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Studies on Production and Kinetic Properties of α-amylase from *Rhizopus* sp.

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ABSTRACT

α-amylase producing Rhizopus was isolated from soil collected from Zoological Garden, University of Nigeria Nsukka, Enugu state. Biochemical test and microscopy mounting was used to confirm the strain as Rhizopus. The α-amylase producing ability of Rhizopus was determined using p-NPG as standard substrates. Rhizopus showed positive colouration on the substrates yellow colouration after two days of incubation. Optimization of the physiologic conditions for α-amylase production from Rhizopus was carried out in solid state fermentation system. α-amylase production peaked at pH 6.0. Studies on the effect of incubation time on α-amylase production showed that the highest α-amylase production was obtained on the 5th day of fermentation. Crude proteins were precipitated using 80% saturation of ammonium sulphate salt with specific activity of 171.52 U/mg. Specific activity of 244.1 U/mg was obtained after gel filtration chromatography. α-amylase was purified upto 1.54 folds after the gel filtration with percentage yield of 2.4 %. The specific activity of the enzyme increased from 160.2 to 244.1 U/mg after gel filtration. Characterization of α-amylase gave optima pH and temperature of 5.0 and 60°C respectively. K_m and V_{max} of 4.5 mg/ml and 250 µmol/min were obtained respectively at various starch concentrations. Ca^{2+} and Co^{2+}enhanced α-amylase activity in the presence of Mn and Fe.

Keywords: α-amylase, Rhizopus, p-NPG, optimization, solid state fermentation.

INTRODUCTION

α-Amylase, (1, 4-α-D-glucano hydrolase) is a wide spread enzyme responsible for hydrolysis of α-1-4 glycosidic bonds found in carbohydrates in a random catalytic manner and other alike sugars with the implicated bonds (Swetha *et al*., 2006). As reported by Vander Maarel *et al*. (2002) they belong to the sub family of endo amylases of clan "GH 13" where they perform the internal glycosidic hydrolysis in saccharrides (Mohammed *et al*., 2007). α-Amylases generally are known for the following catalytic features: they must act on an α-1,4 glycosidic linkages and hydrolyse

them to produce an α- anomeric monosaccharide and oligosaccharide or form an α- glucosidic linkage by trans glycosylation; they have four highly conserved regions in their primary structures consisting of catalytic and substrate binding sites (allosteric); they comprise of the enlisted amino acids at the designated positions: Aspartate (Asp 206), Glutamate (Glu 230) and Aspartate (ASP 297) at corresponding positions in their catalytic domain; they possess a $(Beta/alpha)_8$ or Tim (triose isomerase) barrel catalytic domain (Balkan and Ertan, 2005).

α-Amylases represent one of the three largest groups of enzymes implicated in industrial processing and accounts for approximately 25–33% of the world enzyme market, after proteases (Van der maarel *et al*., 2002).

Among the producers of the enzyme, α-Amylase produced from microorganisms are among the most important hydrolytic glycol hydrolyase that have been widely studied (Singh *et al*., 2014). Among the organisms, fungal strains of *Aspergillus, Rhizopus, Candida, Mucor, Penicillum* are reportedly the highest producer of the enzyme known (Nwagu and Okolo, 2011; Vijayaraghavan *et al*., 2011). *Rhizopus* are filamentous Zygomycete fungi and grow on large varieties of growth medium which are organic in nature (Kirk *et al*., 2008).

Rhizopus is employed in processing of various fermented foods and widely employed in starch hydrolysis and liquefaction (Van der Maarel *et al*., 2002). They are used in the production of alcoholic beverages in parts of Asia and Africa since they are good source of α-amylase

Most limitations in the utilization of enzymes industrially could be attributed to the factors of protein stability at some physiologic conditions of application (Nwagu and Okolo, 2011). Starch liquefaction is an important industrial process catalyzed mostly by α-amylase requires high processing temperature condition; however, metabolites given off during the liquefaction-saccharification process lowers the pH of the liquefaction bioreactor.

Optimization of pH and temperature in an α-amylase catalysed fermentation bioreactor is particularly crucial as a result of wide utility of the enzyme. These parameters affect the ionization of amino acyl sides chains within the activity pocket of the enzyme and effective collisions of enzyme with the substrate (Anosike, 2001). Several reports on α-amylase production from filamentous fungi have been reviewed but there are fewer information about the catalytic properties of the enzyme especially from *Rhizopus* sp.

MATERIAL AND METHODS

Materials

All the chemicals, reagents and equipments used in the present study were of analytical grade, in good working condition and are products of Bristol (BDh), Sigma Aldrich, Merck, England Burgoyne India and May and Baker.

Methods

- **Processing of Starch from Cassava**

Starch from cassava was processed using the method of Corbishley and Miller (1984). The sweet cassava species tubers were harvested, peeled and washed. They were further grated, rasped and mixed with water. The mixture was filtered, screened, allowed to settle with washing. The starch was allowed to settle, dried and milled.

- **Isolation of Amylolytic Fungi from soil**

Soil samples containing debries were pooled together from the site of collections into clean plastic cans and were taken to the laboratory for isolation of fungi according to the method described by Ezeonu *et al*. (2013). 20g of soil sample was used for the process from where serial dilutions were carried out from.

- **Microscopic Screening of the Isolates**

Identification of the isolated organisms was carried out by comparative relating of certain morphology such as colour, texture, spores growth pattern and the micrographs to "Atlas of mycology" by Barnett and Hunters (1972).

- **Screening of *Rhizopus* sp. for α-amylase Production**

Identified *Rhizopus* sp. was evaluated for amylolytic potentials in a supplemented dextrose water containing with 2mM p-NPG as described by Swetha *et al*. (2006). The inoculated broth was incubated at 37°C for 48 hrs.

- **Assay of αAmylase Activity**

α-amylase activity was evaluated by assaying for glucohydrolase (GH) activity of the enzyme in presence of 3, 5-dinitrosalicylic acid (DNS) as described by Miller (1959).

