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Some properties of partially purified alpha amylase from Rice (*Oryza sativa*)

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Abstract
The present study was aimed at determining some properties of partially purified alpha amylase from rice (*Oryza sativa*). Different cultivars of rice were tested for alpha amylase production. The crude α-amylase was obtained by homogenization of day one germinated rice (cultivar 'Election') in sodium acetate buffer pH 4.0. After centrifugation, the crude enzyme was subjected to 20% (w/v) ammonia sulphate precipitation and dialysis, and its biochemical and kinetic properties were analyzed. After dialysis, the specific activity 1715.875 U/mg with purification fold of 3 and 60% recovery was obtained for the enzyme. The K_m and V_{max} were found to be 9 mg/ml and 178. µmol/min for rice starch, 5.150 mg/ml and 50.000 µmol/min for cassava starch, and 14.667 mg/ml and 111.111 µmol/min for maize starch, respectively. The specificity constant was found to be 13.888 min^{-1}, 9.709 min^{-1} and 7.576 min^{-1} for rice, cassava and maize respectively. This shows that α-amylase has greater affinity for rice starch than the other starches used. The optimum pH and temperature were found to be 7.5 and 50ºC respectively. The presence of metal ion such as Ca^{2+}, Co^{2+}, Fe^{3+}, and Zn^{2+} activated α-amylase activity while Pb^{2+} and Mn^{2+} inhibited α-amylase activity. The results obtained from the present study indicated that *Oryza sativa*, is a good source of α-amylase for food industry, hence possess the properties for biotechnological applications.

Keywords: Enzymes, fermentation, catalysis, biotechnology, starch, bioethanol

1. Introduction
Medicinal plants have greatly gained importance in the management and treatment human diseases, globally [1]. It constitutes the main source of primary healthcare in most rural populations, especially in many African countries, due to its affordability and availability. However, as the usage of these medicinal plants becomes more widespread around the world, there are questions and misgivings about the quality, efficacy, and safety of the products utilized in the health sector [2, 3, 4]. These concerns have hampered the sensible use and validation of bio-products derived from these herbs for the treatment and management of disorders [5].

The validation and quality assurance of medicinal plant products has been hampered by a lack of or incorrect plant identification. The correct identification of the starting material is a must for assuring the reproducible quality of herbal medicine, which is important for its safety and efficacy [6, 7]. To assure appropriate identification of medicinal plants, chemical testing, chromatographic procedures, and DNA-based technologies are now being used to enhance traditional botanical identification. These technologies provide unique chemical and DNA fingerprints for the plant [5]. This study is aimed to ensure the appropriate identification of one of the medicinal plants - *Nauclea latifolia* - that has been employed in traditional medicine for the treatment of diabetes [8], by chemo-profiling its contents with GC-MS.

Nauclea latifolia (Rubiaceae), also known as "Bishop's head" in Akwa Ibom and "Mbom-mbog" in Cross River, both in Nigeria, as well as "Ubuluilu" in the Eastern half, "Agbaseagbase" in Yoruba, "Tabashiya" in most parts of the north, and "molsa" in Kilba, is an evergreen multi-stem shrub that grows up to 200m height, with flowers joined to the calyces, while the fruits are syncarpous [9]. Infusions and decoctions of the plant's stem bark and leaves have traditionally been used to cure malaria, stomach aches, fever, diarrhea, and nematode infections in humans and animals in West and South Africa.

It is used as a starch, being the main storage carbohydrate of all higher plants, in some cases, accounts for as high as 70% of the undried plant material, occurring in the form of water insoluble granules. This heterogeneous polysaccharide comprises two high molecular weight entities: amylose and amylopectin. These two polymers have different structures and physical properties (Aiyer, 2005) [2]. Amylolytic enzymes, also known as amylases, are responsible for the degradation of starch (Horvathova, *et al*., 2000) [15]. These molecular machines are widely distributed in microbial, plant and animal kingdoms. Originally, amylase was the designation for enzymes that hydrolyzing α-(1-4) glycosidic bonds of amylose, amylopectin, glycogen and their degradation products. Amylases act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved (Aiyer, 2005) [2]. Specific amylases are designated by different Greek letters: α, β, and γ for α-amylase, β-amylase and γ-amylase, respectively (Oyeleke *et al.*, 2011) [22].

Cereal α-amylases are suitable for biotechnological applications in food supplementation, breweries and saccharification of starch (Muralikrishna and Nirmala, 2005; Adewale *et al*., 2006). They are crucial to starch metabolism in both developing and germinating cereals. The synthesis of these enzymes which are highly expressed and exist in multiple forms is usually under the influence of plant growth hormones like gibberellic acid. In animals, amylases digest starch into sugars, which are subsequently utilized for various metabolic activities (Muralikrishna and Nirmala, 2005).

