixture of 2 mL in the final concentration contains 10 mM sodium phosphate buffer, pH 6.5 containing 1 mM $CaCl_2$, 0.2 mL of 1 % soluble starch and 0.01 mL of the enzyme. The assay mixture was incubated for 5 min at room temperature for enzymatic reaction to occur, after which the reaction was terminated with 1 ml of 0.5 mM 3,5-dinitrosalicylic acid. The solution was boiled for 5 min for colour development, the yellow colour of 3,5-dinitrosalicylic acid turned into reddish brown and was cooled under a running tap and diluted with distilled water to 10 ml. The optical density was taken at 470 nm using a spectrophotometer. Two blanks were set up for the experiment; the first blank consisted of all assay components except the enzyme while the second contained denatured (boiled at 100ºC) enzyme. Glucose was used to prepare the standard curve from which the amount of reducing sugars liberated was estimated.

2.2.4 Protein Concentration Determination

The protein concentration in the crude supernatants was determined using Coomassie dye binding assay, following the method of Bradford (1976) using bovine serum albumin as the standard protein. The method measures the increase in absorbance of Coomassie Brilliant

Blue G-250 dye at 595 nm upon binding to protein.

2.2.5 Determination of Optimum pH and Temperature for Induced Amylases

Optimum pH and temperature were studied and amylolytic activity induced in the malts of *D. exilis* and *D. iburua* were then determined in the crude extracts obtained after the malts were homogenized. The specific activity of the enzyme from each grain malts were calculated and plotted against respective pH and temperature values.

3. Results

3.1 Levels of Amylolytic Activities in *Digitaria exilis* and *Digitaria iburua* Grains

Figure 1 is a summary of the levels of amylolytic activities in the crude homogenates of malted *D.exilis* and *D.iburua* in water and water containing phosphate salts. High amount of amylolytic enzyme was found higher in grains steeped with water containing phosphate salts when compared with water for both grains. The obtained result is the preliminary information for further studies.

3.2 Effect of Days of Germination on Malted *D.exilis* and *D.iburua* grains

Grains of *D.exilis* and *D.iburua* were steeped in water and water containing phosphate salts and were germinated for couple of days in a locally constructed malting chamber. Harvested malts were subjected to homogenization and a specific activity of 17,948 U/mgprotein and 24,337 U/mgprotein for *D.exilis* and *D.iburua* respectively at day two (2) of germination produced the highest amount of amylolytic enzymes for both grains as summarized in Figure 1 and 2.

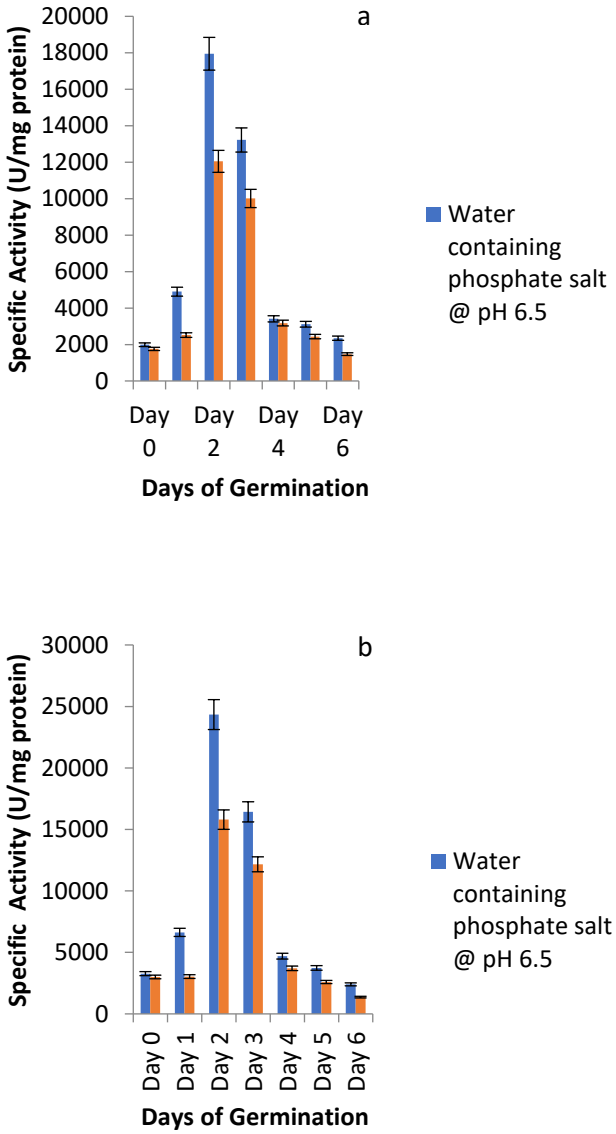

Figure 1: Germination profile of malted grains. (a) Malted *Digitaria exilis* grains (b) Malted *Digitaria iburua* grains