- **Total Protein Content Determination**

Total protein content of the enzyme solution was determined using the description of Lowry *et al.* (1951). Standard protein of Bovine Serum Albumin was used in preparation of the curve.

- **α-amylase Production**

Solid state fermentation (SSF) technique was employed during the enzyme production using a 250ml Erlenmeyer flask containing 100ml of sterile cultivation medium optimized for α-amylase production as described by Silva et al. (2005). Production broth consists of the following: 0.3% NH_4SO_4, 0.6% KH_2PO_4, 0.1% $MgS0_4.7H_2O$, 0.01% $FeSO_4$and 1% cassava starch on matrix of rice bran were utilized during the process. The flask was stoppered with aluminum foil and autoclaved at 121^oC for 15minutes.

Production conditions were also carried out for peak production of the enzyme as described by Silva *et al*. (2005).

- **Purification of α-amylase from *Rhizopus* sp**.

  - **Precipitation of the enzyme using ammonium salt**

Precipitation was carried out as described by Allam *et al*. (2016). Precipitation was carried out using solid ammonium sulphate of 20-90% saturation in the profile setup. Resultant precipitates respectively were redissolved in five (5 ml) of 0.1M sodium sodium acetate at pH 5.0. α-amylases activity was assayed simultaneously as described above.

  - **Dialysis of precipitates**

Precipitated proteins in buffer solution were dialyzed against created physiologic gradients (0.01 M)

created across the dialysis bag for a total period of twelve (12 hours) as described by Chen *et al*. (2008).

- **Size exclusion chromatography**

Gel filtration of the dialysate was carried out as described by Allam *et al*. (2016). The column was packed with sephadex G-100 and equilibrated with 0.1M sodium acetate buffer solution (pH 5.0) before loading the enzyme solution. α-amylase activity of each fraction was also assayed as described above while protein was assayed at 280nm.

- **α-amylase characterization**

- **Effect of pH on α-amylase Activity**

Optimum pH for α-amylase activity was determined using the following buffer: 0.1 M sodium acetate of pHs: 3.5 - 5.5; sodium phosphate of pHs: 6.0 - 7.0 and Tris-HCl of pHs: 7.5 - 9.0 as described by Swetha *et al*. (2006). α-amylase activity was assayed as described above.

- **Effect of Temperature on α-amylase Activity**

This was carried out as described by Sighn *et al*. (2013). Optimum temperature was determined by incubating the enzyme with starch solution at 30-70^{o}C using a thermo regulator bath for 30 minutes and at its optimum pH. α-amylase activity was assayed as described previously.

- **Determination α-amylase kinetic constants**

This was carried out as described by Silva *et al*. (2005). Km and Vmax derived from different concentrations of starch solution was determined by incubating the enzyme with 0.5, 1, 2, 3, 4 and 5 % of starch solutions at the optima pH and temperature recorded in the previous studies. Double reciprocal plot was used to determine the V_{Max} and K_M. Activity of α-amylase was determined as described in above.

- **Impact of divalent metals on α-amylase activity**

Impact of divalent metal ions on the enzyme activity was carried out as described by Li *et al*. (2011). Metal salts of: Calcium (Ca), Manganese (Mn), Magnesium (Mg) and Cobalt (Co) of 30- 50 mM respectively were incubated with the enzyme at their respective optimum pH and temperature; activity of the enzyme at each treatment with the metal salts was determined as described above.

RESULT AND DISCUSSION

Percentage yield of starch from the grated cassava is 78%.

Plate 1 shows the pure isolates of the amylolytic fungi isolated from the soil.

Plate 1: Pure filamentous growth of strains of Rhizopus sp. on a potato dextrose agar media.

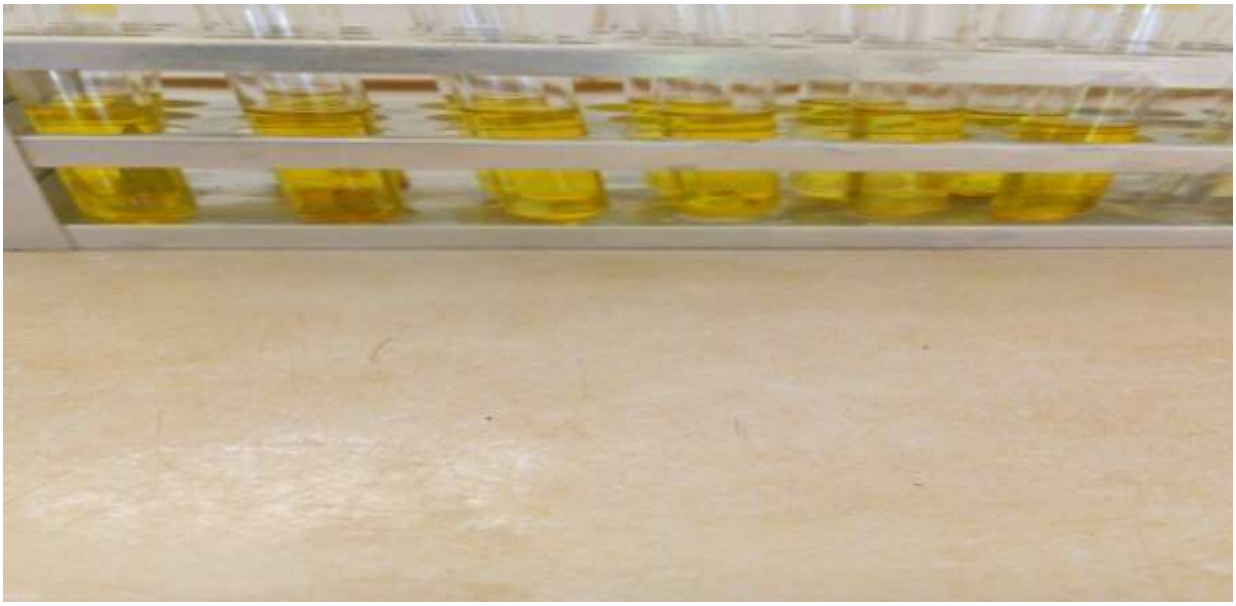

Fig 1: Screening of the fungi isolates in a supplemented dextrose water.

