

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.605.035>

Optimization of Culture Conditions Using One-Factor-at-Time Methodology and Partial Purification of Amylase from *Aspergillus niger* of DTO: H5 under Solid State Fermentation

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ABSTRACT

The aim of this study was to isolate amylase-producing fungi, optimize the cultural conditions using solid state fermentation (SSF) and characterize the partially purified enzyme. Six fungal strains isolated from soil and decayed onion samples were screened for their ability to secrete amylase. Culture medium was optimized using One-factor-at-a-Time (OFAT) methodology under SSF. The crude enzyme was partially purified by ammonium sulphate precipitation and the effect of physicochemical parameters on the amylase was investigated. Out of the six fungal strains, isolate F3 showed highest amylase producing ability. Phylogenetic analysis based on partial sequence of the 18S rRNA gene classified F3 as *Aspergillus niger* DTO: H5. Maximum amylase production was achieved within 48 h of cultivation using 5 % (w/v) wheat bran. Optimal SSF conditions which favoured amylase production were: fermentation period 48 h, initial pH 6.0, initial temperature 30°C, substrate to moisture content ratio 1:5 and inoculum size 1.3×10^{10} spores/ml. Crude amylase was partially purified by 80% ammonium sulphate saturation. The enzyme was purified 3.77-fold with specific activity of 36.65 U/mg and percentage yield of 90.53%. Optimum enzyme activity was noticed at 50°C and pH 4.0. Ca^{2+} had the highest stimulatory effect while Hg^{2+} significantly ($p < 0.05$) inhibited the enzyme activity. Presence of Mg^{2+} , Na^+ and Fe^{2+} increased the amylase activity while Cu^{2+} and Zn^{2+} were slightly inhibitory. This study showed that the fungus could utilize cost effective substrates (wheat bran) for amylase production and could be a promising source of the enzyme for allied and biotechnological industries.

Keywords

Aspergillus niger,
Production,
Amylase, Wheat
bran, Optimization,
Characterization.

Article Info

Accepted:
04 April 2017
Available Online:
10 May 2017

Introduction

Amylases are of ubiquitous occurrence and hold the maximum market share of enzyme sales as they account for about 30% of the world's enzyme production (Van der Maarel *et al.*, 2002). Amylases are one the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, production of cyclodextrins for the pharmaceutical industry, detergent

production, fermentation, brewing and textile to paper industries (Kathiresan and Manivannan, 2006). In order to meet the high demands of these industries, there is therefore need for low cost production of amylase.

Amylase is universally distributed throughout animals, plants and microbial kingdoms. However, due to efficient production strategies, microorganisms have substantial

potential to contribute to a number of industrial applications (Sodhi *et al.*, 2005). Fungal enzymes are more preferable to enzymes from other microorganisms because of their Generally Regarded as Safe (GRAS) status (Sindhu *et al.*, 2009). Studies on fungal amylase especially in the developing countries have concentrated mainly on *Aspergillus* species probably because of ubiquitous nature and non-fastidious nutritional requirement of this organism (Abu *et al.*, 2005; Gomes *et al.*, 2005; Okolo *et al.*, 2000).

Amylases are industrially produced by microorganisms either by submerged fermentation (SmF) or solid-state fermentation (SSF). SSF processes present numerous advantages over SmF. The former not only requires a lower volume of liquid for product recovery and a cheap medium for fermentation, it also poses lower risk of contamination on account of unavailability of free flowing substrates. Enzymes produced by SSF have been reported to possess more stable properties and are less affected by catabolic repression than enzymes produced by SmF (Acuna *et al.*, 1995). However, the contents of a synthetic medium are very expensive and uneconomical, so they need to be replaced with more economically available agricultural and industrial by-products, as they are considered to be good substrates for SSF to produce enzymes (Kunamneni *et al.*, 2005).

Agro-industrial residues pose serious problems of disposal, in spite of them being sources of biomass and nutrients. They are generally considered the best substrates for SSF processes and have been reported to be good substrates for the cost effective production of amylases (Kirankumar *et al.*, 2011). Hence, the present study was aimed to accomplish the objective of production of amylase from various agricultural by-products

by *Aspergillus niger* using solid state fermentation technique, the determination of optimized production conditions and the partial purification and characterization of the amylase produced.

Materials and Methods

Fungi isolation from soil and decayed onion samples

Fungi were isolated from soil and decayed onion samples by serial dilution method wherein Potato Dextrose Agar (PDA) media was prepared, autoclaved and poured in sterile petri-dishes. A 0.1ml from various dilutions (10^{-1} - 10^{-5}) of both samples were plated in duplicate on respective PDA agar (containing 10% lactic acid to prevent bacteria growth) which had solidified. After inoculation the petri-dishes were put in the incubator at $28 \pm 2^{\circ}\text{C}$ for 48 h. Their different physical characteristics were used to differentiate the isolated fungi after which they were named properly. The isolates were sub cultured by point inoculation on sterile PDA plates containing lactic acid and incubation was done at $28 \pm 2^{\circ}\text{C}$ for 48 h so as to get pure fungal isolates.

Screening of fungal isolates for amylase production

The ability of the isolates to produce amylase was studied using starch agar media containing the following (gram/litre): yeast extract 1.5, starch 10, peptone 0.5, agar 15, Sodium chloride 1.5, at pH 5.6. The isolates were inoculated on the starch agar media by streaking after which incubation was done at $28 \pm 2^{\circ}\text{C}$ for a period of 48 h. A control having no inoculation was set up for comparison. All the plates including the control were flooded with iodine solution after incubation and the zone of hydrolysis was observed (Jahir and Sachin, 2011).

Identification of the highest amylase producer

The best amylase producer was identified according to its physical/macrosopic features, microscopic characteristics (Lactophenol cotton blue) and molecular characteristics (Aneja, 2003).