Rice (*Oryza sativa* L.) is the seed of the monocot plant of the genus *Oryza* and of the grass family Poaceae (Oko and Ugwu, 2011) [21]. The genus *Oryza* comprises of 25 species with mainly two cultivated species: *Oryza sativa* L (Asian rice) and *Oryza glaberrima* also known as African rice (Fernando *et al*., 2015) [11]. It is also one of the largest staple foods valued for human food and nutrition across the world.

Rice is a potential source of functional macro and micro components like, gluten, starch, fiber, fatty acids, amino acids, vitamins, phenolic acids, and ferulic esters. Such health promoting constituents of rice can mitigate hypertension, assist in curing cardiovascular diseases, prohibit hormonal imbalance resulting from food, and shield the body from chemical contamination (Zubair *et al*., 2012) [26]. Rice, the most commonly grown species throughout the world today, is grown in all the ecological and dietary zones of Nigeria, with different varieties possessing adaptation traits for each ecology (Sanni *et al*., 2005). *O. sativa* and *O. glabberima* are the two commonly cultivated varieties of rice in Nigeria, with Abakaliki and its environment growing many varieties (Oko and Ugwu, 2011) [21]. Taraba State also grow certain varieties of rice. These varieties (such as Faro 14, Faro 16, Mass, Faro 52, Sipi, Election, Awilo, Canada, and so on) exhibit, on cooking, marked differences in quality (Oko and Ugwu, 2011) [21].

Despite the broad distribution of amylases in microbial, plant and animal sources, past focus has only been on microbial amylases, probably due to their perceived advantages over plant and animal amylases (Gopinath *et al*., 2017) [12]. Due to the high cost of commercially available microbial α-amylases attention has been shifted to studies on cereal malting. Amongst various cereals rice has been suggested to have the highest α-amylase production during malting (Ayernor and Hammond, 2001) [4]. Rice is seemingly more advantageous over other cereals in that it forms a complete set of starch-degrading enzymes: α and β-amylases, debranching enzymes, maltase, and other accessory enzymes needed for the complete degradation of starch. Amongst these enzymes, α-amylase plays a major role in starch degradation (Magneschi and Perata, 2009). This work aimed at characterizing α-amylase purified from locally sourced germinating *Oryza sativa* seeds.

2. Materials and Methods

2.1 Materials

2.1.1 Collection of plant materials

The newly harvested varieties of rice were obtained from Wukari, Taraba State, Nigeria and Abakiliki, Ebonyi State, Nigeria. The seeds have 100% viability. While cassava tuber and maize were obtained from Orba main market, Udenu Local Government, Enugu State, Nigeria.

2.1.2 Reagents

All chemicals used in the study were of analytical grade. 3, 5- dinitrosalicyclic acid (DNS) was obtained from Qualikem, Bovine serum albumin (BSA) and Folin-ciocalteau were obtained from Sigma-Aldrich (USA).

2.2 Methods

2.2.1 Extraction of Rice and Maize Starch

Cereal starches (rice and maize) were extracted using the method described by Agboola *et al*. (1990) [1] with the following modifications. The seeds were milled to fine flour and 600 g of the flours were suspended in 6 L of distilled water for 24 h, after which the suspension was sieved using muslin cloth. The thus extracted starch was allowed to stand for 4 h at room temperature and the supernatant then decanted off, the starch washed with 6 L of distilled water twice, and allowed to stand again for 4 h. The supernatant was then decanted and the resulting wet starch was sun-dried and packaged in an air-tight container which was stored at room temperature for further use.

2.2.2 Extraction of Cassava Starch

Cassava starch was extracted using the method described by Corbishley and Miller (1984) [8] with the following modifications. Freshly harvested cassava tubers were peeled, washed clean and grinded. The ground cassava (2.4 kg) was soaked in 8L of distilled water for 1 h, after which it was sieved (3 times) with muslin cloth. This was allowed to stand for 4 h and the supernatant decanted. The extracted wet starch was sun-dried and packaged in plastic container, which was labeled and kept in a cool, dry place for future use.

