



Production and optimization of alpha amylase from *Aspergillus niger* using TME 419 cassava peel as substrate

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Abstract

Production and optimization of culture medium composition as well as purification and characterization of alpha amylase from *Aspergillus niger* using TME 419 cassava peels as substrate was carried out during the course of this study. Proximate analysis of the dry weight cassava peels was 10.08% moisture content, 6.60% ash content, 11.02% crude fiber, 4.91% crude protein, 1.58% ether extract (fat) and 75.83% total carbohydrate. The optimum process parameter obtained were 120 h for incubation time (0.222 ± 0.033 U/ml), pH of 9 (0.215 ± 0.014 U/ml), gelatin as preferred nitrogen source (0.166 ± 0.144 U/ml), temperature of 35°C (0.048 ± 0.042 U/ml) and TME 419 as favorable carbon source (0.255 ± 0.042 U/ml). Mn and Zn facilitated the activity of alpha amylase while other metal ions like Hg, Tn inhibited the activity of the enzyme. Km and Vmax values of 9.091 mg/ml and 4.431 umol/min/ml were estimated using Lineweaver-Burk plot respectively. TME 419 cassava peels often regarded as agro waste can play a beneficial role in alpha amylase production.

Keywords: Alpha amylase, *Aspergillus niger*, Optimization, Production

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1. Introduction

Amylase is referred to as any member of a class of enzymes that catalyzes the hydrolysis (splitting of a compound by addition of a water molecule) of internal α -1, 4-glycosidic linkages in starch—a polysaccharide into smaller carbohydrate molecules with low molecular weight such as maltose (a molecule composed of two glucose molecules) and glucose or glycogen into sugars (Gupta et al., 2003; and Rajagopalan and Krishnan, 2008). The recent discoveries of starch degrading enzymes have led to increased application of amylases in

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various industrial processes. The need for improved efficiency of industrial processes catalyzed by enzymes has necessitated the use of isolated enzymes as opposed to whole cells.

Alpha-Amylase (EC 3.2.1.1) also named as 4- α -D-glucan glucanohydrolase, has found its application in various industries which includes food, brewing, distilling industry, pharmaceutical, textile, paper and bioconversion of solid waste, etc. (Pandey et al., 2000; and Gupta et al., 2003). Amylase is also used in bioethanol, confectionary and different fermented food (Al-Hindi et al., 2011). Large range of applications is the triggering factor for the industrialization of alpha amylase production and the use of amylase in many industries has made it very important to optimize production process to achieve maximum yield (Obafemi et al., 2018). However, maximal production of amylase at a reduced cost is realized either by strain improvement or optimization of process parameters (Prajapati et al., 2014).

Amylases are produced by plant, animal and microbial sources, although the microbial amylase production has been reported to be most effective. The production of amylase has been demonstrated in bacteria (Coleman, 1967; Cotta, 1988; Rehana et al., 1989; and Ueki et al., 1991) and fungi (Fergus, 1969; Ghandi et al., 1974; Mot and Verachfert, 1982; Ogundero, 1982; Mountfort and Asher, 1988; and Rehana et al., 1989). Fungal enzymes are preferably used over other microbial sources owing to their widely accepted Generally Regarded as Safe (GRAS) status (Karim and Tasnim, 2018). Fungi such as *Aspergillus niger* can produce different cell wall degrading enzymes that break down large polysaccharides into simple reducing sugars which are used up for growth and multiplication (Ajayi and Adedeji, 2014). The production of amylases using synthetic media is capital intensive and researchers are in search of diverse procedures to cut short the cost of production. In most cases, commercial soluble starch is used as the carbon source for the growth of the organisms, although sugars such as maltose, lactose and galactose may be used. These substrates are expensive for large-scale culture of amylase-producing organisms, hence the need for alternative cheaper carbon substrates for amylase production.

Solid state fermentation (SSF) has been reported to be a bit cheaper because the enzyme extraction procedure (Kumar and Duhan, 2011) is a ray of hope. Though, in the case of SSF, the cost of the substrate is important in deciding the cost of production. Agro industrial wastes have been reported to be a good substrate for the cost-effective production of alpha amylases (Pandey et al., 1999; and Kumar and Duhan, 2011) and are thus attractive to researchers.

Nigeria is said to be the world's largest producer of cassava, with a yearly output of about 50 million tons. Cassava production is projected to increase up to 150 million tons by 2020. Nearly half of the quantity of cassava produced are processed into fermented foods like garri, fufu, lafun cassava flour and other cassava-based staple foods. For every 1000 kg of cassava processed, 10%-15%~125 kg/tons, are lost in form of wet peels, which are poorly utilized, dumped as waste or burnt. Cheap and readily available agricultural waste such as cassava peels which presently constitutes a menace to solid waste management may be a rich source of amylase producing microorganisms (Ali et al., 1998).