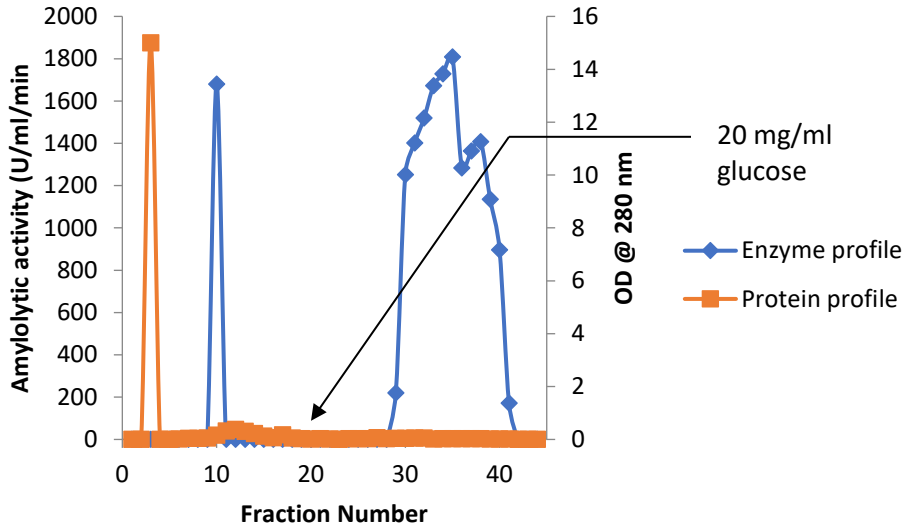

Figure 2: Elution profile of induced amylolytic activity of malted acha layered on Con A Sepharose

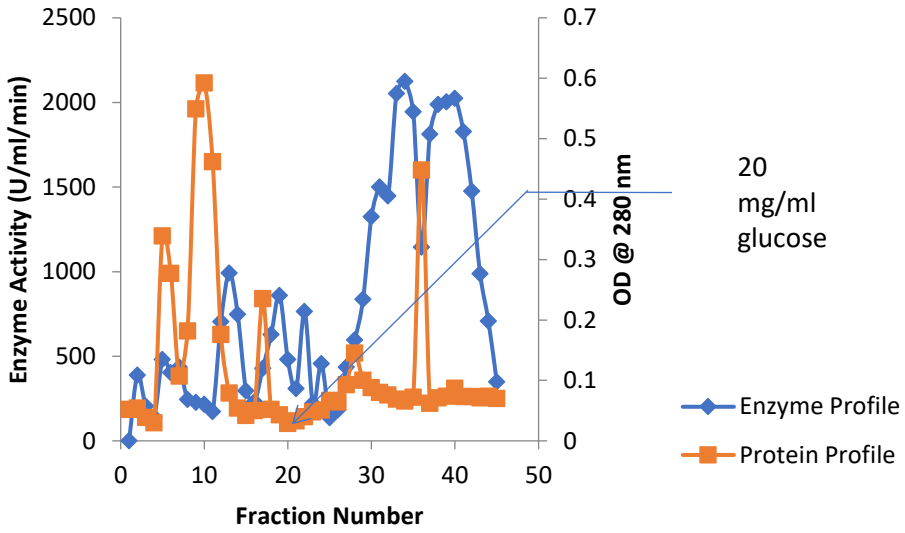

Figure 3: Elution profile of induced amylolytic activity of malted iburu layered on Con A Sepharose