Effect of Incubation Period (days) on α-amylase production

Figures 2 and 3 below show the activity and protein concentrations of the enzyme during the incubation days using starch from cassava as the sole carbon source. Peak α-amylase activity was observed on the 5th day of the 7 days fermentation process. There was also corresponding increase in protein concentration with respect to increased α-amylase activity.

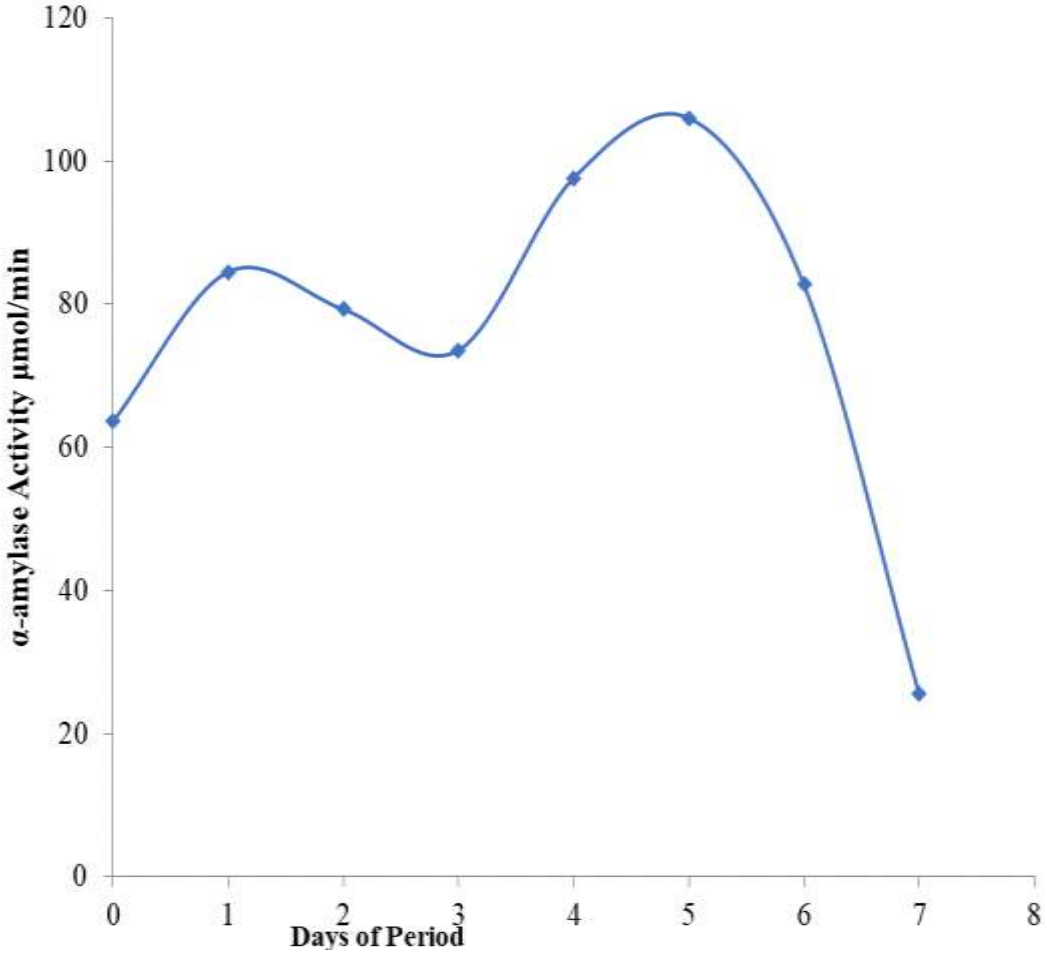

Figure 2: Effect of incubation days α-amylase production from *Rhizopus* Sp. in liquid broth using starch from cassava as the only carbon source.

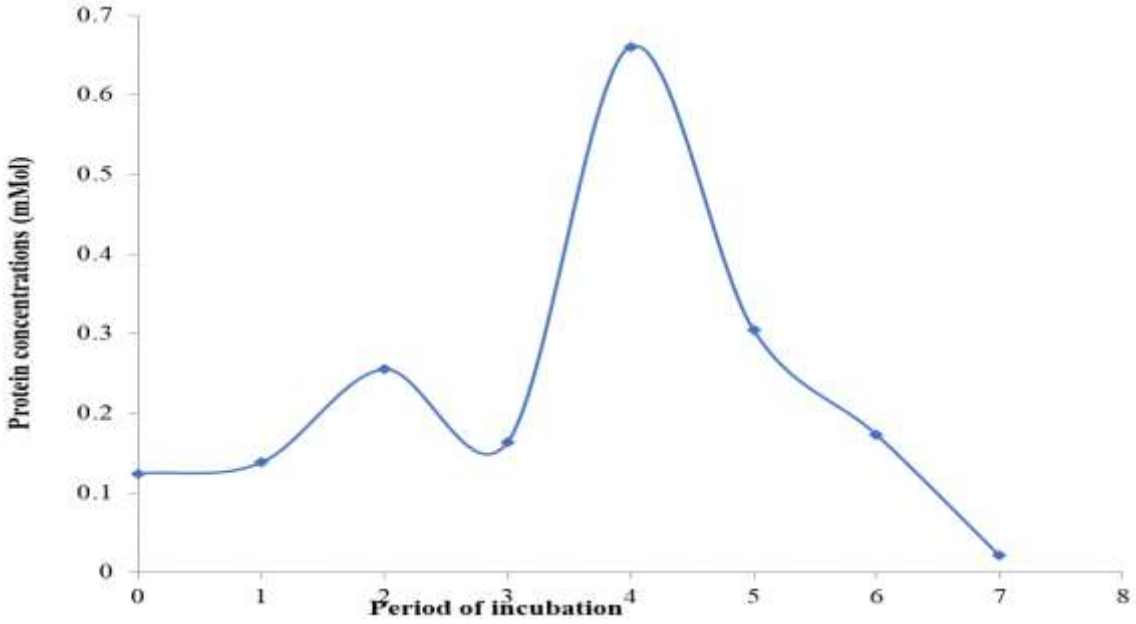

Figure 3: Effect of incubation days on the protein concentration of α-amylase from *Rhizopus* Sp. in liquid broth using starch from cassava as the only carbon source.