Procurement of cheap substrates and their preparation

The cheap substrates used are wheat bran, rice husk, cassava peels and groundnut husk. The substrates were gotten from Oshodi market dump sites in Lagos and agro waste reserve of Federal Institute of Industrial research Oshodi, Lagos. The substrates were prepared by washing, them severally with distilled water, drying them and grinding them using a sterile blender.

Mineral media for enzyme production

The following are the compositions of the mineral media used for enzyme production; Soluble starch (5g/l), Yeast extract (2g/l), Potassium dihydrogen phosphate (1g/l), Magnesium sulphate (0.5g/l), Calcium chloride (0.1g/l), Sodium chloride (0.8g/l). All of the above were dissolved in 1000ml of distilled water after which the preparation was autoclaved.

Preparation of inoculum

Inoculum preparation was done according to the method described by Pandey (1992). Spores from 48 h old slant cultures were used for the inoculation. Spore suspension of the isolate was prepared by pouring ten milliliters (10ml) of sterile distilled water containing two drops of 0.1% Tween 80 to the surface of the slant having copious spore growth. A sterile inoculating needle was used to scrape the spore clumps under aseptic conditions

after which the tube was vigorously shaken to homogenize the suspension.

Production of enzyme by solid state fermentation

Five grams (5 g) of each substrate i.e. wheat bran, rice husk, cassava peel and groundnut husk was transferred into individual Erlenmeyer flasks (250ml) with five milliliters (5ml) of mineral medium (pH 7) i.e. substrate-medium ratio of 1:1. The flasks containing the substrates and the mineral medium were autoclaved and allowed to cool after which they were inoculated with one milliliter (1ml) of 48 h old grown spore suspension of the isolate which gave highest hydrolysis. Incubation was done at $28 \pm 2^\circ\text{C}$ under static conditions. All fermentation set-ups were performed in triplicates. Crude enzymes were extracted after incubation and assayed for their activities. The data represents the mean of three determinations.

Enzyme extraction

After fermentation, the crude enzyme was extracted by shaking the substrate with 0.2 M Phosphate buffer for 30 min in a rotary shaker (250rpm) at a ratio of 1:5 (solid to moistening agent). Filtration of the extract was done using Whatman No.1 filter paper so as to get a clear filtrate which was centrifuged for 20 min at 5000rpm. The supernatant was filtered using Whatman No.1 filter paper to get a cell free supernatant which is the crude enzyme (Kheng and Omar 2005).

Determination of protein concentration in crude enzyme

Proteins in the enzyme preparations were determined by the method of Lowry *et al.*, (1951) with bovine serum albumin as standard.

Assay of enzyme

Determination of amylase activity was done using the method of Miller (1959). Amylase was measured by incubating 1% of soluble starch in 0.2M Phosphate buffer of pH 7.0 at 45°C for 30 min. The enzyme was assayed by using one milliliter (1ml) of crude enzyme solution and adding one milliliter (1ml) of substrate-buffer solution. This mixture was placed in an incubator at 45°C for 30 min after which the enzyme reaction was stopped by adding two milliliters (2ml) of Dinitrosalicylic acid (DNS) reagent. One milliliter of the substrate-buffer solution added to one milliliter (1ml) of distilled water was used as reference blank. All the tubes containing DNS reagent treated reaction products were heated for 15 min in boiling water bath for colour development. The final volume in each case was made to ten milliliters (10ml) by adding distilled water after cooling. Absorbance was read at 540 nm using UV-Visible spectrophotometer and compared with standard curve using 0.1 to 1.0 milligram (mg) of glucose/ml. One unit (U) of enzyme activity was expressed as the quantity of enzyme required to release one micromole (μmol) of glucose per minute per milliliter (ml) under standard assay conditions by using glucose standard curve (Behera *et al.*, 2014).

Production studies

Screening of substrates for amylase production

The impact of the substrates on amylase production were carried out by inoculating five grams (5g) of each substrate containing five milliliters (5 ml) of mineral medium (pH 7) with one milliliter of spore suspension of the organism and incubating at $28 \pm 2^\circ\text{C}$ for a period of five (5) days (Puri *et al.*, 2013) while other parameters were kept constant.

Effect of incubation period on amylase production

Flasks containing five grams (5g) of the substrate were fermented under pre-optimized growth conditions with one milliliter (1ml) of spore suspension and five milliliters (5ml) of mineral medium (ratio 1:1) of pH 7 and incubated at $28 \pm 2^\circ\text{C}$ in rotary shaker. The enzyme was extracted and assayed from 0 h to the 8th day.

Effect of initial moisture on amylase production

The effect of the moisture content on amylase production was tested by varying the substrate to mineral medium ratio (w/v) in ranges of 1:1, 1:2, 1:3, 1:4 and 1:5. Inoculation of the flasks which contained five grams (5g) of the substrate and 5ml, 10ml, 15ml, 20ml and 25ml of mineral medium, respectively was done with one milliliter (1ml) of spore suspension of the organism. The flasks were incubated for 48 h at $28 \pm 2^\circ\text{C}$. Moisture was provided by the medium itself at pH 7.0.

Effect of initial pH on amylase production

The study of the effect of initial pH on amylase production was carried out by varying the mineral medium pH to 3.0, 4.0, 5.0, 6.0 and 7.0. Inoculation of the flasks containing five grams (5g) of the substrate and optimum amount of sterile mineral medium (at various pH, 83.3%) was done using one milliliters (1ml) of spore suspension of the organism. Incubation was done for 48 h at $28 \pm 2^\circ\text{C}$.

Effect of temperature on amylase production

The effect of temperature on amylase production was examined by incubating the inoculated flasks containing a mixture of five

grams (5g) of the substrate with optimized quantity of mineral medium (83.3%) having the optimized pH at various temperature ranges of 20°C - 40°C for 48 h.

