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Effect of Salt and Inhibitoron the Isolation, Purification and Characterization of α -Amylase from *Aspergillusniger* Produced from Pigeon Pea

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ABSTRACT

α-Amylase an industrially used enzyme can be obtained from *Aspergillusniger* and can be produced from food sources such as pigeon pea. α-Amylase was produced from *Aspergillusniger* isolated from pigeon pea, purified and characterized. This process was achieved using ammonium sulphate, ion exchange DEAE column and gel filtration (Sephadex A-50 and sephadex G-100) chromatography. The effect of salt and inhibitor was determinedAmmonium sulphate precipitation results showed that the highest specific α-amylase activity was (1.01 U/ml. mg) obtained at 11.27% saturation level, with a purity of 1.81-fold of the crude extract and yielding 1.00%. Further purification using gel filtration increased the enzyme purity and yielding 8.94-fold relative to the crude extract 3.01% and yielding Specific activity after purification was 4.99 U/mg. The effect of salts on α-Amylase activity increased to 258.09% when in $MgSO_4$, while decreased to 7.71% and 21.07% when in $MnSO_4$ and $CuCl_2$ respectively and yielded no result when in $PbNO_3$.Its reaction with chemical inhibitors such as Bromosuccinimide was activated to 136.465% and was inhibited at Mercaptoethanol to 0%. All these were determined using a visible spectrophotometer with an absorbance of 540nm, against the control that contains 100µL of the enzyme and 100µL of 1% starch solution.Therefore α -amylase produced from *Aspergillusniger* can be exploited for potential usage for industrial applications of enzymes in a wide range of production and its application in food processing.

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Introduction

Aspergillusniger is a common fungus in nature that belongs to the Aspergillus genus. It is an important industrial fermentation microorganism that is widely used for the production of industrial enzymes and organic acids (Schuster *et al.,*2002). According to Oludumila*et al*. (2015), the fungus can be grown on inexpensive substrate and is capable of producing high yield and relatively stable at the operating condition. Major advantage of using fungi for the amylase production is the economical bulk production capacity (Shah *et al.,* 2014).

α-amylases are wide spread in occurrence which can be obtained by different resources e.g. microorganisms, animals and plants etc. However, fungi and bacteria are used for commercial production of amylases (Mathew *et al*., 2016; Singh *et al.,* 2016), because of a few advantages i.e. reliability, less time and space, low cost required for enzyme production, ease of manipulation and economical bulk production capacity (Khan *et al.,* 2019; Mahmood*et al*., 2016).

Pigeon pea (Cajanuscajan (L.) Huth) is one of the most common tropical and subtropical legumes cultivated for its edible seeds. Pigeon pea is fast growing, hardy, widely adaptable, and drought resistant (Bekele-Tessema, 2007). Fasoyiro*et al*. (2009), Amaefule and Nwagbara (2004) and Odeny (2007) reported that pigeon pea is still underutilized as food due to its tough texture, long cooking duration and lack of education on its nutritional potentials.

The objectives of this research is to produce, isolate, purify, characterize and to determine the effect of salt and inhibitor on α-Amylase produced from *Aspergillusniger* isolated from Pigeon pea.

Materials and Methods

Collection of the sample and preparation of seed culture

1. Pigeon pea was purchased from Anaye market, odo-ora, Ekiti state, Nigeria. The chemicals used were of analytical grade. The pigeon pea was fermented for 4 days (96 hours) and serial

dilution was done in triplicate according to Chakrabortyet *al.,* (2000).

Isolation of α-Amylase from *Aspergillusniger*

This was carried out according to Chakrabortyet *al.,* (2000) with slight modifications.

Assay of α- amylase activity

Estimation of α- amylase activity was carried out according to the dinitrosalicylic acid (DNS) method of Bernfeld (1955).

Enzyme purification

Ammonium sulphate precipitation.

The cell-free supernatant after centrifugation was subjected to ammonium sulphate precipitation and dialysis and each fraction was checked for enzyme activity as well as protein concentration.This was carried out according to Chakrabortyet *al.,* (2000).

Ion exchange chromatography

The dialysate was further purified by applying 25 ml of dialysate on DEAE-Sephadex A50 column (1.5 x 24 cm) equilibrated with 50 mM phosphate buffer pH 6.8 at a flow rate of 1 ml/min. All the fractions were checked for enzyme activity. This was carried out according to Chakrabortyet *al.,* (2000).

Gel filtration.

The active fractions were pooled and applied on sephadex G-100 Column (1.5 x 75 cm The resulting enzyme was utilized for the characterization of the extracellular α-Amylase. This was carried out according to Chakrabortyet *al.,* (2000).