Peak production of α-amylase from *Rhizopus* sp. was seen at pH 6.0 which decreases as the pH increases.

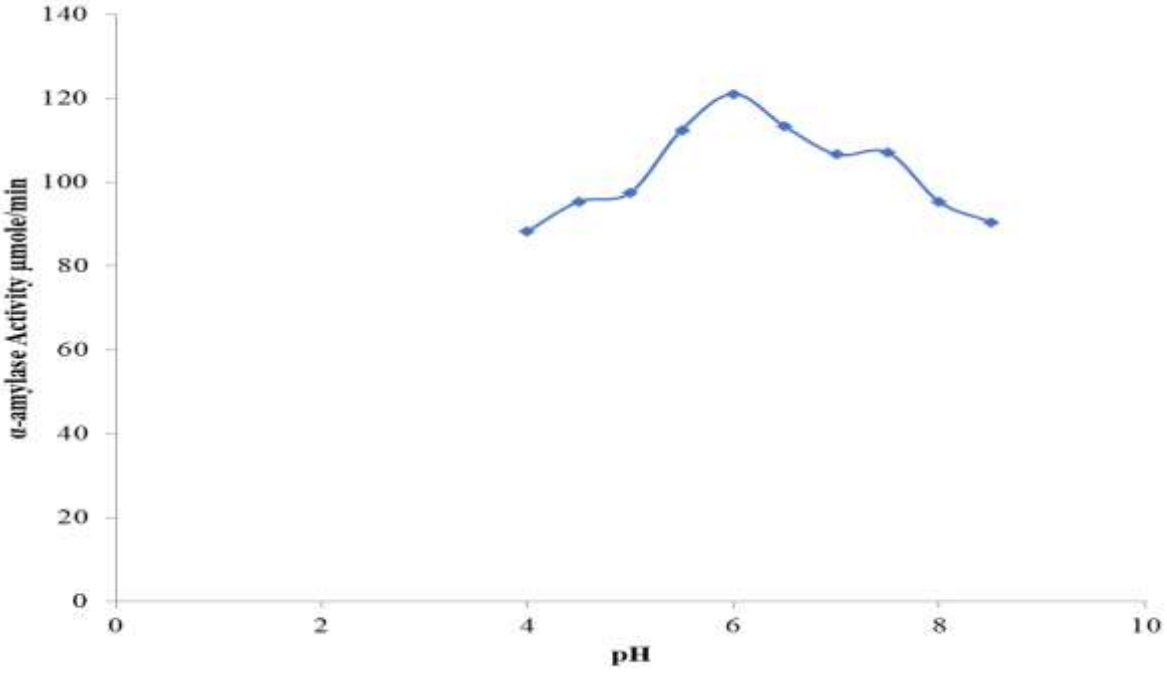

Figure 4: Impact of pH on production of α-amylase produced from *Rhizopus* Sp. in liquid broth using starch from cassava as the only carbon source.

Purification of α-amylase from *Rhizopus* Sp.

Ammonium sulphate precipitation of α-amylase produced from *Rhizopus* Sp showed peak precipitation of enzyme with highest α-amylase activity with 70% of the salt saturation.

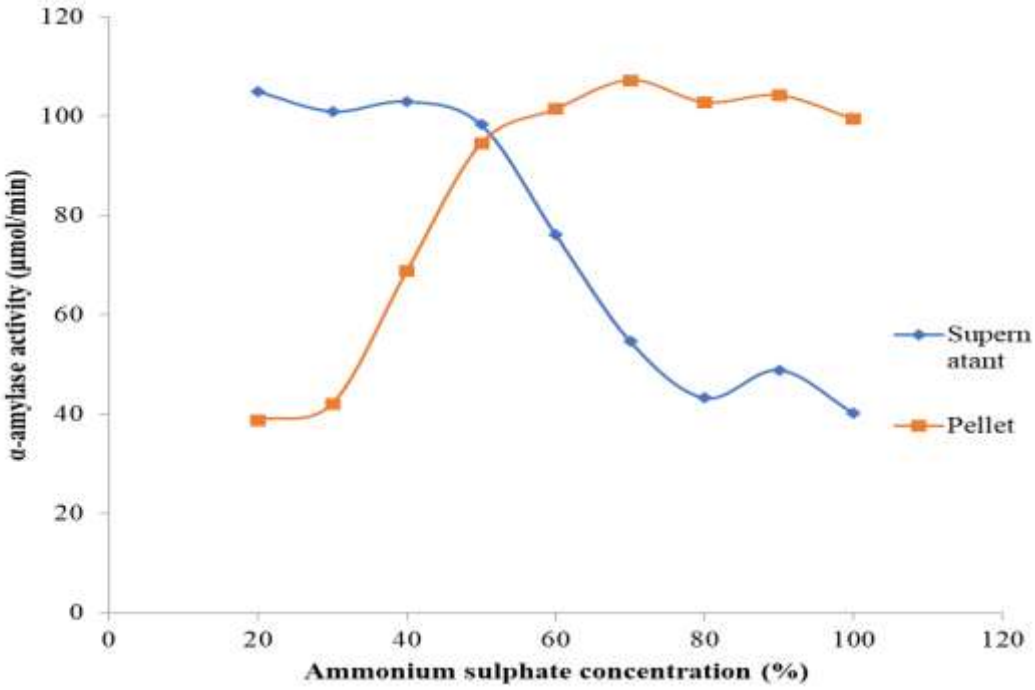

Fig 5: Precipitation profile of α-amylase from *Rhizopus* sp using ammonium salt.