Effect of inoculum concentration on amylase production

The effect of the inoculum concentration (based on the number of spores/ml) on amylase production was studied by inoculating the substrate with different inoculum levels from the dilutions 10^{-5} , 10^{-6} , 10^{-7} and 10^{-8} respectively. SSF was carried out for 48 h with 83.3% moisture and incubated at 30°C.

The determination of the inoculum size was done by counting the number of cells per milliliter using serial dilution and plating techniques. One milliliter (1ml) from 48 h old grown culture was taken, serially diluted (10^{-1} - 10^{-8}) and 0.1ml from the dilutions 10^{-5} - 10^{-8} were spread aseptically on PDA agar. The number of spores for each dilution plated was counted and thereafter multiplied by the dilution factor.

Time course study

A time course study was carried out on amylase production using the optimized fermentation parameters. Flasks containing five grams (5g) of the substrate (wheat bran) and twenty five milliliters (25ml) of the mineral medium having pH 6 were inoculated with one milliliter (1ml) of spore suspension of the organism containing 1.30×10^{10} spores/ml and incubated for varying periods of time (1-6 days) at 30°C.

One flask was withdrawn each day for the period of six (6) days and the crude enzyme was extracted using 0.2 M phosphate buffer after which amylase activity was determined using DNS method as earlier described.

Partial purification of amylase

Crude amylase produced from the time course study after a period of two days was purified with ammonium sulphate. Crude amylase (10ml) was concentrated with different concentrations of ammonium sulphate i.e. 60%, 70% and 80% saturations (6g/10ml, 7g/10ml and 8g/10ml respectively). The mixture was chilled at 4°C in a refrigerator overnight to prevent denaturation after which the precipitate was separated by centrifugation at 10,000 rpm for 15 min and the residue dissolved with 0.2 M Phosphate buffer of pH 7.0 in order to obtain the partially purified enzyme, which was assayed for its activity.

Effect of pH on partially purified amylase activity

The enzyme activity was assayed over a pH range of 3.0-10.0 using 0.2 M phosphate buffer. The buffer solution was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme activity. The assay was done in duplicates for each pH using standard assay procedure. The control blank was set up using one milliliter (1ml) of substrate-buffer solution and one milliliter (1ml) of distilled water under standard assay conditions (pH 7.0, temperature-45°C).

Effect of temperature partially purified amylase activity

The enzyme activity was assayed at different temperatures (30°C-70°C). The buffer solution of pH 4.0 was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme activity. The assay was done in duplicates for each temperature using standard assay procedure. The control blank was set up using 1ml of substrate-buffer solution and one milliliter (1ml) of distilled water under standard assay conditions (pH 7, temperature-45°C).

Effect of metal ions on the partially purified amylase activity

The enzyme activity was determined in the presence of various ions at 10m M concentration. The ions studied were Mg^{2+} , Cu^{2+} , Na^+ , Hg^{2+} , Zn^{2+} , Ca^{2+} , Fe^{2+} . The buffer solution of pH 4 was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme activity at temperature of 50°C.

The assay was done in duplicates for each metal ion using standard assay procedure. The control was set up using one milliliter (1 ml) of the enzyme and one milliliter (1 ml) of the substrate-buffer solution (pH 4.0) while the blank was set up using 1ml of substrate-buffer solution and 1ml of distilled water under standard assay conditions (pH 7, temperature-45°C).

Results and Discussion

Identification of the highest amylase producer

The best amylase producer strain was identified using conventional and molecular methods as *Aspergillus niger* DTO: 133-H5 with accession number KX786646.

Screening of substrates for amylase production

The result represented in table 1 shows that out of the four agricultural residues screened, wheat bran gave the highest amylase excretion (305.26±0.00 U/ml) followed by cassava peels (114.45±0.16 U/ml) with a relative enzyme yield of 37.49%.

Rice bran gave the lowest amylase production (36.14±0.33 U/ml) with a relative enzyme yield of 11.84%. Thus, wheat bran was selected as substrate for further study.

Effect of different cultural conditions on enzyme production

Effect of incubation period on amylase production

In figure 1, the effect of various periods of incubation (0-8th day) on amylase production is shown. The results revealed that amylase production was highest after 48 h of incubation with 324.93±0.16 U/ml enzyme activity after which enzyme production decreased with increase in incubation period. Thus, incubation period of 48 h was optimum for amylase production.

Effect of moisture content on amylase production

The effect of moisture content on amylase production is shown in figure 2. The results revealed that as moisture content increased, amylase production also increased. Maximum amylase production (317.31±0.33 U/ml) was observed when the moisture content was twenty five milliliters (25 ml). Hence, twenty five milliliters (25 ml) of the medium/5gram of the substrate (1:5 ratio) was taken to be the optimum moisture level.

Effect of initial pH on amylase production

The impact of the initial pH on amylase production is depicted in figure 3. The synthesis of enzyme increased with increase in pH. The result showed that enzyme production was highest at pH 6.0 after which production decreased with further increase in pH. pH 6.0 was taken as the best for amylase production with activity 304.01±0.16 U/ml.

Effect of temperature on amylase production

The effect of different temperatures of incubation on the production of amylase is

presented in figure 4. The results showed that *Aspergillus niger* DTO: 133-H5 (KX786646) yielded maximum amylase production of 319.47 ± 0.16 U/ml at 30°C . There was a decrease in amylase production at 20°C while further increase above 30°C led to reduction in amylase production.

Effect of inoculum concentration on amylase production

Figure 5 shows the impact of different concentrations of *Aspergillus niger* DTO: 133-H5 (KX786646) on the production of amylase. The results indicated that as the inoculum size decreased from 3.90×10^7 to 1.30×10^{10} cells/ml, there was increase in amylase production from 200.48 ± 0.32 U/ml to 284.69 ± 0.16 U/ml. Thus, inoculum size of 1.30×10^{10} cells/ml was optimum for amylase production.