1.1. Statement of problem

Demand for microbial alpha amylases has increased due to their specificity of the reaction, mild conditions required for the reaction and also because they require less energy consumption than the conventional chemical methods. Amylases for industrial activities are relatively non-existent in local Nigerian industries. Some local industries that can import these enzymes, do so at a high cost. Furthermore, the commercial enzyme when stored for some time also tends to lose its stability and potency due to the epileptic power supply in the country. Also, the economic bulk production of enzymes on cheap substrates such as agro wastes in solid state fermentation using *Aspergillus niger* has been reported and these agro wastes are abundant in Nigeria and underutilized; they could be put to an alternative use as solid support (substrate) for amylase production. This has called for the local commercial production of amylase for these local industries upon urgent demand.

1.2. Aim

This study aims to produce alpha amylase from *Aspergillus niger* through solid state fermentation using cassava peel as substrate.

1.3. Objectives

- (i) To determine the proximate analysis of cassava peels.

- (ii) To Isolate and optimize process parameters for *Aspergillus niger* from cassava peels
- (iii) To carry out solid state fermentation
- (iv) To purify and characterize alpha amylase

2. Materials and methods

2.1. Substrate preparation and proximate analysis

TME 419 cassava species was collected from plant and propagation unit, BIODC Oka Akoko, Ondo State. Cassava was washed before peeling, air-dried for 14 days and grounded to a fine powder. The powder was sieved, stored in labeled airtight plastic containers at room temperature and proximate analysis was determined using AOAC (1987 and 2005) methods.

2.2. *Aspergillus niger* isolation

1 g of the soil sample from simulated dumpsite of cassava peels was serially diluted. The serial diluent was aseptically inoculated on plates of sterile Potato Dextrose Agar (PDA). Sub-culturing was carried out until pure cultures of *A. niger* was obtained. The fungus was grown on slants of potato dextrose agar (PDA) for five days before storage and maintained at 4 °C on PDA.

2.3. Screening for amylolytic activity

The amylolytic activity of the test isolates was determined by using the starch agar plate method as described by Alfred (2007) by inoculating the test organism into 1% starch PDA medium. The agar plates were then incubated at room temperature for five days. After the incubation period, Lugol's iodine solution was added to the culture plate to identify the zones around the cultures. The diameter of the halozone formed after the addition of iodine solution was measured to represent the amylolytic activity.

2.4. Solid-state fermentation

5 g of cassava peel amended with 10 ml of mineral salt solution containing (g/l) KH_2PO_4 0.4 g, H_2O 0.4 g, NaCl 0.02 g, KCl 0.02 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04 g, casein 0.4 g and MnSO_4 0.1 g was taken in 200 ml cotton plugged Erlenmeyer flask, mixed homogenously and sterilized at 121 °C for 15 min in an autoclave. Thereafter, the flask material was cooled at room temperature and inoculated with 1 ml spore suspensions in the laminar airflow with the help of a sterilized pipette. The flasks were then incubated at 30 °C for five days.

2.5. Optimization of process parameters

Various process parameters were optimized for maximal enzyme production as follows: incubation period (24-144 h), incubation temperature (20-50 °C), initial pH (3-9). Experiments were also performed to evaluate the influence of different carbon sources (maltose, glucose, galactose, lactose, soluble starch, cassava peels) and nitrogen sources (yeast extract, peptone, urea, casein, NH_4NO_3 , gelatin and KNO_3 on α -amylase production under the optimized fermentation conditions (Shahzad, 2016)

2.6. Extraction of amylase enzyme

50 ml distilled water was added to each of the fermented media and placed on a rotary shaker at 150 rpm for 60 min. Afterward, the mixture was centrifuged at 4000 rpm for 15 min and used as crude enzyme to measure α -amylase activity.

2.7. Alpha amylase activity

Enzyme activity was measured by using a modified procedure based on the method of Okolo et al. (1995) with modification of Okonji et al. (2019) The reaction mixture containing 1 ml enzyme extract and 1 ml soluble starch solution (1%) was incubated for 10 min at 50 °C in a water bath. Afterwards, the reaction was stopped by adding 1 ml of DNSA reagent and boiled for 5 min. The reaction mixture was allowed to cool at room temperature and absorbance was measured by spectrophotometer at 540 nm (Miller, 1959). One unit (IU) α -amylase activity was defined as the amount of enzyme that releases 1 μg of glucose per minute under the standard reaction conditions. The amount of glucose produced was calculated by referring to the standard plot using glucose as the reducing sugar (The standard plot was prepared simultaneously).