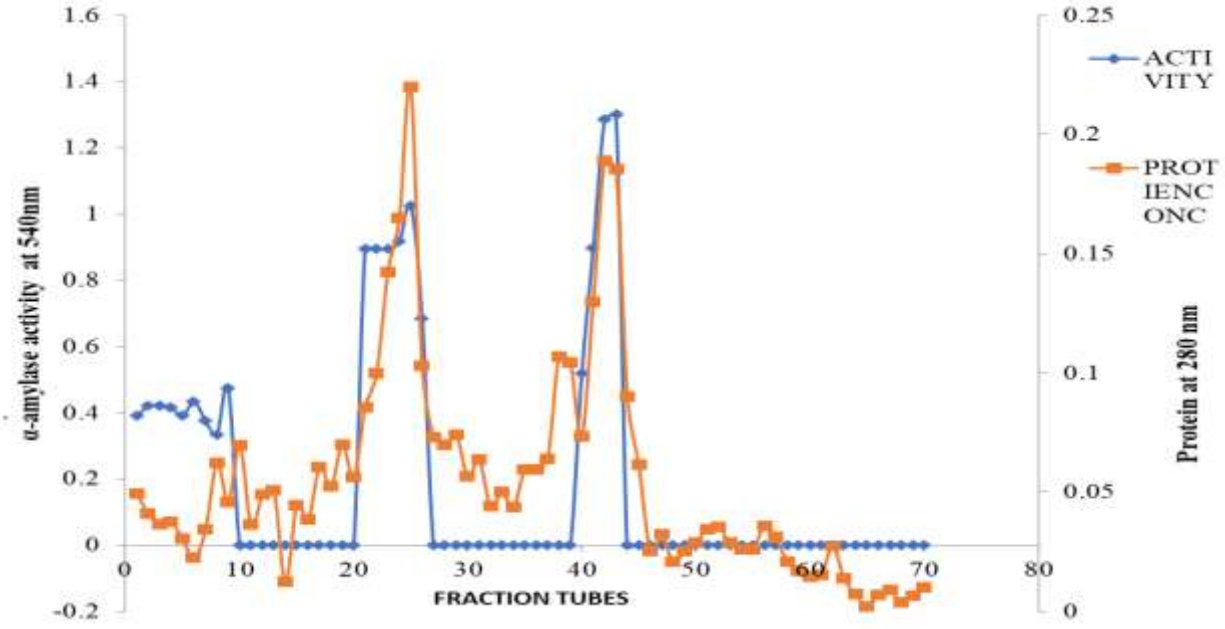

Fig 6: Gel chromatogram of α-amylase using sephadex G-100.

Table 1: Profile of Purification of α-amylase Produced from *Rhizopus* sp.

Enzyme	Vol. (ml)	Protein (mg/ml)	Total protein	Activity (μmol/min)	Total activity	Specific activity (U/mg)	Purification fold	%yield
Crude enzyme	1000	0.662	662	106.05	106050	160.2	1	100
Ammonium sulphate precipitation	300	0.433	129.9	74.27	22281	171.52	1.07	21.1
Dailysis	160	0.298	47.68	54.87	8779.2	184.1	1.15	8.3
Gel filteration (G100).	60	0.176	10.56	42.96	2577.6	244.1	1.54	2.4

Kinetic Properties of α-amylase from *Rhizopus* sp.

Fig 7 and 8 respectively below show the effect of pH and temperature on the activity of α-amylase produced from *Rhizopus* sp. from the figures, the optimum pH for α-amylase harvested on the fifth day was 5.0 as shown in figure 7 when α-amylase activity was monitored with 1% cassava starch. Increase in temperature up to 60°C was accompanied by a corresponding increase in the activity of α-amylase as shown in figure 8, after which a decline in activity was observed up to 80°C which was steady as the temperature was increased to 90°C.

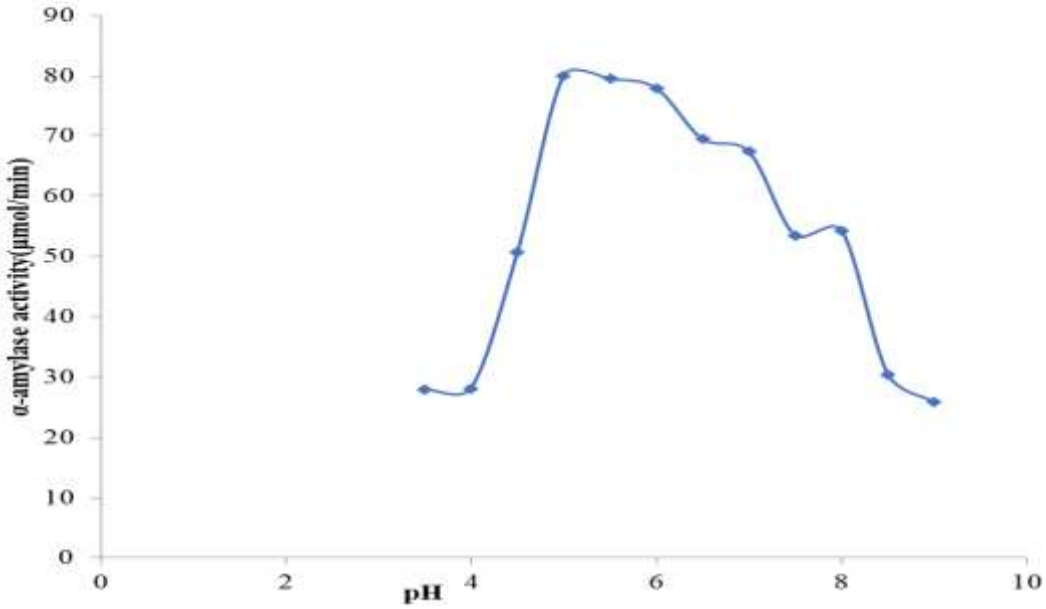

Fig. 7: pH-activity profile of α-amylase produced from *Rhizopus* sp.