Time course study of amylase production by *Aspergillus niger* DTO: 133-H5

The result for the time course study carried out on amylase production from wheat bran by *Aspergillus niger* DTO: 133-H5 (KX786646), using optimized fermentation parameters for a period of six (6) days is illustrated in table 2. The crude amylase extract had highest activity of 299.01 ± 0.16 U/ml after 48 h. This was partially purified using ammonium sulphate and used for further studies.

Partial purification of amylase by ammonium sulphate precipitation

As shown in table 3, of the various ammonium sulphate fraction used for the partial purification of amylase, 80% fraction gave the highest activity of 270.5 ± 0.13 U/ml while 70% and 60% fractions gave activities of 258.29 ± 0.38 U/ml and 232.02 ± 0.17 U/ml respectively. The summary of partial

purification of amylase using 80% ammonium sulphate saturation is shown in table 4. The percentage yield from 80% fraction was 90.53% and the purification fold obtained was 3.77. Hence, characterization studies of amylase were done using this partially purified fraction.

Table 4 shows the purification summary of the partial purification of crude amylase using 80% ammonium sulphate concentration. The specific activity of the partially purified amylase was 36.65 U/mg with purification fold of 3.77 and percentage yield of 90.58%.

Effect of pH on partially purified amylase activity

The impact of various pH from 3.0 to 10.0 on partially purified amylase (80% concentration) is illustrated in figure 6. From the observations, partially purified amylase had highest activity at pH 4.0 and the relative enzyme activity was taken as 100%. There was 2.52% decrease in activity as pH increased to 5.0 and 18.73% decrease in activity as pH increased to 6.0. When the pH was reduced to 3.0, there was a decrease in activity by 11.17%.

Effect of temperature on partially purified amylase activity

Highest amylase activity was obtained at 50°C (Figure 7) when partially purified amylase preparation was incubated at different temperatures for a period of 30 minutes using the optimized pH (4.0).

At lower temperatures of 40°C and 30°C , amylase activity decreased by 11.10% and 23.63% respectively while at higher temperatures of 60°C and 70°C , amylase activity decreased by 5.55% and 36.88%, respectively.

Effect of metal ions on partially purified amylase activity

The effect of metal ions on partially purified amylase activity is shown in figure 8. Result obtained indicate that amylase activity was stimulated by the presence of the metal ions; Ca²⁺ (230.88% relative activity), Mg²⁺

(155.02% relative activity), Na⁺ (141.76% relative activity) and Fe²⁺ (139.72% relative activity) while the presence of Hg²⁺, Cu²⁺ and Zn²⁺ ions inhibited amylase activity with mercury ion (Hg²⁺) having the highest inhibitory effect and the least relative enzyme activity of 60.34%.

Table.1 Screening of substrates for the production of amylase

Serial No	Substrates	Amylase activity (U/ml)	Relative enzyme yield (%)
1	Wheat bran	305.26±0.00	100
2	Cassava peels	114.45±0.16	37.49
3	Groundnut husk	101.61±0.32	33.29
4	Rice bran	36.14±0.33	11.84

Table.2 Time course study of amylase production by *Aspergillus niger*

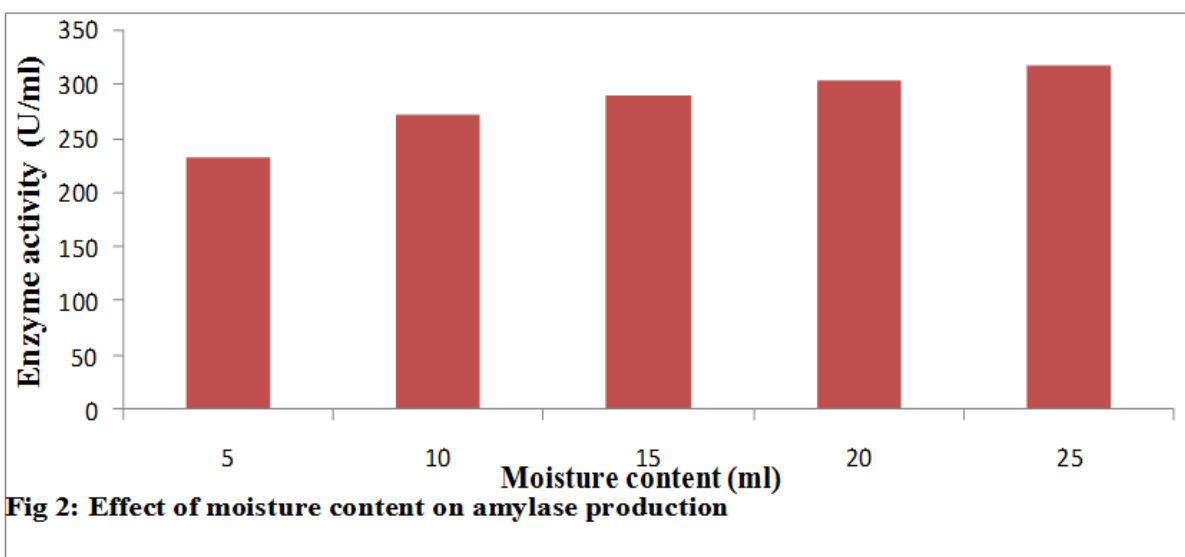
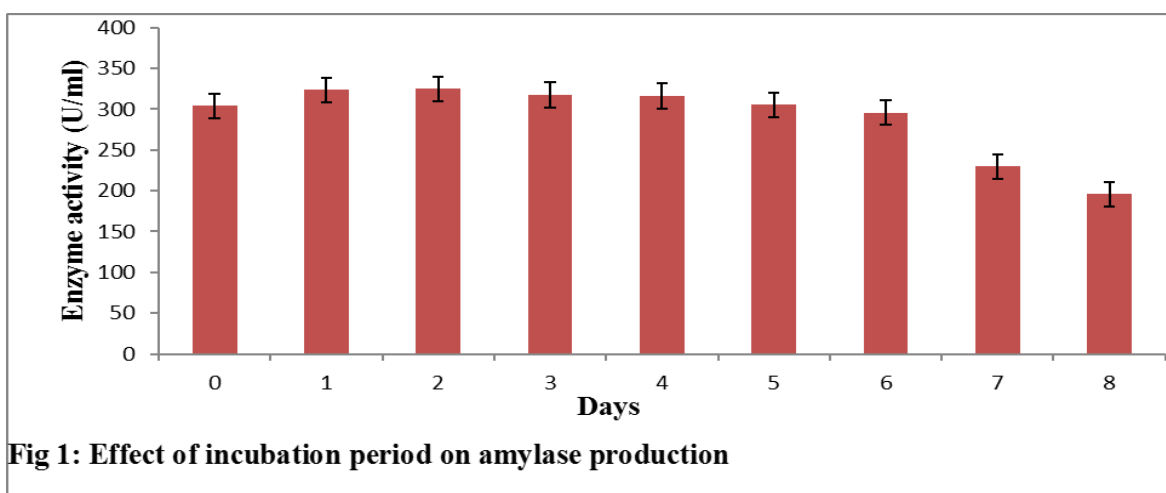
Serial No	Days	Activity (U/ml)
1	1	297.31±0.32
2	2	299.01±0.16
3	3	281.85±0.00
4	4	284.92±0.16
5	5	285.72±0.32
6	6	285.60±0.48