2.2.3 Enzyme Extraction

Seed Germination and Enzyme Extraction

The soaked rice grains were germinated and the enzyme was extracted using the method of Warner *et al*. (1991), with the following modifications. Ten grams (10 g) of samples of rice seeds were washed with 1% NaOCl (sodium hypochlorite) solution, rinsed and imbibed in water for 12 h. The steeped grains were allowed to germinate in a cardboard box lined with a sterilized jute sack treated with antibiotics for seven (7) days at a temperature of 25 ^{o}C, distilled water treated with antibiotics were used to water the plants two to three times daily. Ten (10) grams of germinating seeds were taken from the cardboard box on

each day of the germination period and were soaked in 0.02 M sodium acetate buffer (pH 4.0) at 4 ºC for 24 h. The endosperm tissues were homogenized with 60 ml of 0.02 M sodium acetate buffer (pH 4.0) at 4 ºC using pestle and mortar surrounded by ice. The homogenates were allowed to stand for 30 min., sieved using muslin cloth, and the filtrates centrifuge at 4000 rpm for 15 min. The supernatant, in each case, was carefully decanted and used as the crude enzyme for ammonium sulphate precipitation while the pellet was discarded.

2.2.4 Assay of α-amylase Activity by DNSA Method

Alpha amylase activity was determined by the method of Bernifield (1955) [5]. The reaction mixture contained 0.5 ml of the enzyme preparation and 0.5 ml of (1%) starch solution in 20 mM sodium acetate buffer (5.0). The reaction mixture was then incubated at 55ºC for 60 min., after which the reaction was stopped by the addition of 1 ml 3, 5-dintrosalicyclic acid (DNSA) reagent and boiled for 10 min. 1 ml of sodium potassium tartarate was added to stabilize the red colour produced. The mixture was allowed to cool and the glucose released was measured using a UV/VIS spectrophotometer at a wavelength of 540 nm. One unit of α-amylase activity (U) was represented as one micromole of glucose formed per minute under assay conditions.

2.2.5 Protein Determination using Lowry Method

Protein content of the enzyme was determined by the method of Lowry *et al*. (1951) [18], using Bovine Serum Albumin as standard. For protein standard curve, the reaction mixtures contained 0.0-1.0 ml (at interval of 0.1 units) of protein stock solution (2 mg/ml BSA) in test tubes in duplicates. The volume was made up to 1ml with distilled water and in the blank tube. For the test mixture, 0.5 ml of buffer was added to 0.5 ml of the enzyme. The component reagent for protein determination (solution A-E) was prepared according to standard methods. Solution E (5 ml) was added to each tube and allowed to stand at room temperature for 10 min. To each test tube, 0.5 ml of solution C (diluted Folin-Ciocalteeu Phenol reagent) was also added with vigorous shaking. It was then allowed to stand for 30 min, and the absorbance was read at a wavelength of 750 nm using UV spectrophotometer. Absorbance values were converted to protein concentrations by extrapolation from protein standard curve.

2.2.6 Production of Alpha Amylase

After seven (7) days of pilot study, the day of highest α-amylase activity was selected for mass production as well as the cultivar of rice seeds with highest amylase activity. Two hundred and fifty grams (250g) of the seeds were used to produce 1000 ml of the crude α-amylase as described above.

2.2.7 Partial Purification of Alpha Amylase

2.2.7.1 Determination of Percentage Ammonium Sulphate Saturation Suitable for Alpha Amylase Precipitation

To nine test-tubes, 5 ml of the crude enzyme was added to each and made to 20-100% saturation with solid ammonium sulphate with gentle stirring. The mixture was then allowed to stand for 30 h at 4ºC. The supernatant was carefully decanted off, which were centrifuged at 3500 rpm for 30 min. The pellets (precipitates) were re-dissolved in equal volumes of buffer. The α-amylase activity in both precipitates and the supernatant was determined, 20% ammonium sulphate saturation was found suitable for the precipitation of α-amylase activity.

2.2.7.2 Ammonium Sulphate Precipitation of Alpha Amylase

A volume, 800 ml, of crude enzyme preparation was precipitated with 20% ammonium sulphate saturation by adding 84.8 g of the salt. The precipitation was carried out using standard methods where one hundred and sixteen (116) ml of enzyme was recovered, and the α-amylase activity and protein concentration determined.

2.2.7.3 Dialysis

The ammonium sulphate precipitates were dialyzed for 12 h against 20 mM sodium acetate buffer pH 5.0. The dialyzed enzyme was also assayed for α-amylase activity and protein content determined. After dialysis, the partially purified enzyme was frozen at -10ºC for further usage.

2.2.8 Characterization of Partially Purified Alpha Amylase

2.2.8.1 Effect of Enzyme Concentrations on Alpha Amylase Activity

This experiment was performed to investigate the effect of α-amylase concentrations on the enzyme activity. 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml and 0.5 ml of the partially purified α-amylase enzyme were used.