Determination of protein concentration

The Protein concentration was determined according to Bradford (1976) using Bovine Serum albumin and the absorbance was measured at 595nm with a spectrophotometer.

Physicochemical properties of purified enzyme.

Effect of some salts on the α-Amylase activity

An assay mixture containing a final concentration of each salt were carried out according to the standard assay procedures of Ojo and Ajele (2011).

Effect of inhibitorson purified α-Amylase activity

The effect of different inhibitors on α-Amylase activity were determinedaccording to Ojo and Ajele (2011).

Results and discussion

Production of α-Amylase from *Aspergillusniger*

α-Amylase activities increased with the increase in time. It has its peak at 25 h fter which it begins to decline as the starch solution has been exhausted. During the incubation incubation of *A. niger* in 1% starch solution, the growth increased with time and at the 30^{th} h, the microorganisms has its optimum growth here produced its peak number of cells due to the exhaustion of the available starch and the growth begins to retard. The protein concentration also increases with time at the inception but is stationary at the 10^{th} to 20^{th} h and increased a bit till it gets to 25^{th} h and has a sharp increase from 25^{th} h to the 35^{th} h where it has its optimum protein concentration and then begin to decline.Figure 1 shows the production of α-amylase from *A. niger*in a medium containing 1% starch solution.

Purification of α-Amylase from *Aspergillus. Niger*

The elution profile of α-amylasefrom ion exchange chromatography on DEAE sephadex A50 was shown in Figure 2. The elution profile shows one protein and activity peaks and the fraction containing α-amylase activity was pooled and used for further purification on gel filtration chromatography while in Figure 3, the elution profile of α-amylase from gel filtration chromatography produced one activity peak which was also pooled for further analysis, the results were similar with Samson *et al.,* (2001), who purified *A. niger* from food substrate. The summary of purification of alpha amylase from *A. niger*is as shown in Table 1. After the purification, the activity of the enzyme increased from 8.85 to 32.7 mmol/min, the specific activity was 4.99 mmol/min/mg, the yield of the enzyme was 13.01% and the purification fold was 8.94. This was in accordance with Shafieiet *al.,* 2010, which carried out enzyme activity, specific activity and purification fold of enzyme purified from *Aniger*.