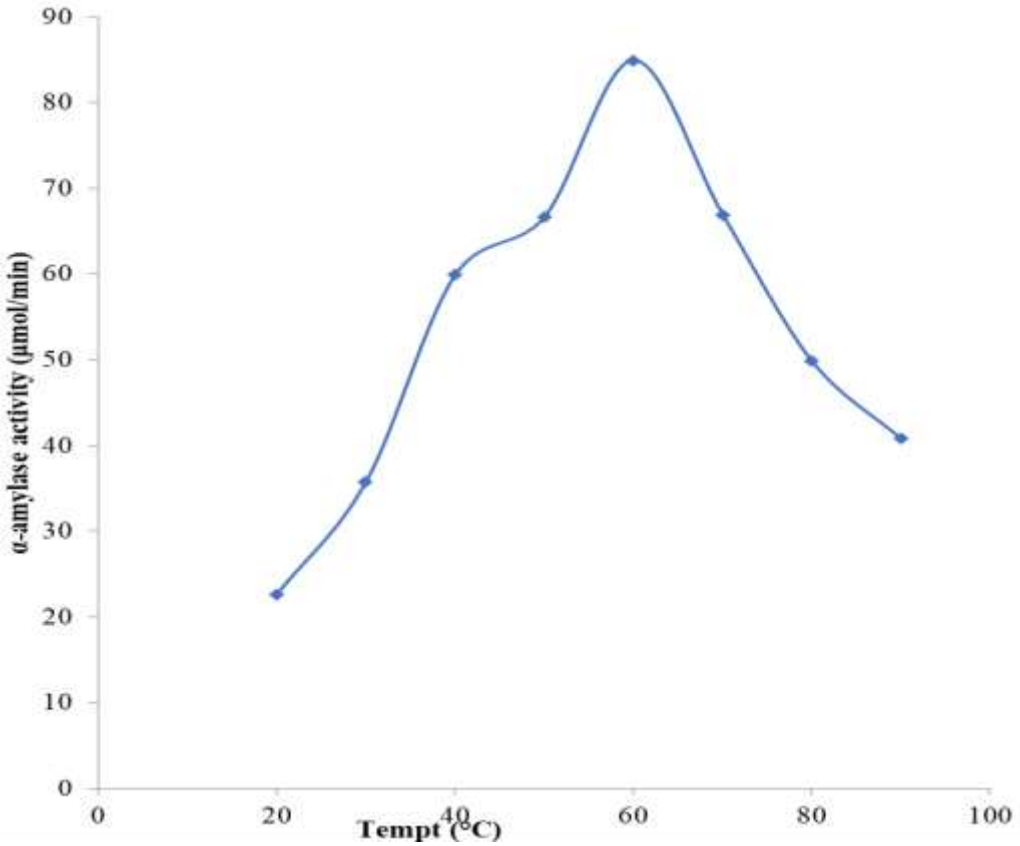

Fig. 8: Temperature (°C) optimization on the activity of α-amylase produced from *Rhizopus* sp.

Determination of Kinetic Parameters

K_M and V_{max}, which gave information about the affinity and reaction velocity of the produced α-amylase calculated from the Lineweaver-Burk plot shown in Figure 9 and 10 below. α-amylase K_M value obtained was 3.46mg/ml while for V_{max} of 270.3µmol/min was obtained.

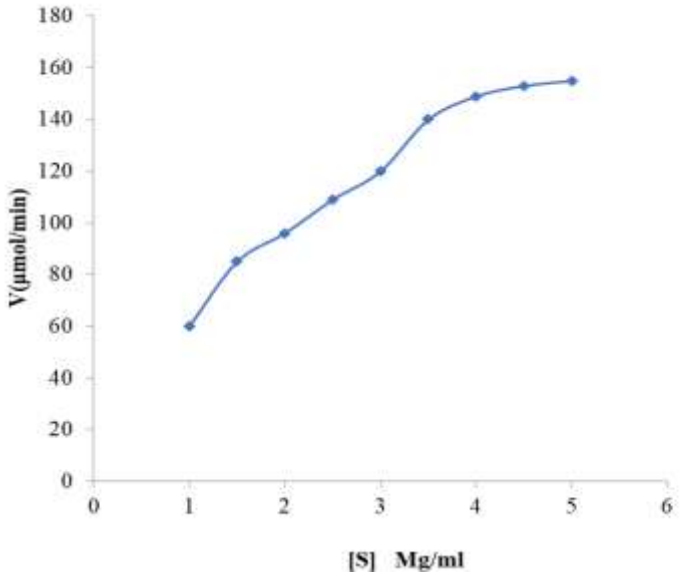

Fig 9: Michealis-Menten plot of α-amylase from *Rhizopus* sp. cultured for 5 days using different concentrations of starch as sole carbon source.