2.2.8.2 Effect of pH on Alpha Amylase Activity

The optimum pH for α-amylase activity was determined using 20 mM sodium acetate buffer (pH 3.5 - 5.5), 20 mM phosphate buffer (pH 6.0 - 7.5) and 20 mM Tris-HCl buffer (pH 8.0 - 10.0) at intervals of 0.5. Each buffer with its respective pH was used in the assay. A volume (0.5 ml) of 1% starch solution was mixed with 0.5 ml of enzyme solution and the enzyme activity was determined.

2.2.8.3 Effect of Temperature on Alpha Amylase Activity

The optimum temperature of α-amylase activity was determined by assaying for amylase activity at temperatures of 30-90ºC at intervals of 10ºC.

2.2.8.4 Effect of Substrates Concentration on Alpha Amylase Activity

The effect of substrate concentration on the α-amylase activity was determined by incubating 0.5 ml of enzyme with 5, 10, 20, 30, 40, 50, and 60 mg/ml of starch solution using optimum conditions of pH and temperature. The maximum velocity (V_{max}) and Michaelis constant (K_m) values of the enzyme were determined using the Lineweaver-Burk plot of initial velocity data.

2.2.8.5 Effect of Metal Ions on Alpha Amylase Activity

Metal salts ($CaCl_2$, $CoCl_2$, $MnCl_2$, $FeCl_3$, $ZnCl_2$ and $PbCl_2$) of varied concentrations (20, 30, 40 and 50 mM) were prepared in 20 mM sodium acetate buffer pH 5.0. The reaction mixtures contained 0.5 ml of enzyme solution, 0.5 ml of 1% starch solution and 1ml of the metal ion solutions (Ca^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+} and Pb^{2+}). The mixtures were incubated for 1h at optimum conditions of pH and temperature. The control contained 1 ml of both enzyme and starch solution but no metal ion. The reactions were carried out in duplicates and α-amylase activity was determined.

3. Results and Discussion

3.1 Experimental Studies

Figure 1 showed that the cultivars of rice 'Election' had the highest enzyme activity on day one (1) during a pilot study of 7 days. This result is in contradicts the findings of Asante *et al*. (2013) [3] where day 8 was the optimum period for α-amylase production from germinating seedlings of malted rice. The variation could be due to genetic difference in the varieties of rice. Increased α-amylase production during germination is brought about by activation of aleurone layers resulting in α-amylase production for the hydrolysis of the reserved starch in the endosperm. A decline in α-amylase production could be attributed to the downregulation of α-amylase production as most of the starch reserved in the endosperm had already been hydrolyzed to reducing sugars. Furthermore, the gradual rise in α-amylase production after day five (5) might be as a result of the synthesis of sucrose to keep pace with its great demand for tissue (shoots and roots) development. Figure 1 also showed protein concentration during day one, the protein observed may be due to protein synthesis during the growth.

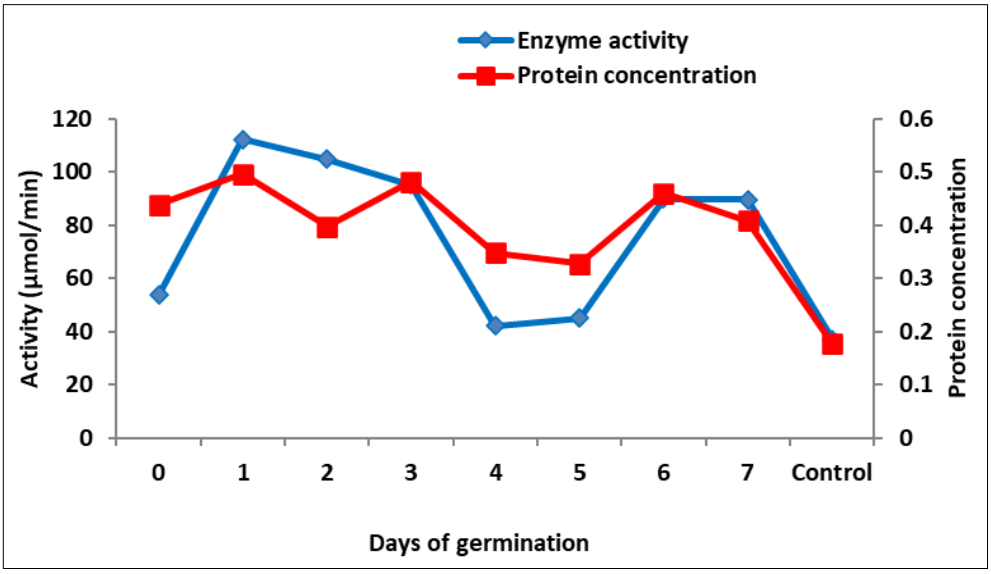

Fig 1: Seven (7) days germination of rice seeds for α-amylase production. Alpha amylase activity and protein concentrations were monitored for seven days using DNS and Lowry method, respectively.