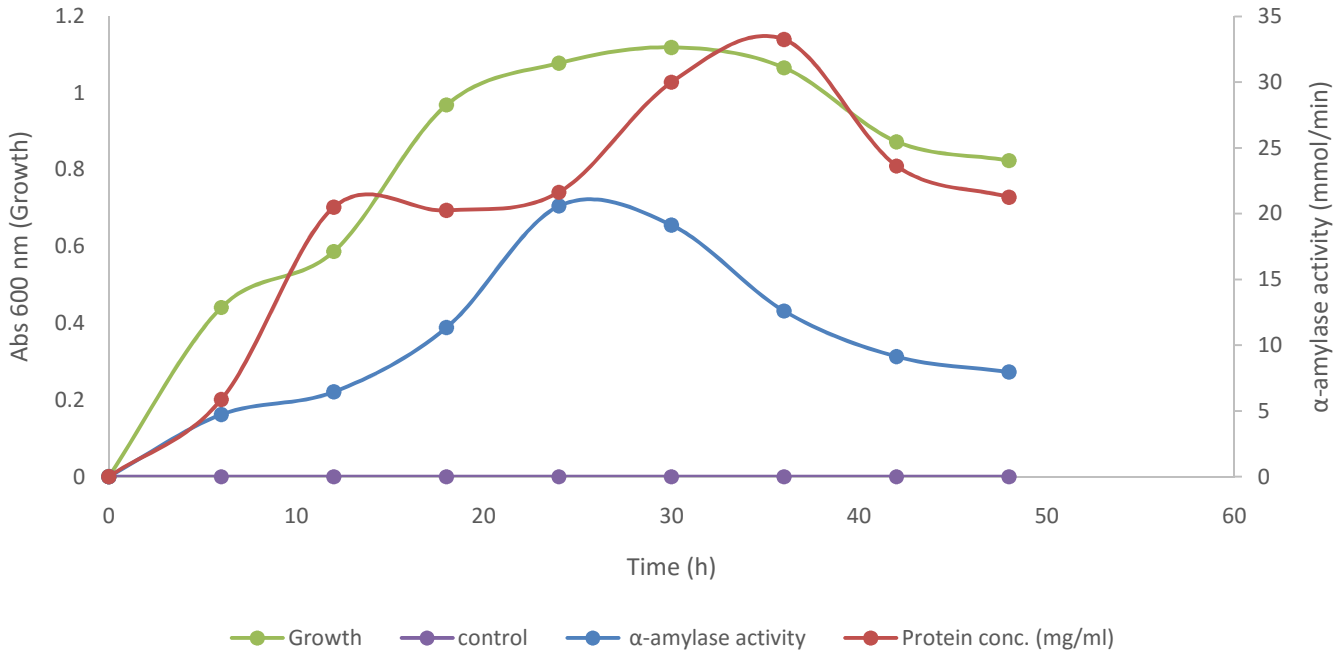

Figure 1: Alpha amylase production from *Aspergillusniger*in a medium containing 1% starch solution.

