Acta Scientific NUTRITIONAL HEALTH (ISSN:2582-1423)

Volume 5 Issue 12 December 2021

Research Article

Effect of pH and Temperature on the Isolation, Purification and Characterization of α-Amylase from Aspergillus niger Produced from Pigeon Pea

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Received: September 22, 2021

Published: November 24, 2021

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Abstract

α-Amylase an industrially used enzyme can be obtained from *Aspergillus niger* and can be produced from food sources such as pigeon pea. α-Amylase was produced from *Aspergillus niger* 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 temperature and pH was determined. Ammonium 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. Having varied temperature from 30-80°C, the optimum temperature was obtained at 50°C. The pH ranged from 3-10, at a concentration of 0.01 M which was prepared using different buffer systems, the enzyme was found stable at pH of 6. Therefore α -amylase produced from *Aspergillus niger* can be exploited for potential usage for industrial applications of enzymes in a wide range of production and its application in food processing.

Keywords: α-Amylase; *Aspergillus niger;* Pigeon Pea; Purification; Characterization; pH; Temperature

Introduction

The demand for industrial enzymes has been steadily increasing. This is primarily due to an increase in the demand for long-term solutions. Microbial sources have been the most important and useful sources of many of these enzymes [1] and the use of cheap and underutilized crops that is drought resistant such as pigeon pea has a huge advantage in reducing enzyme production cost. *Aspergillus niger* 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 [2]. It is usually found as saprophytes and are most commonly found on stored grain, dead leaves, and other decaying vegetation. It appears in the form of very dark brown patches or carbon black and a very important group of microorganisms that dominate solid state fermentation. It possesses some very special properties such as good tolerance to less availability of water and capacity to spread over and into the solid substrate, therefore, it is extensively grown and used in food industry for the production of a variety of enzymes such as α-amylases, amyloglucosidases, cellulases, lactase and acid proteases [3,4]. According to Oludumila., *et al.* [5], 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 production of amylase is the economical bulk production capacity [6].

α-Amylases are wide spread and can be obtained by different resources such as microorganisms, animals and plants. However, fungi and bacteria are used for commercial production of amylases [3,7], because of their advantages such as reliability, less time and space, low cost, ease of manipulation and economical bulk production capacity [8,9]. Fungal enzymes are preferred over other microbial sources owing to their widely accepted Generally Regarded as Safe (GRAS) status [10].

Pigeon pea (*Cajanus cajan* (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 [11].

The objectives of this research are to produce, isolate, purify, characterize and to study the effect of pH and temperature on α-Amylase produced from *Aspergillus niger* cultured on substrate.

Materials and Methods

Collection of the sample and preparation of seed culture

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 h) and serial dilution was done in triplicate according to Chakraborty., *et al.* (2000). Pure culture of *Aspergillus niger* was activated in petri-dish containing potato dextrose agar (PDA). The seed culture was done by suspending the fungus in potato dextrose agar (PDA) using water bath shaker for 72 h in order to make the organism active prior to inoculating the α – amylase screening medium.

Isolation of α-Amylase from *Aspergillus niger*

This experiment was carried out according to Chakraborty., *et al*. 2000 with modifications. Pure fungus colony from the agar slant was aseptically transferred into 100 ml potato dextrose broth prepared in 250 ml Erlenmeyer flask. The flasks were incubated for 48 h at 37°C in a rotary shaker incubator at 150 rpm. This mixture was regarded as the seed culture. Basal medium (100ml) containing 0.9 g/L K_2HPO_4; 0.2 g/L KCl; 0.2 g/L $MgSO_4.7H_2O$; 1.0 g/L NH_4NO_3; 0.002 g/L$ZnSO_4$;0.002 g/L $MnSO_4$; 0.002 g/L $FeSO_4$. $7H_2O$ and 1 g of corn starch was prepared. The medium was autoclaved at 121°C at 15 atm for 20 min. The medium was allowed to cool. The seed culture (5 mL) was aseptically transferred into the basal medium containing the starch. The culture was incubated in a rotary shaker incubator at 37°C for 72 h at 150 rpm. At the end of the incubation period, the mixture was centrifuged at 5,000 rpm at 4°C for 20 min. The supernatant was collected after centrifugation and preserved at 4°C until required while the residue was discarded.

Assay of α- amylase activity

This was carried out according to Bernfeld, 1955. One hundred microliter of 1% starch in 50 mM phosphate buffer pH 6.8 was added to 100 µl of enzyme extract in reaction tubes and incubated for 3 min at room temperature. 100 µl of dinitrosalicylic acid colour reagent was added. The mixture was boiled in a water bath for 5 min, cooled to room temperature and 1000 µl of distilled water was later added. The mixture was vortexed and the absorbance was read at 540 nm, against the reagent blank.

Enzyme purification

Ammonium sulphate precipitation

The cell-free supernatant after centrifugation was subjected to ammonium sulphate precipitation and dialysis. Solid ammonium sulphate was added to supernatant to bring the solution to 60% saturation. The mixture was centrifuged to obtained protein pellets. The precipitates (pellets) were dissolved in 5 ml of 50 mM phosphate buffer pH 6.8 and dialyzed against 2l of the same buffer. Each fraction was analysed or enzyme activity and protein concentration. Ammonium sulphate precipitation was carried out according to Chakraborty., *et 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 tested for enzyme activity. Ion exchange chromatography was carried out according to Chakraborty., *et al.* (2000).