3.2 Purification Studies

The partial purification of alpha amylase from *Oryza sativa* is summarized in table 1 below. Ammonium sulphate saturation (20%) was found suitable for precipitation of α-amylase from *Oryza sativa*. The result agrees with the report of El-Safey and Ammar (2004) [9] who precipitated α-amylase from *Aspergillus flavus var. columnaris* at 20% ammonium sulphate saturation. This low concentration of $(NH_4)_2SO_4$ indicates that the enzyme is highly hydrophobic as opposed to hydrophilic proteins which precipitate at higher salt concentrations. After dialysis, alpha amylase was found to have been purified 3.214 times; with the enzyme activity being 41.181µmol/min and the specific activity being 1715.875(U/mg). The decrease in enzyme activity (as observed in table 1) after dialysis might be due to the loss of some cofactors in the enzyme that enhances its activity. Intercaltion of ammonium sulphate particles in the active site of enzymes may also interfere with enzyme-substrate interactions. The increase in specific activity may be due to the fact that the purification procedure was successful, with a greater specific activity of the desired enzyme as compared with the crude (Okwuenu *et al*., 2017).

Table 1: Purification Table for partially purified Alpha Amylase

Enzyme	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U)	Total Activity	Specific Activity (U/mg)	Purifition Fold	Percentage Yield
Crude	1000	0.497	497	114.553	114553	230.489	1	100
$(NH_4)_2SO_4$	116	0.100	11.600	53.389	6193.124	533.890	2.316	5.406
Dialyzed Amylase	90	0.024	2.160	41.181	3706.290	1715.875	3.214	59.845

3.3 Characterization of Alpha amylase

3.3.1 Determination of Optimum pH

Figure 2 shows the variation of activity of partially purified α-amylase produced by germinating rice seeds with pH ranging from 5.0 to 7.5. The activity of the enzyme increased with an increase in pH until an optimum at pH 7.5 when using rice starch as a substrate. The results agree with the findings of Uchino and Katano (1981) [25]. Maryada and Anoop (2011) [19] reported α amylases from *Dolichos biflorus* with maximum activity between pH 5.5 to 7 and pH optimum of 6.1, whereas Chessa *et al*. (1999) [7] reported α-amylase with optimum pH 7.5. The amylase from this study had higher activity in slightly alkaline medium. Enzymes are proteins consisting of both acidic and basic groups located on their active site surfaces. Change in pH affects the ionic state of the substrates, the amino acid residue at the active site, as well as those responsible in maintaining the active site conformation. These changes affect the enzyme activity by changing the ionization state of the amino acid responsible for substrate binding and catalysis.