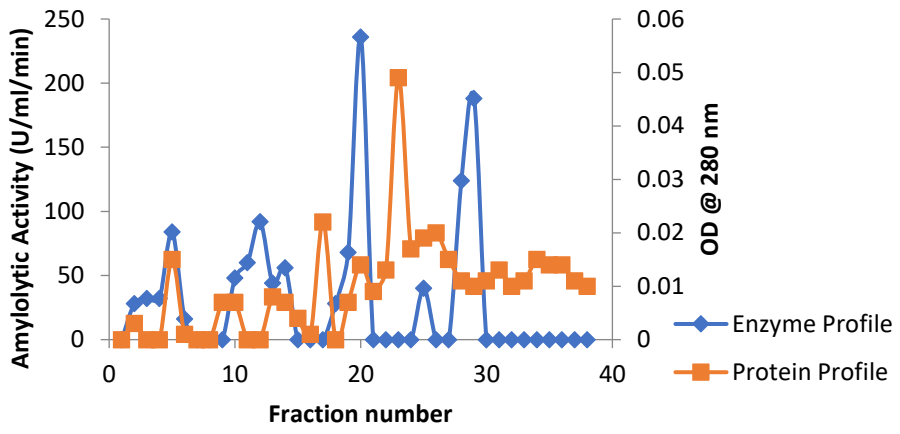

Figure 4: Gel filtration elution profile of post affinity acha fraction layered on Bio-gel P100

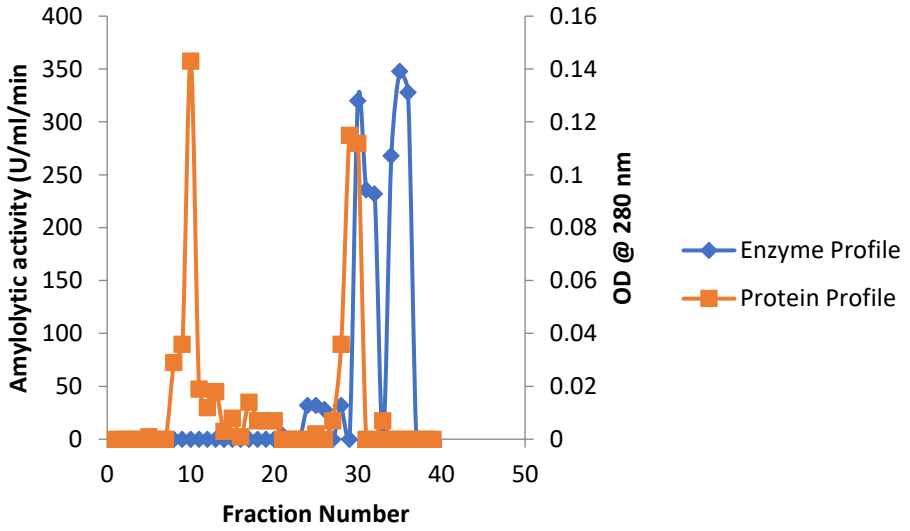

Figure 5: Gel filtration elution profile of post affinity iburu fraction layered on Bio gel P100