Gel filtration

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

Determination of protein concentration

The total protein content of the supernatants and fractionated protein solutions was determined according to the method described by Bradford (1976) using bovine serum albumin(BSA) as standard protein. Two hundred microgram per liter (200 µg/ml) of bovine serum albumin (BSA) was prepared as the standard solution. 10 µl, 20 µl, 30 µl, 40 µl, 50 µl of the standard protein solution was pipetted into clean test tubes according to the varied BSA concentrations of 2, 4, 6, 8, 10 µg/ml respectively from the stock solution. The corresponding amount of distilled water was pipetted into the test tubes and 200 µl of the Bradford reagent was added to each of the test tubes. The blank contained water and Bradford reagent instead of the protein solution. The absorbance of the content in each tube was read at 595 nm against the blank. The quantity of the protein in the dialysate solutions was determined from the graph of absorbance against concentration plotted as the standard calibration curve.

Physicochemical properties of purified enzyme

Effect of pH on α-amylase activity

The α - amylase assay was carried out according to the standard assay procedure (Ojo and Ajele, 2011).

Buffers of different pH values ranging from 3-10 at a concentration of 0.01 M were prepared using different buffer systems: acetate buffer pH 3, 4 and 5; phosphate buffer, pH 6, 7 and 8; Tris -HCL buffer pH 9 and 10. Each buffer solution was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme.

Effect of temperature on α-amylase activity

The optimum temperature was determined using the method of Ojo and Ajele (2011). α- Amylase enzyme was estimated at pH 7 and between the temperature range of 30-70°C for 5 min. Colour reagent (3, 5-dinitrosaliccyclic acid) was added to the mixture after incubation. After incubation the mixture was cooled, 1000 µl of distilled water was added and the absorbance was taken at 540nm against the blank.

Results and Discussion

Production of α-Amylase from *Aspergillus niger*

α-Amylase activities increased with the increase in time. It has its peak at 25 h after 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 30th hour, 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 10th to 20th hour and increased a bit till it gets to 25th hour and has a sharp increase from 25th hour to the 35th hour 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-amylase from 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.* [12], 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 Shafiei., *et al.* [13], which carried out enzyme activity, specific activity and purification fold of enzyme purified from *A. niger*

Figure 1: Alpha amylase production from *Aspergillus niger* 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.

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
Ammonium ppt/ Concentrate (mmol/min/mg)	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

Table 1: Purification of α-Amylase.

Effect of temperature on α-amylase

The optimum temperature was taken as 100% and the rest of the temperature was calculated relative to the optimum activity. Assay mixture was incubated at temperature 30-80 °C and the activity was determined using standard assay procedure. The optimum temperature of **α** -amylase activity as shown in figure 4 was observed at 50 °C. A steady decline in relative activity was observed at temperatures from 50°C to 80°C. The optimal temperature for maximum activity of fermented pigeon pea amylase (50°C) is in accordance with what was reported in β-amylase from *C. thermocellum* SS8 (Swamy., *et al.* 1994), *Bacillus circulans* (Kwan., *et al*. 1993) and *Bacillus megaterium* (Ray., *et al.* 1995). The result also agrees with the temperature for optimal activity reported for *A. flavus* and *M. pusillus* by Alli., *et al*. (1998). However, there was a rapid decline in the activity of the enzyme at temperature above 50°C, which agrees with the report of Alli., *et al.* (1998).

Figure 4: Effect of temperature on purified α-Amylase.

Effect of pH on purified α-Amylase

The incubation of enzyme-substrate reaction mixture was carried out at various pH values which ranges from pH 3-10. At pH 6, maximum enzyme activity (979.71U/g) was observed. Enzyme stability was also assessed by pre-incubating enzyme at various pH buffers and enzyme was found stable in pH range of 6-7. It was studied in previous literature that α-amylase was best active at pH 6.8 (Kanwal., *et al*. 2004), pH 5 (Patel., *et al*. 2005), pH 7 (Tiwari., *et al*. 2007), pH 7 (Wang., *et al*. 2016) and pH 5.6 (Yahya., *et al*. 2016), respectively.

The effect of pH on the activity of the purified α-amylase which was studied over a range of pH 3 to pH 10 showed that the activity increased gradually over a range of pH 3 to pH 6 and was constant and stable at pH 6 - 7 with the maximum level at pH 6 as shown in figure 5 and the α-Amylase activity gradually decreased until pH 10.

Figure 5: Effect of pH on α-Amylase.

Conclusion

The results revealed that α -amylase produced from *Aspergillus niger* can be exploited for potential usage for industrial applications of enzymes in a wide range of production and its application in food processing. Conclusively, with the use of pigeon pea as substrate for this experiment, the following has been observed: the activity of α-amylase depends on pH, which is usually between the range of 6-7 and on temperature, in which the optimum temperature is 50°C.

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Volume 5 Issue 12 December 2021

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