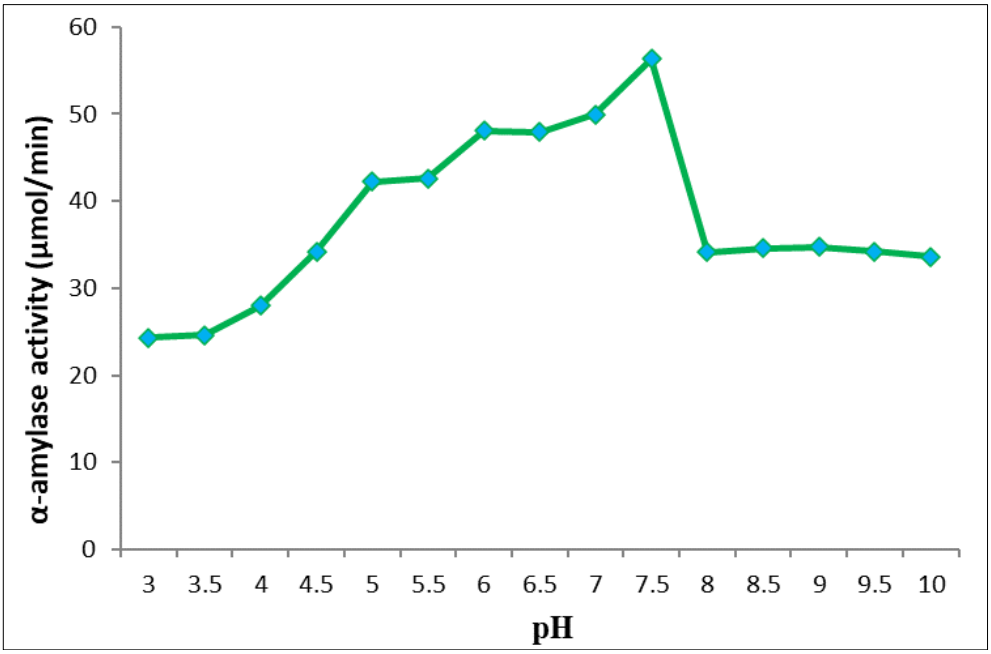

Fig 2: Effect of pH on α-Amylase Activity

3.3.2 Determination of Optimum Temperature

Figure 3 shows the effect of temperature on alpha amylase activity Increase in temperature from 30-50°C increased the enzyme activity until it gradually declined with further rise in temperature, indicating loss in the active conformation of the enzyme due to thermal denaturation. This value is closely related to that of wheat α-amylase with optimum temperature of 50°C as reported by Mohamed *et al*. (2009) [20]. Chakraborty *et al*. (2000) [6] also reported 50 °C as the maximum activity of a thermostable α-amylase, and Sundarram and Thirupathihalli (2014) [24] reported 50°C as the observed optimal temperature when values were varied within a range of 30-90°C for production of α-amylase by *Aspergillus oryzae*.

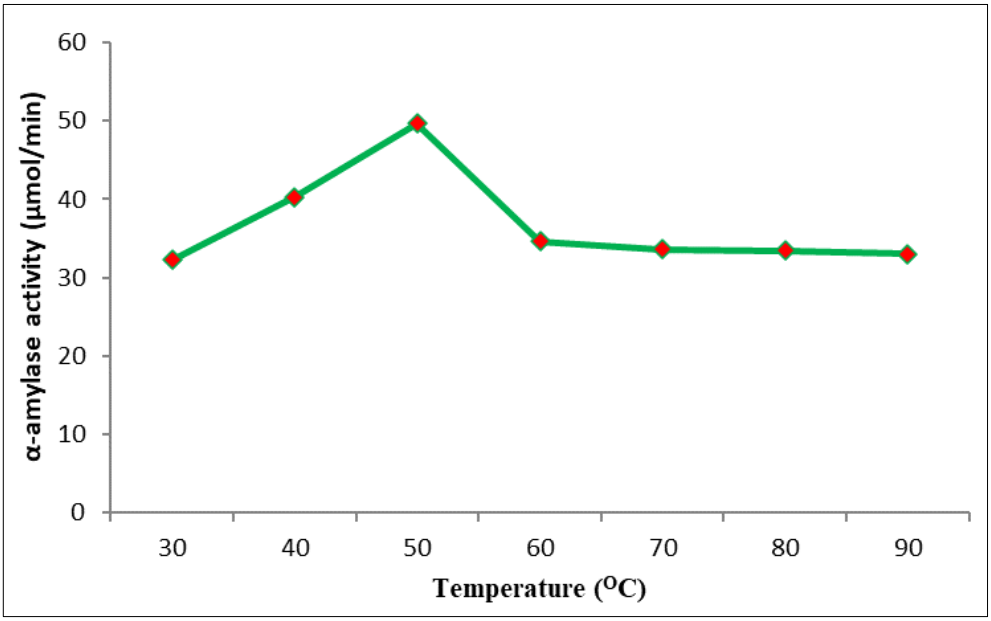

Fig 3: Effect of Temperature on α-Amylase Activity

3.3.3 Effect of Substrate Concentrations on Alpha Amylase Actitivy

Figure 4a shows the effect of substrate concentrations on alpha amylase activity. The kinetic parameters such as Michealis-Menten constant (K_m) and maximum velocity (V_{max}) were obtained from Lineweaver-Burk plots of initial velocity data using different concentrations (10 mg/ml – 100 mg/ml) of the different starches (Figure 4b**)**. The K_m and V_{max} were found to be: 16.804mg/ml and 178.571µmol/min; 5.150mg/ml and 50.000 µmol/min; 14.667 mg/ml and 111.111 µmol/min; using rice, cassava and maize starch as substrates, respectively. It was observed that the specificity constant (V_{max}/K_m) of rice starch was higher than the value for the other substrates, meaning that rice starch is the most sensitive and preferred substrates for α-amylase from *Oryza sativa* than other starches.