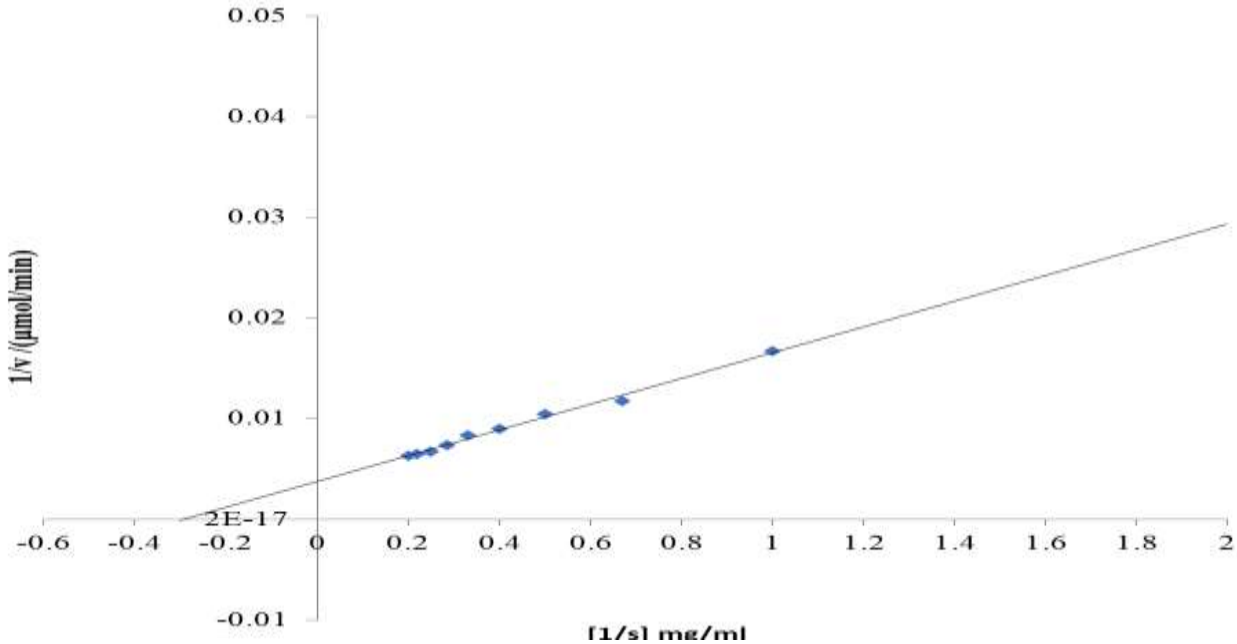

Figure 10: Lineweaver-Burk plot for α-amylase from *Rhizopus* sp. cultured for 5 days using different concentrations of starch as substrate and sole carbon source

Figure 11 below shows the effect of divalent metal ions on the activity of α-amylase from *Rhizopus* sp. From the figure divalent metals of Ca, Co and Mn served as catabolite effector to the activity of the enzyme in the presence of the control experiment.

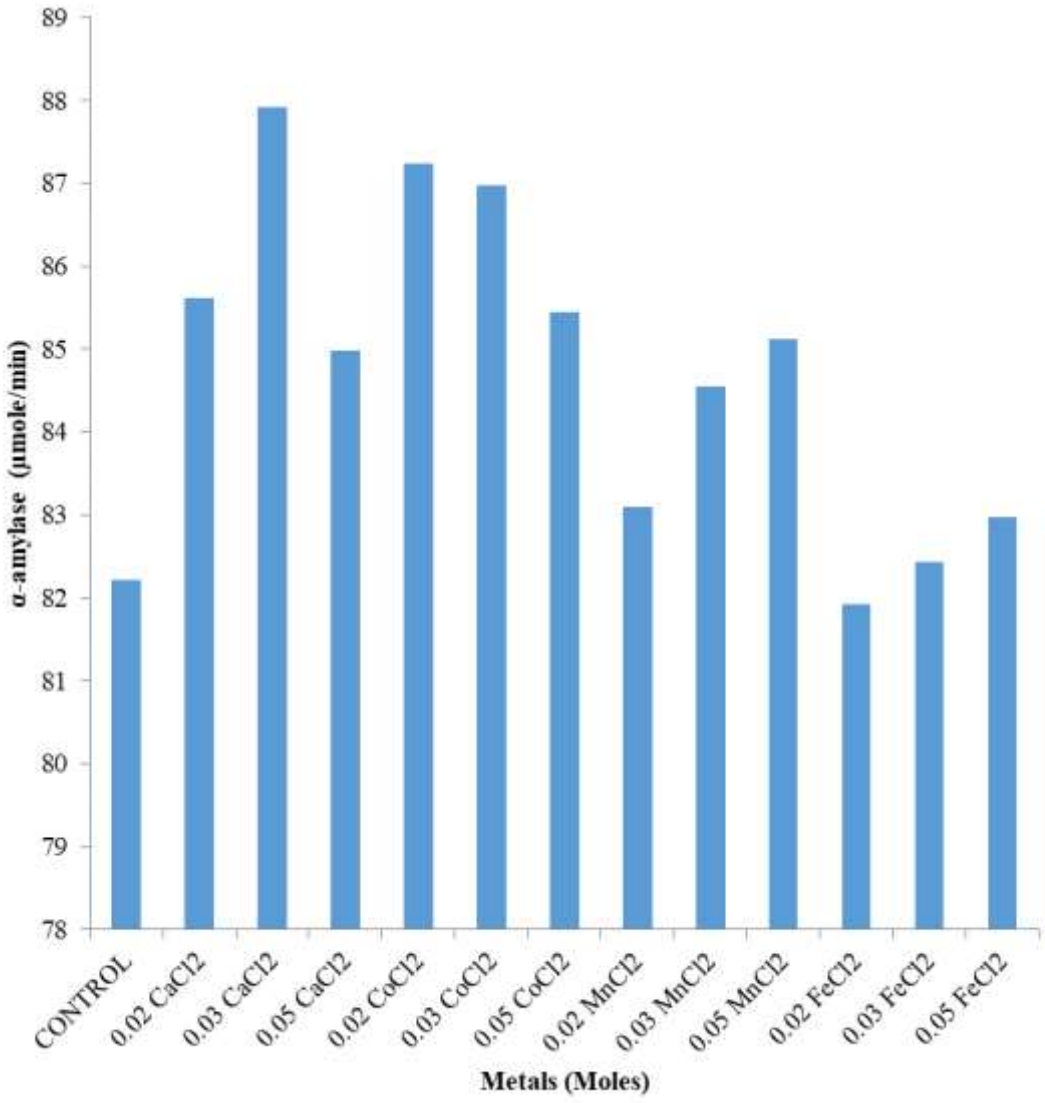

Figure 11: Divalent metal ions-activity profile stabilization of α-amylase from *Rhizopus* sp. cultured for 5 days using different concentrations of starch as substrate and sole carbon source.