Table.3 Partial purification of amylase using various fractions of ammonium sulphate

Fraction	Amylase activity (U/ml)
60% ammonium sulphate saturation	232.02 ± 0.17
70% ammonium sulphate saturation	258.29 ± 0.38
80% ammonium sulphate saturation	270.5 ± 0.13

Table.4 Summary of partial purification of amylase using 80% ammonium sulphate saturation

Fraction	Enzyme Vol (ml)	Protein Conc. (mg/ml)	Amylase activity (U/ml)	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Purification fold	% yield
Crude extract	1	30.77	298.81	30.77	298.81	9.71	1	100
Ammonium sulphate	10	7.38	270.5	73.8	2705	36.65	3.77	90.53



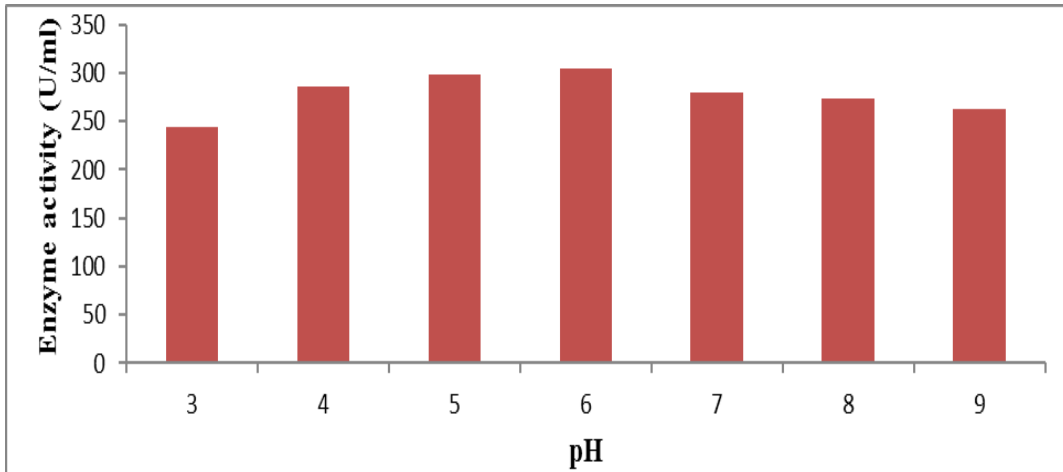