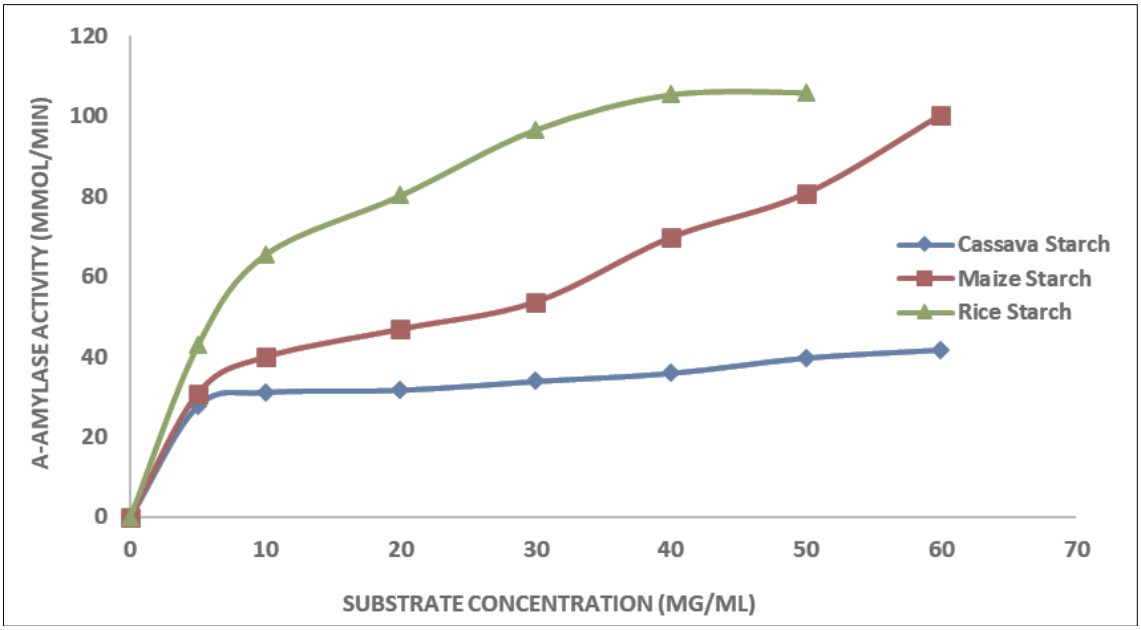

Fig 4a: Effect of Substrate Concentrations on Alpha Amylase (Using Rrice, Cassava, and Maize Starches as Substrates)

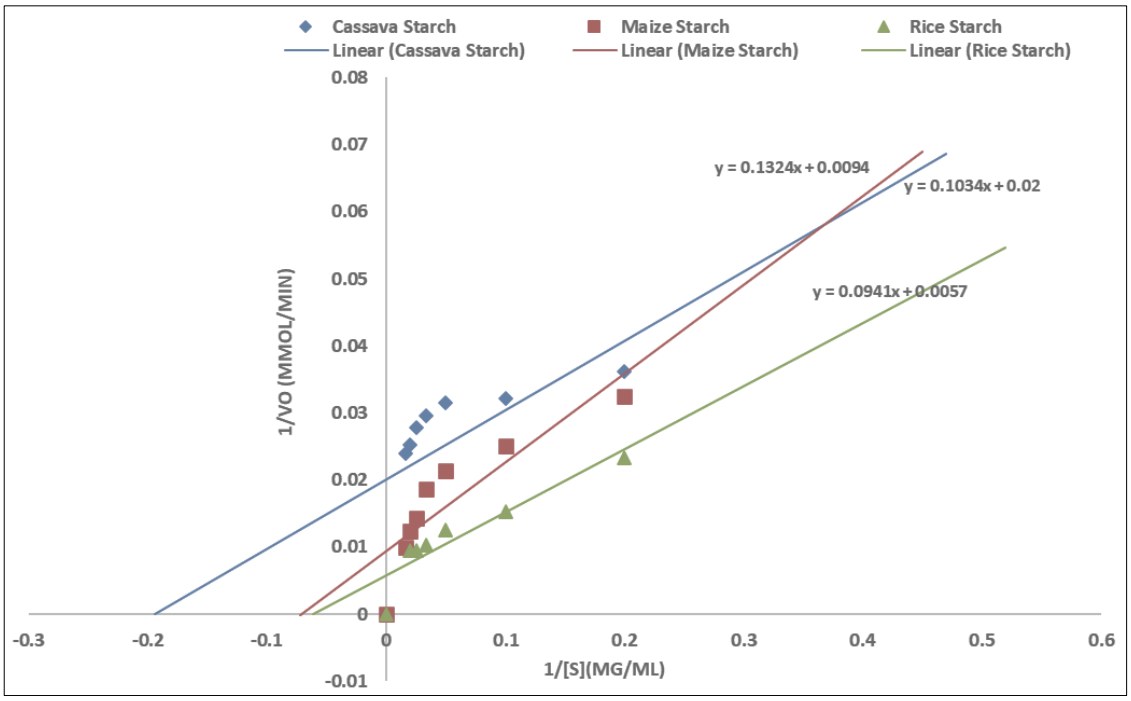

Fig 4b: Lineweaver-Burk Plots for Effect of Substrate Concentration on Alpha Amylase Activity (Using Rice, Cassava and Maize Starchs as Substrates)