2.8. Estimation of total protein

A method of Bradford (1976) was used to measure the total protein in the aqueous extract of fermented matter.

2.9. Purification of α -amylase

2.9.1. Ammonium sulphate precipitation

For the crude enzyme, 65% ammonium sulphate saturation was suitable to precipitate the enzyme proteins. This was kept at 4 °C for 24 h; thereafter it was centrifuged at 4000 rpm for 30 min. The precipitate was collected and dialysed in 0.1 M phosphate buffer pH 7.5. The alpha amylase activity and protein were determined as described.

2.9.2. Ion exchange chromatography

A volume (2 ml) of the precipitated enzyme was layered into activated CM- Sephadex resin ion exchange chromatographic column and was afterwards washed with 20-25 ml of 0.1 M phosphate buffer pH 7.5. Fractions were collected at a flow rate of 2 ml/4 min. The protein concentration of each fraction was monitored using a UV/VIS spectrophotometer (Beckman Instruments, Inc. Huston Texas) at 595 nm. The alpha amylase activity of each fraction was assayed as earlier discussed with the active fractions pooled together and stored at 4 °C.

3. Results and discussion

In this study, alpha amylase was produced from *Aspergillus niger* by solid state fermentation using cassava peels; food waste as substrate. Various process parameters were optimized and characterization of alpha amylase was carried out.

3.1. Proximate analysis of cassava peel

Raw dried cassava peel was analyzed and was found to have 10.08% moisture content, 6.60% ash content, 11.02% crude fiber, 4.91% crude protein, 1.58% ether extract (fat) and 75.83% total carbohydrate. The percentage of total carbohydrate accounts for three-quarters of other nutritional components thus making TME 419 a good source of substrate for the production of bioethanol. Also, several authors have reported high carbohydrate content in TME tubers with about 83.63% (Uchechukwu et al., 2015), 88.22% (Iwe et al., 2017) 85.44% (Oyeyinka et al., 2019) and as a matter of fact from this study, the peel which is been considered as wastes also contain a considerable amount of carbohydrate making it a suitable substrate for the proliferation of *A.niger* and hence alpha amylase production.

3.2. Optimization parameters

Three isolates were obtained from this study and screened for alpha amylase activity using the Plate assay method. Isolates A, B and C had halozones of 0.2 cm, 0.9 cm and 0.9 cm respectively.

3.2.1. Effect of incubation time

Alpha amylase activity measured for each day of the incubation period in solid state fermentation is shown in Table 1. Alpha amylase was produced by *Aspergillus niger* 24hrs after incubation and there was a progressive

Table 1: Effect of Incubation Time on Alpha amylase activity

Incubation Time (h)	Activity (U/mol)
24	0.002 ± 0.004
48	0.036 ± 0.016
72	0.192 ± 0.021
96	0.221 ± 0.016
120	0.222 ± 0.033
144	0.214 ± 0.019

increase in activity with the peak achieved after 96 h at 0.682 U/ml and thereafter a decline in alpha amylase production. This corresponds to the findings of Gupta *et al.* (2008) where it was reported that the increase in the incubation period resulted in a decrease in the production of α -amylase by the culture of *Aspergillus niger*. This may be as a result of depletion of essential nutrient and the further production of other by-products after maximum production of alpha amylase was achieved. These byproducts are capable of inhibiting the proliferation of fungi and ultimately the production of desired enzymes (Duochaun *et al.*, 1997).

3.2.2. Effects of pH

The effect of pH on alpha amylase activity and stability produced by *Aspergillus niger* as shown in Table 2. The pH for the enzyme reaction was adjusted ranging from 3-9 with the addition of different buffers. This is in agreement with Kirti (2012), who reported that amylases are generally stable over a wide range of pH from 4-11. In this study, the optimum enzymatic activity of 0.215 u/ml was observed at pH 9.0 while the least activity of -0.013 u/ml at pH 7.0. The enzyme activity was seen increasing towards the basic region of the pH while the activity was decreasing towards the acidic portion of the pH. This finding agrees with Senthilkumar *et al.* (2012) who reported that *Bacillus* spp. used for the production of alpha amylase by submerged fermentation have an optimum pH between 6.0 and 7.0 for growth and enzymatic production. However, this differs from Nyamful (2013) experiments who had crude enzymes from *Aspergillus niger* with the highest enzymatic activity at pH 5.0 and when the pH increases, the activity decreases.