Fig 3: Effect of initial pH on amylase production

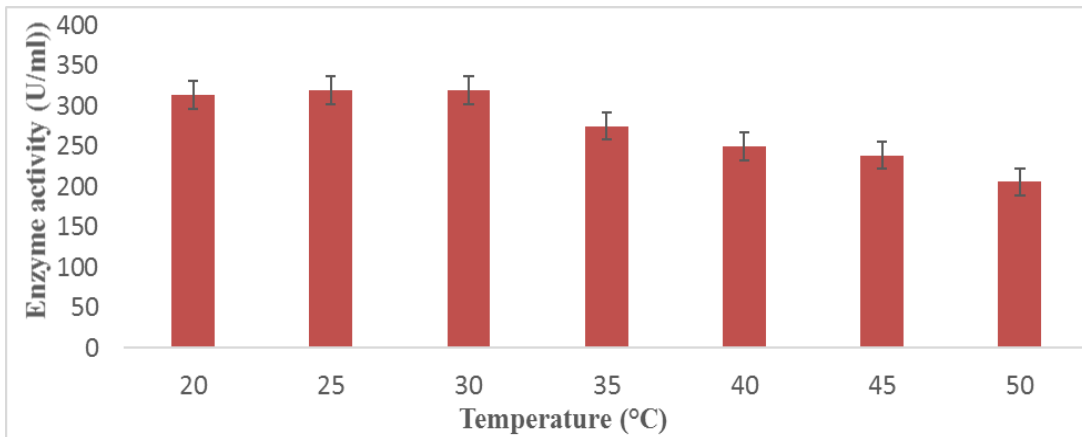


Fig 4: Effect of temperature on amylase production

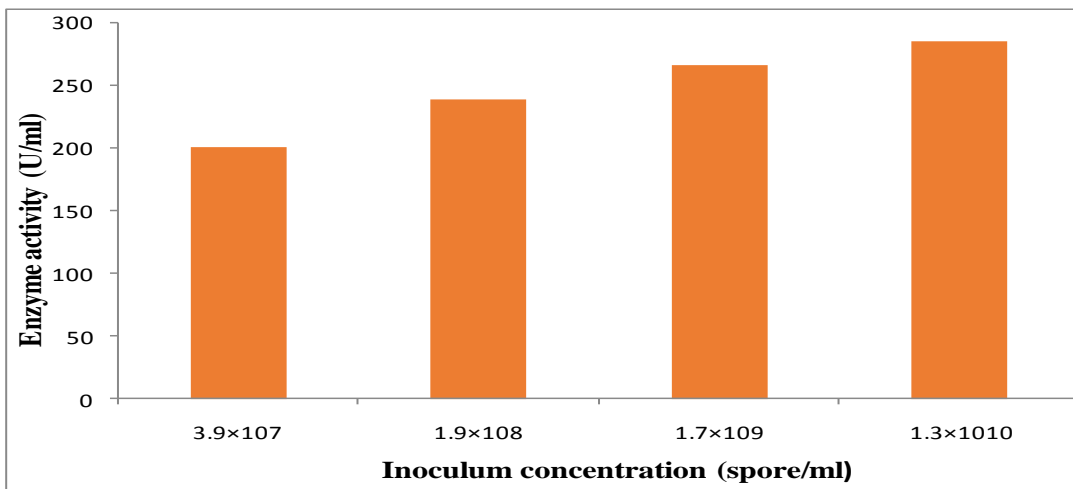


Fig. 5: Effect of inoculum concentration on amylase production

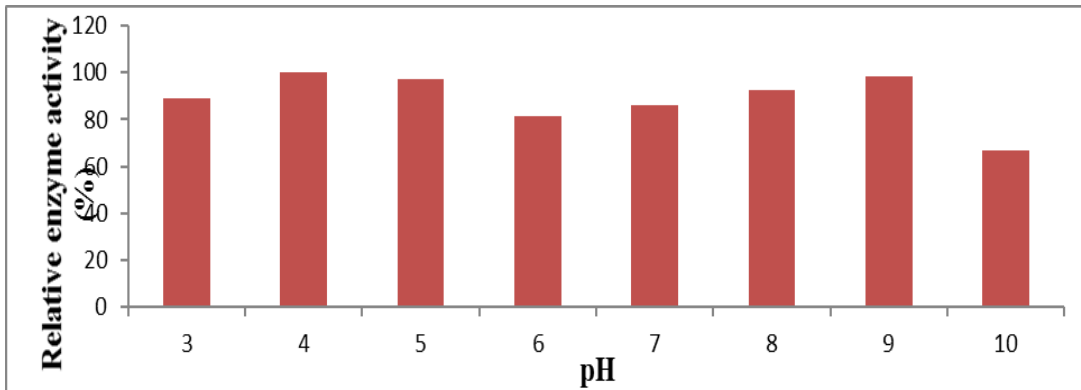


Fig 6: Effect of pH on partially purified amylase activity

(Each pH is expressed in relative enzyme activity with the highest taken as 100%)

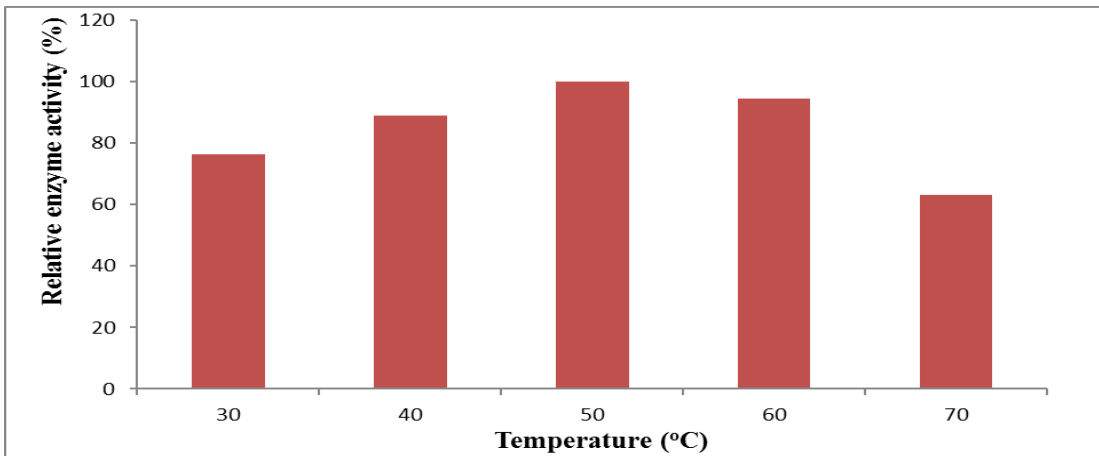


Fig 7: Effect of temperature on partially purified amylase activity

(Each temperature is expressed in relative enzyme activity with the highest taken as 100%)