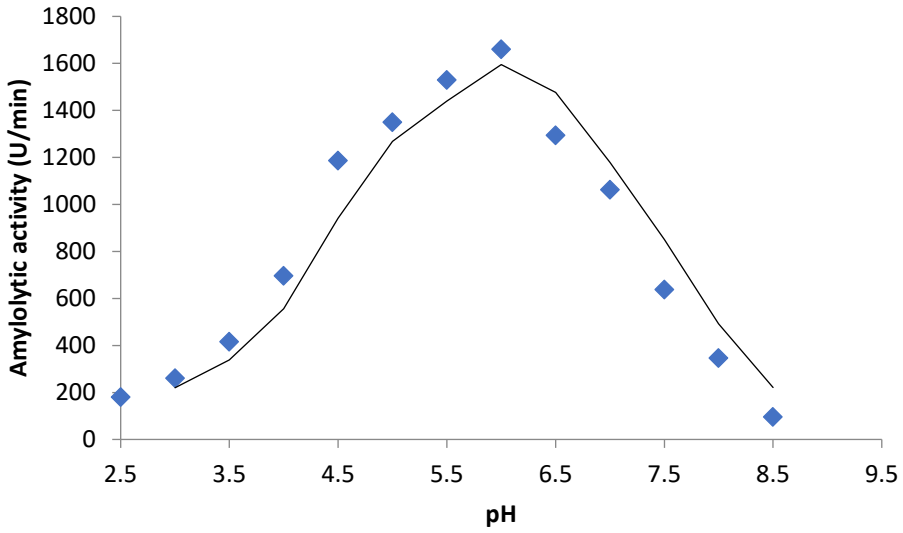

Figure 6: Effect of pH on the activity of acha-induced amylolytic enzyme

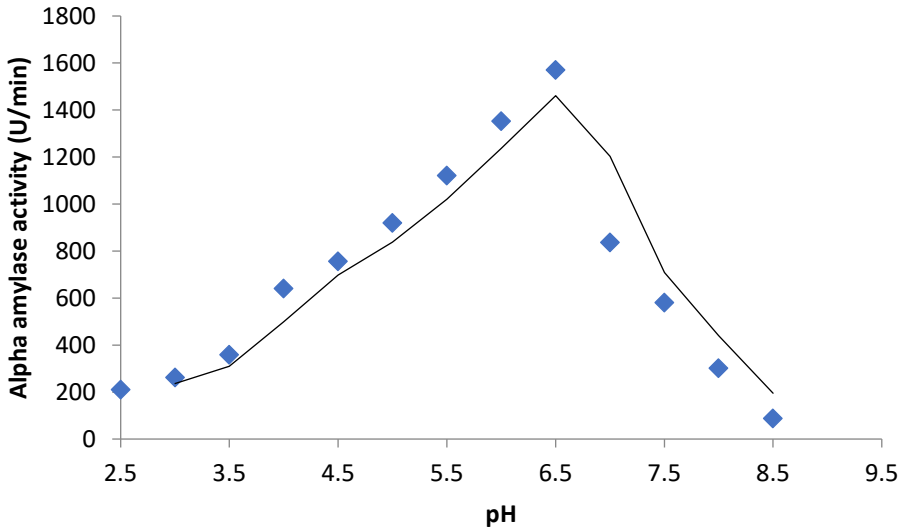

Figure 7: Effect of pH on the activity of iburu-induced amylolytic activity

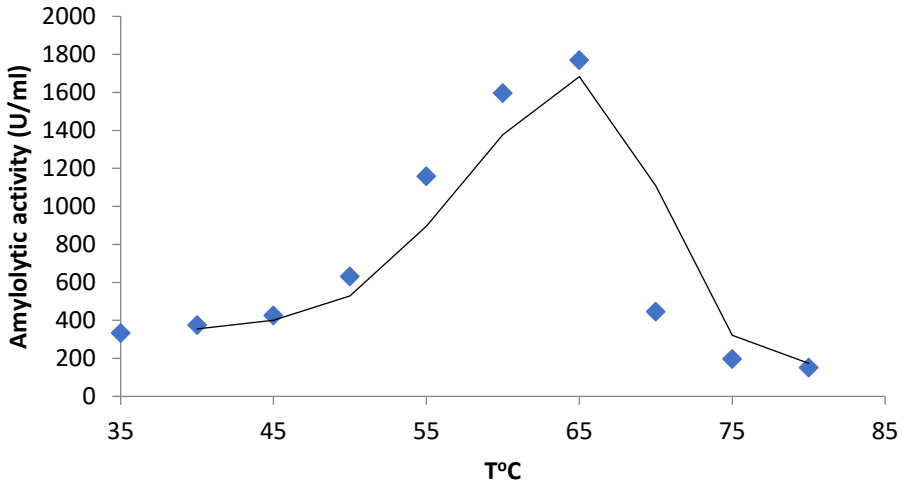

Figure 8: Effect of temperature on the activity of acha-induced amylolytic enzyme