3.3.4 Effect of Metal Ions on Alpha amylase Activity

Figure 5 showed that the activity of α-amylase was enhanced by Ca^{2+}, Co^{2+}, Fe^{3+}, and Zn^{2+}. The positive effect of these metal ions could be based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acid (Linden *et al*., 2003) [17], which resulted in stabilization, as well as maintenance of enzyme conformation. The affinity of Ca^{2+} to amylase is much stronger than that of other ions (Gupta *et al*., 2003) [14]. This is because amylase is a metallo-enzyme containing at least one activating Ca^{2+}. These results are related to the findings of Sudha (2012) [23]. The stabilizing effect of Ca^{2+} on thermostability of the enzyme might be due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thereby, causing the adoption of a compact structure (Goyal *et al*., 2005) [13], thus, inducing resistance to extreme pH and temperatures (Kareem *et al*., 2014) [16].

Metal ions may stimulate enzyme activity by acting as a binding link between enzyme and substrate, combining with both and so holding the substrates in proper orientation at the active site of the enzyme (Ezugwu *et al*., 2015) [10]. Alpha amylase in the presence of Ca^{2+} ion can be utilized in liquefaction processes. Stimulation of α-amylase from rice seeds by metal ions makes it suitable for utilization in industrial starch bioconversion processes.

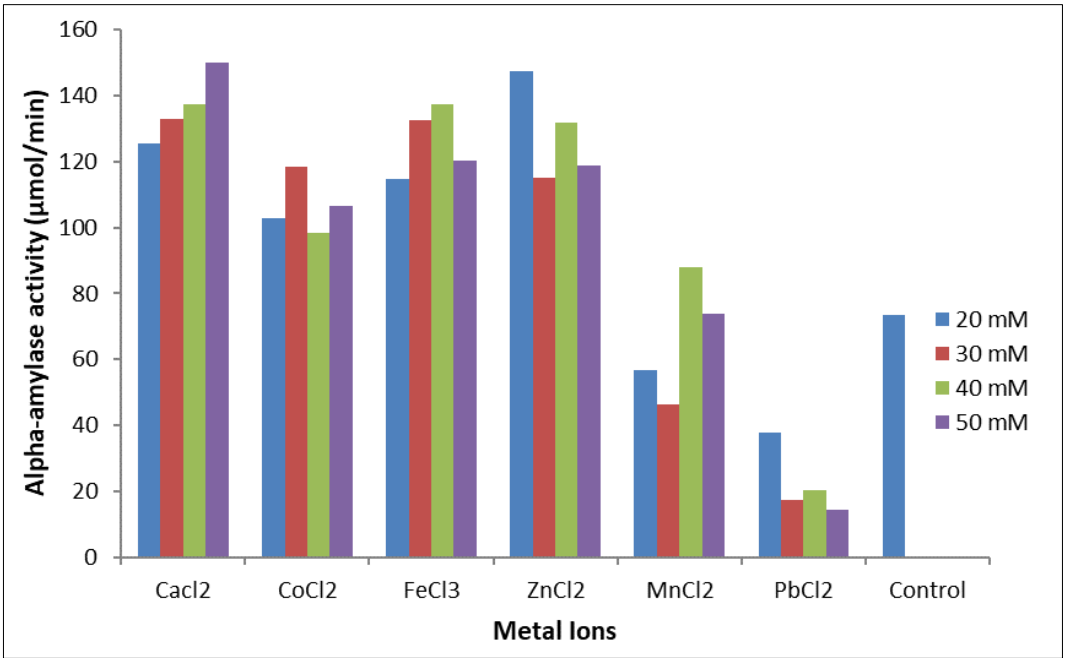

Fig 5: Effect of Different Concentrations of Metal Ions on Alpha-amylase Activity

4. Conclusion

The result of the present study indicates that α-amylase obtained from the variety of *Oryza sativa* ('Election') could be utilized in biotechnological application as well as in starch degradation. This is because the enzyme met the prerequisites needed for industrial application, which includes broad pH optima, high optimum temperature, low K_m value with high affinity towards its natural substrate; the enzyme activity was also enhanced by some metals such as calcium, zinc and cobalt etc. A large number of enzymes are available commercially which are very costly, and due to its increasing demand in industries, rice serves as a potential target for industrial production of α-amylase and a good substrate for the enzyme activity due to its greater affinity with rapid hydrolysis.

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