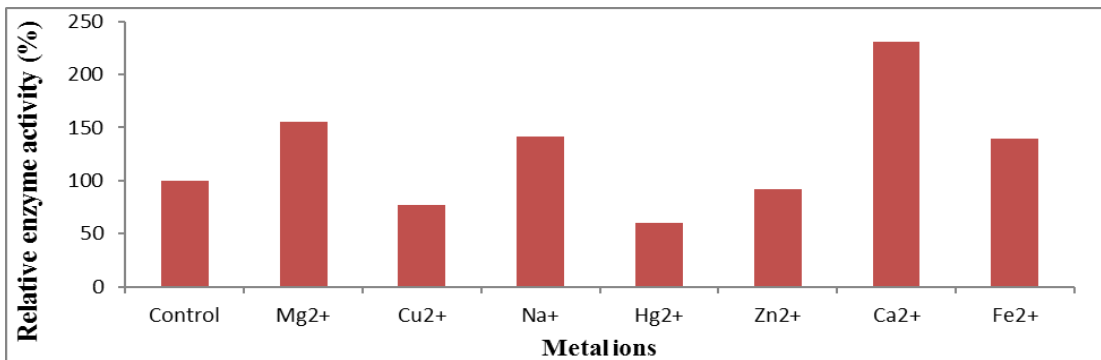


Fig 8: Effect of metal ions on partially purified amylase activity

The result of this study revealed that wheat bran yielded maximum enzyme production thus was selected as substrate for further studies. This result notably corroborates with the reports of Singh *et al.*, (2014) and Ferreira *et al.*, (2015), who stated that maximum amylase production were observed when wheat bran was used as the substrate under SSF by *Aspergillus fumigatus* NTCC122 and *Rhizopus oryzae* respectively. Also, when wheat bran was used as solid substrate there was high production of amylase by the isolate W74 (Tsegaye and Gessesse, 2014). Furthermore, it was reported that wheat bran was the best substrate for the synthesis of amylase by other researchers (Balkan and Ertan, 2007; Singh *et al.*, 2010).

Maximum yield of amylase was observed on the second day (48 h) of incubation with further increase in the period of fermentation leading to a reduction in the production of amylase. The reduction in yield may be because the culture has entered death phase of growth, reduction of nutrient (Arzumanov *et al.*, 2000), the build-up of by-products and different toxins and also deterioration in the enzyme system (Sikander *et al.*, 2003). This result is in contrast with the reports of Chimata *et al.*, (2010), Farid and Shata (2011) and Puri *et al.*, (2013), where other amylase producing fungi gave maximum yield at a period of 5 days. However, this observation corroborates with the findings of George-Okafor *et al.*, (2013) where a period of 48 h was enough for *Aspergillus oryzae*-SR2 to give maximum amylase production in submerged fermentation. When the incubation period was increased, there was a decrease in amylase production. Singh *et al.*, (2012) also reported that *Streptomyces* sp. MSC702 produced maximum quantity of amylase after an incubation period of 48 h. According to Gupta *et al.*, (2008), the decrease in activity at the later phase of growth probably was

because of catabolite repression by glucose which was released from starch hydrolysis. The incubation period differs depending on the enzyme. Short incubation creates room for cheap enzyme production (Somjoy *et al.*, 1995). However, the kind of media used, rate at which microorganism grow on a carbon source and its pattern for enzyme synthesis all affect the incubation time for maximum enzyme production (Grover *et al.*, 2013).

Moisture is very important in the regulation and optimization of solid state fermentation process (Laukevics *et al.*, 1984). The results illustrated that twenty five milliliters (25ml) of medium to five grams (5g) of substrate i.e. ratio 1:5 (83.3%) was the optimum moisture level. In a related manner, the production of amylase was maximum when moisture content was 80% under SSF of rice bran using *Aspergillus oryzae* MTCC 3107 (Puri *et al.*, 2013). Also, in previous studies on the production of extracellular amylases using *Thermomyces lanuginosus* ATCC 58157 by solid state fermentation (SSF) of wheat bran, it was discovered that yield was highest at 90% initial moisture content as compared to 83.3% moisture content in our study (Kunamneni *et al.*, 2005).

Kundu *et al.*, (1984) stated that there were enzyme inhibition and greater diffusion of enzyme from the substrates when the moisture levels were below and above the determined optimal levels respectively. Small quantity of moisture reduces the growth of the organism and the activity of the enzyme and also the level at which the organism access the nutrients, while excess moisture compacts the substrate, obstructs the penetration of oxygen and makes it possible for fast growing bacteria to cause contamination (Laukevics *et al.*, 1984). However, the substrate used affects the optimum moisture level because various types of substrate have different capacities at

which they hold water (Grover *et al.*, 2013).

Maximum amylase production was obtained at initial pH value of 6.0. Results reveal that when the initial pH of the medium was of value higher than 6.0, there was reduction in amylase production. The pH of the fermentation medium is the most critical parameter for amylase production process therefore maintenance of the medium pH is paramount for successful fermentation of amylase. Fungal strains tend to thrive best in acidic medium ranging from 3 to 6 (Fawole and Odunfa, 2003), hence the pH range of 2 to 6 is frequently utilized for submerged and solid state fermentation (Adham, 2002; Lesuiak *et al.*, 2002). Sivaramakrishnan *et al.*, (2006) also opined that slightly acidic pH is needed for fungi growth. Our observations are in corroboration with the reports of Zambare (2010), who similarly reported initial pH 6.0 for maximum yield of amylase of 0.198 IU by solid state fermentation of wheat bran using *Aspergillus oryzae*. Singh *et al.*, (2014), in a study on amylase synthesis and supernatant protein concentration from *Aspergillus fumigatus* NTCC1222 reported maximum yields at pH 6.0 (339.1 U/ml, 8.1 mg/ml respectively). Furthermore, Vidya *et al.*, (2012) in their study on the effect of different initial pH on the synthesis of amylase from *Penicillium chrysogenum* stated that maximum production was at pH of 6.0. Maintenance of the pH value at the beginning of fermentation is necessary for specific biomass formation (Kareem *et al.*, 2010).

Temperature of 30°C gave the highest amylase production from our findings. This result agrees with the reports of Sivaramkrishnan *et al.*, (2007) who stated that maximum amylase production of 15095U/gds for fourteen (14) agro industrial residues was achieved at temperature of 30°C. Temperature for incubation is a very important factor in enzyme production (Seyis and Aksoz, 2003).