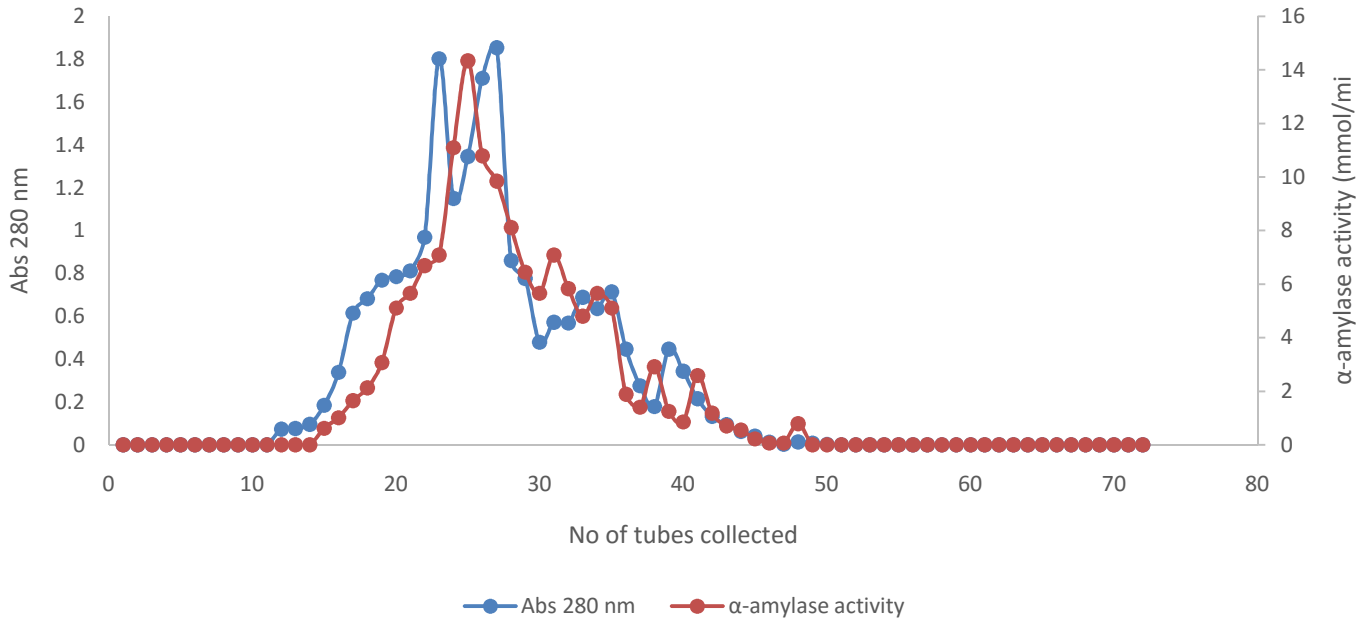

Figure 2: Elution profile of α-amylase on DEAE-sephadex A50

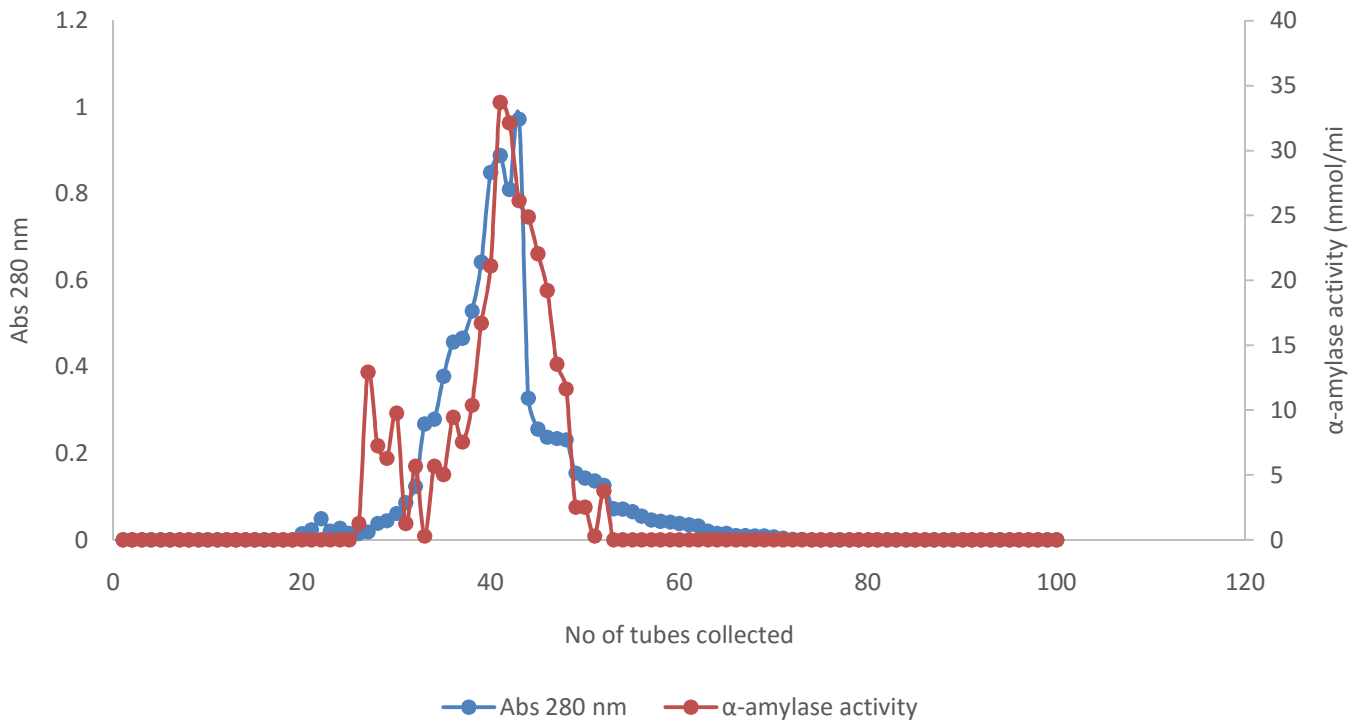

Figure 3: Elution profile of α-amylase on sephadex G-100

Table1: purificationofα-Amylase

Step	Vol. of solution	α-amylase activity	Protein Concentration	Total α-amylase activity	Total Protein Concentration	Specific activity	Yield	Fold
Crude extract	850	8.85	15.87	7529.52	13493.75	0.56	100.00	1.00
Ammppt/ Concentra	63.6	13.34	13.25	848.83	842.72	1.01	11.27	1.81
Ion exchange	41.8	17.44	9.62	729.03	402.32	1.81	9.68	3.24
Gel	32.7	29.96	6.00	979.71	196.21	4.99	13.01	8.94

Effect of salts on α- Amylase activity

Effects of metal ions of nitrate salts on the partially purified α-amylase activity is shown in

Table 2. Metal such as $CuCl_2$ and $MnSO_4$decreased the α-amylase activity; however, $PbNO_3$ reduced the activity of purified α-amylase to zero. $MgSO_4$ increased the activity of α-amylase to over 200% followed by the control (100%) and $CoCl_2$(77.12%). The results were similar to the report of Bajpai *et al*., (1992).

Effects of Inhibitors on α-Amylase Activity

When inhibitor binds reversibly to the active side of the enzyme it is known as a competitive Inhibitor. Often a competitive inhibitor is a similar shape to the substrate. Its association with the active site of the enzyme reduces the rate of binding between the substrate and the enzyme, thus lowering the rate of reaction. However in comparison to literature, this type

of inhibition can be overcome by increasing the substrate concentration as this will decrease the chances of enzyme and inhibitor binding (Reece *et* al., 2011).