Table 2: Effect of pH on alpha amylase activity

pH	Activity (U/mol)
3	0.068 ± 0.128
4	0.040 ± 0.277
5	0.017 ± 0.130
6	0.014 ± 0.120
7	-0.013 ± 0.015
8	0.120 ± 0.029
9	0.215 ± 0.014

3.2.3. Effect of nitrogen source

A suitable nitrogen source is essential for the production of a high amount of α -amylase by fungi. The requirement may be explained by the organism's preference for protein as the nitrogen source for growth and

Table 3: Effect of nitrogen source on alpha amylase activity

Nitrogen source	Activity (U/mol)
Casein	0.041 ± 0.008
Potassium nitrate	0.062 ± 0.001
Gelatin	0.166 ± 0.155
Yeast extract	0.022 ± 0.017
Urea	0.090 ± 0.033
Peptone	0.090 ± 0.018
Ammonium nitrate	0.022 ± 0.003

also for extracellular enzyme production. The amylase synthesis by several microorganisms has been correlated to the presence or absence of various amino acids and complex nitrogen sources in the culture medium (Fogarty and Kelly, 1980; and Hillier et al., 1997). In this study, the tested nitrogen sources of inorganic and organic nitrogen were KNO₃, NH₄NO₃, urea, peptone, yeast extract, casein and gelatin. Among the tested nitrogen sources, potassium nitrate was the best inorganic nitrogen source with amylase activity of 0.062 μ /ml as shown in table 3, while gelatin was the best organic nitrogen source with 62.65% more yield than potassium nitrate. α -amylase activity in presence of gelatin as a nitrogen source was 0.166 μ /ml. Our results confirm an earlier observation that complex nitrogen sources like peptone, gelatin, yeast extract, casein and urea results in the production of a higher amount of amylase than simple inorganic sources of nitrogen like various salts of ammonia and potassium (Thippeswamy et al. 2006).

3.2.4. Effect of temperature

Varying temperatures of 25, 30, 35, 40, 45 and 50 °C of the reaction mixture on the activity of alpha amylase was evaluated. Highest enzyme activity (0.048 u/g) was obtained at 35 °C as shown in Table 4 while the activity of the enzyme showed a declining trend with the increase in temperature. The lowest activity (0.001 u/g) was obtained at 50 °C. This report buttressed the claim of Ayansina and Owoseni (2010), that as the temperature gradually increases, the enzyme activity decreases over time. Also, Gupta et al. (2010) reported similar findings stating that *A. niger* had an optimum temperature of 35 °C when assayed for enzyme activity.

Table 4: Effect of temperature on alpha amylase activity	
Temperature	Activity (U/mol)
25	0.011 ± 0.086
30	0.035 ± 0.015
35	0.048 ± 0.042
40	0.012 ± 0.008
45	0.025 ± 0.03
50	0.001 ± 0.006

3.2.5. Effects of carbon sources

Carbon sources evaluated include Lactose, Soluble starch, Glucose, Maltose, TME 419 and Sucrose as shown in Table 5. The most suitable carbon source was found to be TME 419 which has the highest activity with 0.255 U/mg, followed by Soluble starch while the lowest activity was obtained from sucrose with -0.001 U/mg. This report follows the trend of Oyeyinka et al. (2019) that has been reported that starch gives the

Table 5: Effect of carbon source on alpha amylase activity	
Carbon source	Activity (U/mol)
Lactose	0.019 ± 0.006
Soluble starch	0.127 ± 0.119
Glucose	-0.041 ± 0.167
Maltose	0.08 ± 0.005
TME 419	0.255 ± 0.042
Sucrose	-0.001 ± 0.003

Note: Uncolored rows depict the highest value for each of the parameters in each table.

highest activity of various carbon sources in the study on the optimum condition for alpha amylase production by *Aspergillus niger* mutant isolates using solid state fermentation. Interestingly, TME 419 cassava variety has been proven to contain higher starch content compared to other varieties and the findings of this report also buttress this fact.

3.3. Enzyme purification

The results of the purification of alpha amylase from *A.niger* is summarized in Table 6.

Table 6: Summary of purification of alpha amylase								
	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total activity (U)	Total protein (U)	Specific activity (U/mg)	Purification fold	% Yield
Crude extract	425	0.058	0.1443	24.65	61.33	0.401	1	100
Ammonium sulphate	100	0.00907	0.5758	0.907	57.58	0.0157	0.0392	3.680
Ion exchange	400	0.0338	0.09131	13.52	36.524	0.370	0.921	54.85

4. Conclusion

It was concluded that nutritional and environmental conditions are necessary for the optimum production of Alpha amylase from *Aspergillus niger*. Also, cassava waste that is considered as a waste can be used as a substrate for growing *A. niger* and hence the conversion of waste to wealth.

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