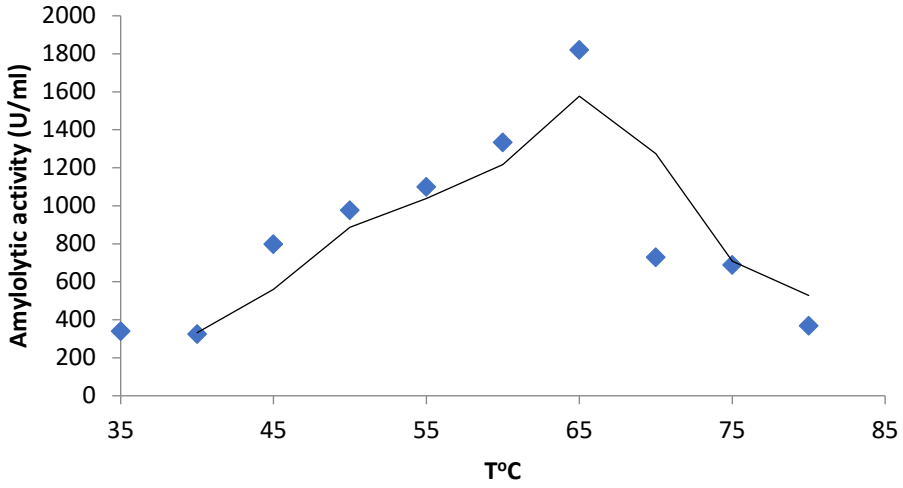

Figure 9: Effect of temperature on the activity of iburu-induced amylolytic enzyme

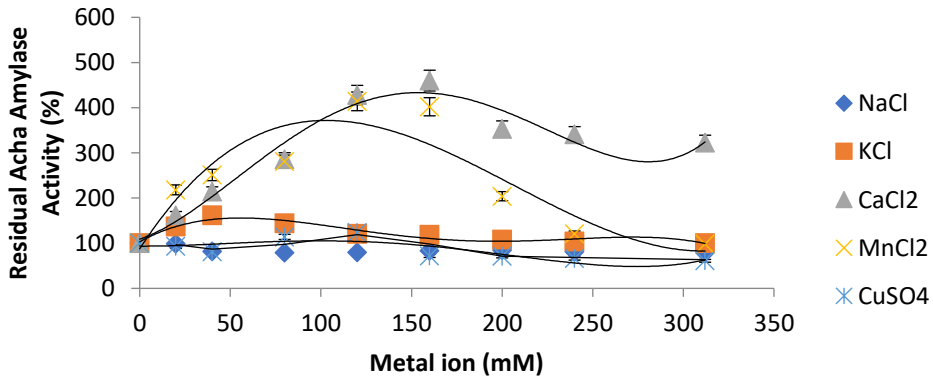

Figure 10: Effect of metal ions on the activity of acha-induced amylolytic enzyme

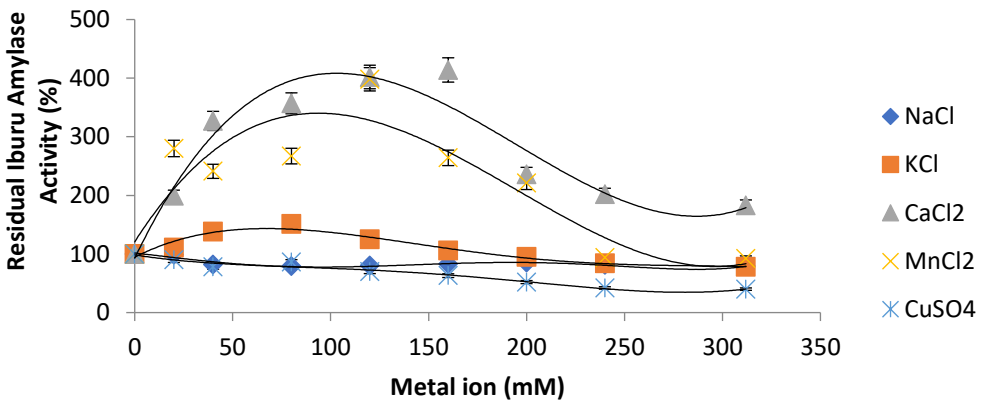

Figure 11: Effect of metal ions on the activity of iuru-induced amylolytic enzyme

4. Discussion

This study optimized germinating conditions for the induction of amylolytic enzymes in *D. exilis* and *D. iburua* grains. High malting loss has previously been reported with these grains, which was experienced in the preliminary experiments in this work. High malting loss in *D. exilis* and *D. iburua* grains was prevented by steeping grains in large volume of water (1:20 (w/v) of water). After 24 h *D. exilis* and *D. iburua* grains became swollen this could be as a result of higher water absorption capacity of these grains, probably because of their thin seed coat. As such, *D. exilis* and *D. iburua* require high moisture content for germination. To ensure uniform germination grains were sprinkled at 12 h interval contrary to the 6 h usually employed for other grains such as sorghum. However, studies have shown that the rate of water diffusion in grains depends on some factors like steeping duration, water temperature, grain dimension, and protein content of grains and possibly the quantity of available oxygen (Francis, 2013).