Discussion

Percentage yield of starch from cassava stem (sweet variety) used as a substrate in this experiment was 83.6%, which as described by Corbishely and Miller (1984) indicated a high starch content. Plate one shows the pure whitish filamentous hyphaels of strains of α-amylase cultured on a sabourand dextrose agar media (SDA). Analysis of the potentials of the organism for α-amylase production showed a positive test as the there was an intense yellow colouration in the dextrose water infused with 2mM of p-NPG after 36th hour of incubation (fig 1). During the 7days pilot study carried out to produce the enzyme from the fungi, α-amylase activity increased from Day1of the fermentation before declining in activity between Days 2-3, thereafter there was a steady increase in activity from Days 4 to the peak day of day 5 (fig 2); thereafter enzyme activity progressively decreases. The protein concentrations followed the same trend as in the activity profile. The rise and fall in the activity and protein concentrations of the enzyme as reported by Adefila *et al.* (2012) which stated that α-amylase are specific to α-1,4 gylcosidic linkages in starch and other polysaccharides and its activity diminishes on meeting the branching point (1,6 linkages) in sugars and secondly that this could be attributed to the presence of isoforms of the same enzyme present in the crude extract. Peak production of α-amylase from *Rhizopus* sp was observed at pH 6.0. Strains of *Rhizopus* are among the members of zygomycete that survive relatively low pH. As reported by Nwagu and Okolo (2011) most α-amylase optimum production occur at moderate lower pH.

α-amylase produced from *Rhizopus* was also partially through precipitation using sulphate of ammonium ions before gel filtrations using Sephadex G 100 gel packed in a column. 70% ammonium sulphate saturation precipitates protein with highest α-amylase activity. Shen *et al*. (2012) reported 80% ammonium sulphate saturation as the best for protein precipitation with highest α-amylase activity. Dialysis was carried out for 12 hours using dialysis bag (with pore size of 2mm). Activity per protein concentration of the dialysate was found to be 184.1 U/mg.

Column chromatography was used for further purification of the dialysate. The chromatogram showed double activity peaks of enzyme activity and protein concentrations. Two distinct peaks were seen from tubes number 21-29 before a drop in activity of the enzyme and then peaked from tubes number 41-47. Protein concentrations of the enzyme showed the same trend as that of the activity in the chromatogram. Zero fractions (Vo) of the elutions were seen from tube number 0-18. As reported by Chilaka *et al*. (2002), they stated that dialysate shows isoenzymes forms especially from precipitated salts. They narrated in furtherance that replacement of dialysis by gel filtration during protein purification takes away isoenzymes in the enzyme solution. Multiple enzyme activity peaks shown in the chromatogram could be attributed to ionic scrambling which is encouraged by dialysis and this leads to formation of aggregates with unordered pairing of ions. These chemical scrambling show ionic heterogeneity on any column chromatography (Chilaka *et al.,* 2002). α-amylase was purified upto 1.54 folds after the gel filtration with percentage yield of 2.4 %.

The specific activity of the enzyme increased from 160.2 to 244.1 U/mg after gel filtration. Adefila *et al*. (2012) reported a purification fold of 4 and a specific activity of 11,500 on a three purification steps using ammonium sulphate precipitation, ion exchange chromatography and CM- sepharose, Singh *et al*. (2014) reported a purification of 2.9 fold on a two-step purification using ammonium sulphate and dialysis.

As reported by Singh *et al*.(2014), pH and temperature stabilities are key physiological factors to be considered for maximum enzyme activity and applications in industries. α-amylase, when examined at different pH ranges, showed that the partially purified enzyme was active over a broad pH range of 4.5-8.0 with maximum activity at pH 5.0. Shen *et al*. (2012) reported a maximum enzyme activity at pH 5.0. Also they reported a maximum relative stability of its purified α-amylase in the pH range of 3-8 where the enzyme retained about 75% of its activity at pH range of about 4-6 and 50% at 3.0. Nwagu and Okolo (2011) reported an optimum pH of 6.5 for α-amylase isolated from *Fusarium spp* and that the enzyme was relatively stable and retained 60% of activity at pH 4.5-7.0. α-amylase when examined at different temperatures shows an optimum activity at 60ºC.

Shen *et al*. (2012), Singh *et al*.(2014)and Nwagu and Okolo (2011) reported an optimum temperature of temperature of 70,55 and 50ºC respectively for their isolated α-amylase. Kinetic constants (K_m and V_{max}) of α-amylase determined during the study showed K_m of 4.5 mg/ml. K_m which is the substrate concentration at half the maximum velocity during enzyme catalyzed reaction shows the affinity of the enzyme to its available substrates (Chilaka *et al.,* 2002). Singh *et al*., (2014) reported a K_M of 2.407 mg/ml for α-amylase from *Streptomyce sp* strain MSC 702 while Shafiei *et al*. (2010) reported a K_M of 4.5mg/ml for α-amylase from *Nesterenkonia sp* strain F. V_{max} of 250 µmole/min was obtained at the different concentration of p-NPG. Metal ions of calcium, cobalt, manganese and iron with concentrations of 0.03-0.05M showed stabilization on α-amylase activity in the presence of the control experiment. The stabilization of the protein by the divalent metals is seen to be concentration dependent. Calcium and cobalt ions showed relatively greater stabilization impact on α-amylase activity relative to the control while Iron II ion showed the least effect on the enzyme activity.

CONCLUSION

The present study has shown the production of α-amylase with stable catalytic potentials from strains of *Rhizopus* sp. isolated from the soil. The study has demonstrated that the enzyme shows stable activity over physiologic pH and temperature; this has demonstrated the promising features of the enzyme for maximum industrial applications.

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Author's Contributions

Oparaji Emeka Henry: conceived and designed the experiments, performed the experiment and processed the data, analyzed the data and wrote the manuscript.

Arazu, Amara V.: Guided the experimental design, performed the experiment and processed the data.

Chukwudozie, Izuchukwu, C.: Guided the experimental design, performed the experiment and processed the data.

Oyibo, Okpanachi N: Analyzed the research design and methodology, interpreted the data.

Nworie, Martin B.: Guided the experimental design, performed the experiment and processed the data.

Eze, Sabinus O: Supervised research, guided the research design and methodology and interpreted the data.

Chilaka Ferdinand, C.: Supervised the research, analyzed the data, interpreted the data and revised the manuscript.

Ethics

Authors declared no ethical issues that may arise after the publication of this manuscript.

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