Table 2: Effect of metal ion on the partially purified activity of α-amylase

Metallic salts	Activity	R.A (%)
KCl	8.30	54.24
$PbNO_3$	0.00	0
$MgSO_4$	39.52	258.09
$MnSO_4$	1.18	7.712
NaCl	13.70	89.46
$CuCl_2$	3.22	21.07
CoCl	11.81	77.12
Control	15.31	100.00

The relative activity of some chemical inhibitors and their effects on the purified α-Amylase activity is shown in Figure 4. This was determined using a visible spectrophotometer with an absorbance of 540nm, against the control that contains 100µL of the enzyme and 100µL of 1% starch solution, after testing the chemical inhibitors against the control, the results gotten are as follows; Mercaptoethanol 0%, Triton X-100 9.172%, Bromosuccinimide 136.465%, EDTA 12.527%, Urea 54.809%, Sodium Dodecyl Sulfate (SDS) 12.751%. The values of the chemical inhibitors higher than 100% of the control activated the activity the enzyme, while the values lower than the control inhibited the activity of the enzyme. Putting this into consideration, this makes Mercaptoethanol with 0% the chemical compound with the highest inhibiting power, followed by Triton X-100 with 9.172%, EDTA with 12.527%, Sodium Dodecyl Sulfate (SDS) with 12.751%, and Urea with 54.809%. This makes Bromosuccinimide, with 136.465%, an activator.

Therefore, when the structural resemblance of the competitor matches that of the substrate, it binds to the active site competitively, but when competitive inhibitor's concentration exceeds that of the substrate, competitive inhibitor binds to the active binding site to form enzyme-inhibitor complex (EI) and finally no product is formed (Thoma and Koshland, 1960; Hegyi*et* al., 2013) which makes all the compounds but Bromosuccunimide inhibitors in respect to their mechanism of actions.

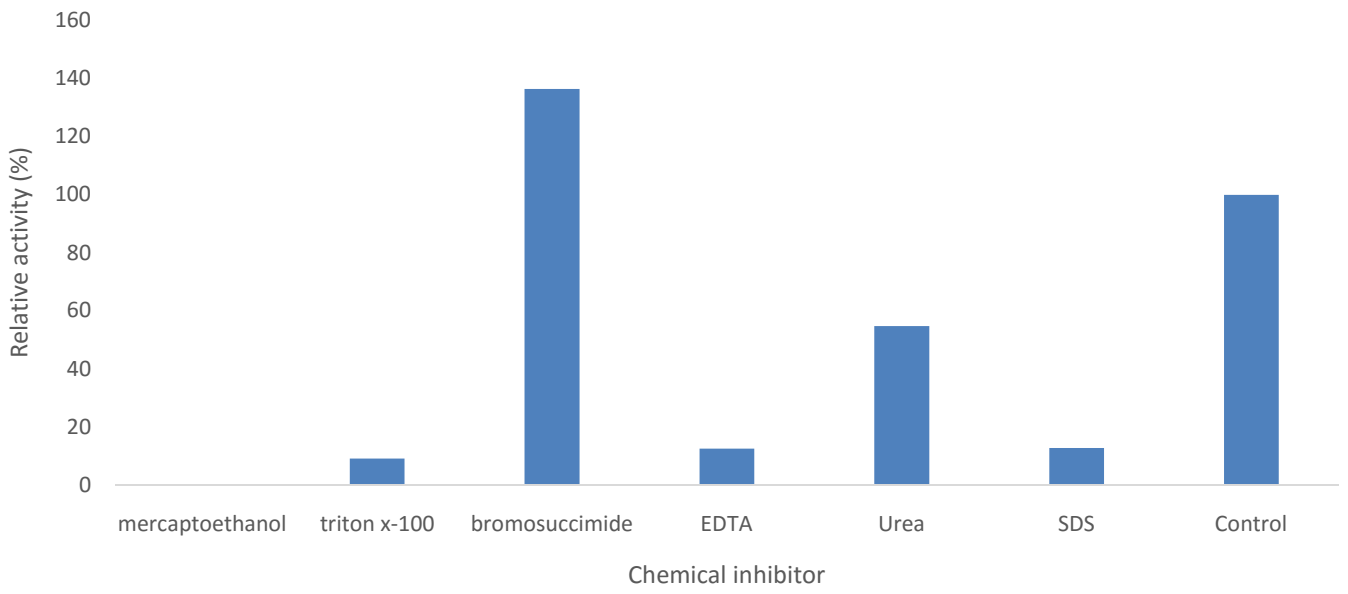

Figure 4: Effect of some inhibitors on purified α-amylase activity

Conclusion

Conclusively, with the use of pigeon pea as substrate for this experiment, the following has been observed: the activity of α-amylase depends on increased activity in the presence of $MgSO_4$, and a decrease in $MnSO_4$ and $CuCl_2$. The activity was inhibited using EDTA, SDS, Urea, Mercaptoethanol, and Triton X-100 while Bromosuccinimide activated the enzyme, increasing its activity. The experiment shows that the activities of enzymes can be activated/increased or inhibited/decreased, which are useful in fermentation, and in prolonging the shelf life of foods respectively. These could be achieved with the use of suitable mineral salts, or inhibitors.

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