Maximal enzyme activity was obtained in 2 days which is lower than the number of days required to achieve the same result in other grains (Adewale *et al*., 2006; Adefila *et al*., 2012). The enzyme was extracted and the resulting supernatants were assayed for amylolytic enzyme activity. The amylolytic enzyme induced is a function of the days of germination as the highest amylase activity was obtained on second day (48 h) of germination with 17498 U/mg protein and 24337 U/mg protein for *D. exilis* and *D. iburua* respectively (Figure 1 a and b) for grains steeped in water containing phosphate salt. About 5000 U/mg protein was the difference in the amylolytic enzyme activity induced under the same condition but with only water as the medium of steeping. Water is traditionally the steeping medium in most industries. The observed reduction in the amylolytic enzymes activity after 48 h is an indication that induced enzymes have possibly been degraded to produce other biomolecules required by the growing plant.

This study therefore established that more amylolytic enzymes were induced in *D. exilis* and *D. iburua* grains within a very short germination when compared with other grains such as sorghum and millet which require 3-5 days for maximal amylolytic activity (Egwim and Oloyede, 2006). This implies that *D. exilis* and *D. iburua* grains would generate far larger quantity of amylolytic enzymes which will invariably increase the economic value of these underutilized African grains.

Optimum pH of 6.5 was obtained for amylases from *D. exilis* while pH 6.0 was obtained for *D. iburua* malts. These results are similar to that obtained by Valaparia (2018). Similarly, acidic pH range of 4.5-6.5 was obtained for amylases from finger millet(Nirmala and Muralikrishna, 2019) as well as shoots and cotyledons of peas seedlings (*Pisum Sativum* L) (Beers and Duke,

1990; Norman *et al*. 2006). Mohamed *et al*. (2009) equally reported pH from 5.0 to 7.0 for α-amylase from wheat.

The influence of temperature on amylolytic activity from *D. exilis* and *D. iburua* were measured at different temperature ranging from 35-85°C at their respective established optimum pH. The results revealed that amylase activity increased with increase in temperature and highest activity was attained at 65°C for amylases from *D. exilis and D. iburua*. Above these optimum temperatures, a reduction in amylases activity implies enzymatic denaturation process. These results correspond with that reported by Mawalub *et al*. (2010). They found optimum temperature for amylases activity isolated from sorghum malt to be 70°C and 50°C respectively. The optimum temperature obtained in this study is equally consistent with that of Kumar *et al*. (2015) who got an optimum temperature of 50°C for amylase from malted jowar (*Sorghum bicolor*).

Cereal amylases are known to be metal ion dependent enzymes containing at least one Ca^{2+} per molecule (Janecek and Belex, 2012). The effect of metal ions on amylases from *D. exilis* and *D. iburua* were measured in the presence of various metal ions at concentration between 0-312 mM. Activation of these amylases was stimulated in the presence of Ca^{2+} and Mn^{2+}ions (Figure 10 & 11). Enhancement of amylase activity by Ca^{2+} ion is based on its ability to interact with positively charged amino acid residues such as aspartic and glutamic acid which resulted in stabilization as well as maintenance of enzyme conformation. Furthermore, calcium is known to have a role in substrate binding (Sprinz, 2019). This was established in this study as $CaCl_2$ gave about 4 fold activation of purified amylases from malts of *D. exilis and D. iburua*.

This study therefore concluded that about 7-fold increased amylolytic enzymes were induced when malts were produced from *D. exilis* grains steeped in water containing phosphate salts at pH 6.0 and temperature of 20°C and pH 6.5 at 22.5°C for malts of *D. iburua* when compared to water. The visual assessment of the germinated grains did not correlate well with the amount of amylolytic enzymes induced, as some malts that visually appeared to be uniformly germinated tend to have lower amylolytic enzymes.

Amylolytic enzymes are important in many industrial processes and the search for amylase from new sources is a continuous process. This study has thus demonstrated conclusively that malts of *D. exilis and D. iburua* contain amylases which could be suitable for industrial use because of their physicochemical properties. They could be useful as additives in detergent making industries and also in some breweries in the production of wort.

5.0 Conclusion

D. exilis and D. iburua are good sources of amylases. Amylolytic enzymes induced from these grains have similar properties and their thermal stabilities could be enhanced through immobilization and as such could be alternative sources to those currently utilized in most starch-based industries.

Further studies however could be done on how the stability of amylases from *D. exilis* and *D. iburua* could be further enhanced by chemical modification and protein engineering as this could increase the economic value of these underutilized grains.

References

Adefila, O.A., Bakare, M.K. and Adewale, I.O. (2012). Characterization of α-amylase from sorghum (*Sorghum bicolor*) obtained under optimized conditions. *Journal of the Institute of Brewing*, 118(1): 63-69.

Adewale, I.O., Agumanu, E.N and Otith-Okronkwo, F.I. (2006). Comparative studies on α- amylase from malted maize (*Zea mays*), millet (*Eleusine coracana*) and Sorghum (*Sorghum bicolor*). *Carbohydrate Polymers*, 66: 71-74.

Aiyer, P.V. (2005). Amylases and their applications. *African Journal of Biotechnology*, 4(13): 1525-1529.

Bernfeld, P. (1951). Enzymes of starch degradation and synthesis. In: *Advanced*

*Enzymo*logy. *Interscience Publications Inc*, New York. 12(2): 379-428.

Bradford, M.M. (1976). Rapid and sensitive method for the quantification of microorganism quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*. 72:248-254.

Dehkordi-Mobini, M. and Javan, F.A. (2012). Application of alpha-amylase in biotechnology. *Journal of Biology and Today's World*, 1: 39-50.

Drauz, K., Gröger, H. and May, O. (2010). *Enzyme catalysis in organic synthesis: a comprehensive Handbook, John* Wiley and Sons, Souza, P. M. D. (2012). Application of microbial α-amylase in industry-A review, *Brazilian Journal of Microbiology*, 41: (4):850-861.

Egwim, E.C. and Oloyede, O.B. (2006). Comparison of α-amylase activity in some sprouting Nigerian cereals. *Biokemistri*, 18(1): 15-20.

El-Fallal, A., Dobrara, M.A., El-Sayed, A. and Omar, N. (2012). Starch and microbial α-amylases: from concept to biotechnological applications. *In Technology Publisher*, Croatia. pp. 459-488.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K. and Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective, *Process Biochemistry, 38* (11): 1599-1616.

Hashemi, M., Shojaosadati, S.A., Razavi, S.H. and Mousav, S.M. (2014). Different catalytic behaviour of α-amylase in response to the nitrogen substance used in the production phase. *Journal of Indian. Engineering Chemistry*, 21: 772-778.

Janeček, Š. and Belex, S. (2012). α-amylase and their approaches leading to their enhanced stability. *Federation of European Biochemistry Sciences Letters*. (FEBS). 304: 1-3.

Kumar, R.S., Singh, S.A. and Rao, A.G (2015). Thermal stability α-amylase from malted jowar (*Sorghum bicolor*). *Journal Agricultural Science*. 53 (17): 6883-6888.

Nirmala, M. and Muralikrishna, G. (2019). Three amylases from malted finger millet Ragi, *Eleusine coracana*, elndaf-15). Purification and partial characterization. *Phytochemistry*.62: 21-30.

Valaparia, V.K. (2018). Purification and properties of a thermostable α-amylase by *Acremonium sporosulcatum*. *International Journal of Biotechnology. Biochemistry.* 6: 25-34.

Santana, A.L. and Meireles, M.A.A. (2014). New starches are the trend for industry applications: a review. *Food and Public Health*, 4(5): 229-241.

Saxena, R.K., Malhotra, B. and Batra, A. (2004). Commercial Importance of Some Fungal Enzymes. In: Handbook of fungal biotechnology. Arora, D.K. (ed) Marcel Dekker, New York, USA, pp. 287-298.

Shah, I.J., Gami, P.N., Shukla, R.M. and Acharya, D.K. (2014). Optimization for α amylase production by *Aspergillus oryzae* using submerge fermentation technology. Basic *Research Journal of Microbiology*, 1(4): 1-10.

Sivaramakrishna, S., Gangadharand, D. and Nampoothiri, K.M. (2006). α-amylase from microbial sources. *Food Techno Biotechnology* 44: 173-184.