Similarly, Nwagu and Okolo (2010) in a study on the synthesis of amylase observed that temperature of 30°C was optimum for maximum amylolytic activity of *Aspergillus fumigates*, which is a thermophilic fungi. Chimata *et al.*, (2010) also reported that amylase production by *Aspergillus* sp. MK07 was highest when the temperature was 30°C. Optimal temperature for efficient production of amylase was reported to be 30°C (Gupta *et al.*, 2008; Alva *et al.*, 2007) and 30-37°C (Ueno *et al.*, 1987, Kundu *et al.*, 1973). According to Simoes *et al.*, (2009), very low and high temperature reduces the synthesis of enzyme due to inhibition of the growth of the organism. Furthermore, when the temperature is low there is membrane solidification while there is damage of the microorganisms by enzyme denaturation which results to low enzyme production when the temperature is high (Willey *et al.*, 2008).

Maximum amylase secretion (284.69 U/ml) was obtained when the inoculum size was 1.30×10^{10} spores/ml. Enzyme production reduced as inoculum size increased. This may be as a result of the limitation of nutrients at higher inoculum size (Tsegaye and Gessesse, 2014). Our results corroborated with the findings of Esfahanibolandbalaie *et al.*, (2008), who observed that increase in inoculum size yielded a gradual decrease in the production of amylase by *Aspergillus oryzae*. This reveals that there is reduction of the surface area available in the medium in the presence of high inoculum; thus, affecting the amount of oxygen needed by the organism to carryout fermentation (George-Okafor *et al.*, 2013). Previous reports showed that high fungal load adversely affected enzyme production (Acharya *et al.*, 2008; Chimata *et al.*, 2010).

Our result indicated that maximum enzyme secretion was obtained after 2 days (48 h) of cultivation with optimized SSF conditions.

The crude enzyme was partially purified using different fractions of ammonium sulphate. Eighty percent (80%) fraction gave highest amylase activity and was used for characterization studies. The purification by ammonium sulphate precipitation (80% saturation) gave 3.77 purification fold and a yield of 90.53%. The specific activity of the purified amylase was 36.65 U/mg while the crude enzyme had a specific activity of 9.71 U/mg. A thermo stable amylase from novel thermophilic actinobacteria *Streptomyces sp.* MSC702 which had 2.98 fold purification and a yield of 56.58% by ammonium sulphate precipitation (40-60% saturation), was reported by Singh *et al.*, (2014). Fifty-five percent (55%) recovery of amylase from *Thermobifida fusca* NTU22 having a purification fold of 1.3 by ammonium sulphate precipitation was reported by Yang and Liu (2004). Purification fold of 1.3 and 4.29% yield of amylase produced by *Geobacillus* LH8 strain was obtained (Mollania *et al.*, 2010). In comparison to the previous reports, a higher purification fold and yield were obtained in this study.

The influence of pH on the activity of the partially purified amylase was studied. Highest amylase activity was observed at pH 4.0 when the activity of the enzyme was measured at different pH (3.0-10.0). Enzyme activity declined between pH 5.0 to 8.0 and rose again at pH 9.0, retaining 98.18% activity. At pH 10.0, amylase activity was least with 66.85% activity retained. Amylases which maintain high activity at low pH are very relevant in industrial processes (Sajedi *et al.*, 2005). Natural starch slurry has pH of 4.5 and its pretreatment in such extreme condition requires using an enzyme which can withstand low pH (Sivaramakrishnan *et al.*, 2006). Furthermore, using amylase that perform very well at lower pH value helps in reducing the formation of certain by-products which are formed at high pH (Goyal *et al.*,

2005). Our result is in corroboration with the reports of Singh *et al.*, (2014) who recorded that that partially purified amylase from *Streptomyces sp* MSC702 had highest activity at pH 5.0. Similarly, Ojo and Ajele (2011) obtained purified amylase which had highest activity at pH 5.0 from cassava peels.

The effect of temperature in other to know the thermo-stability of the partially purified amylase was carried out. Maximum amylase activity was achieved at 50°C when enzyme activity was measured at various temperatures (30°C-70°C). Enzyme activity showed gradual increase from 30°C to 50°C after which there was a decline from 60°C to 70°C, which was the least with 63.12% activity retained. Our result revealed that the partially purified amylase is thermo tolerant in nature, hence having great potential compatibility with conventional industrial processes. In a similar manner, Sexena and Singh (2011) reported that partially purified amylase from *Bacillus sp.* recorded highest activity at 50°C. Singh *et al.*, (2014) reported a thermo stable partially purified amylase from *Aspergillus fumigatus* NTCC1222 which had maximum activity at 55°C. A study by Doss and Anand (2012) reported that partially purified amylase by *Aspergillus flavipes* showed maximum activity at temperature of 60°C to 70°C while partially purified amylase gotten from cassava peels had optimum activity at 60°C (Ojo and Ajele, 2011).

The effect of some metal ions (Mg^{2+} , Cu^{2+} , Na^+ , Hg^{2+} , Zn^{2+} , Fe^{2+} and Ca^{2+}) on the activity of the partially purified amylase was studied. Our result indicated that the metal ions; Hg^{2+} , Cu^{2+} and Zn^{2+} inhibited amylase activity with Hg^{2+} being the most potent inhibitor by up to 39.66%. Inhibition of Hg^{2+} is indicated by residues of indole amino acid being present in the enzyme (Chakraborty *et al.*, 2012). Syed *et al.*, (2009) and Chakraborty *et al.*, (2009) also reported the inhibition of amylases from

Streptomyces sp. D1 and *Streptomyces gulbargensis* by Hg^{2+} ion. The inhibition of amylase activity by Cu^{2+} ion was also reported by Ojo and Ajele (2011). Amylase activity inhibition as a result of the presence of Zn^{2+} ions shows the nature of the thermo stability of the enzyme (Gessesse and Mamo, 1999) while the inhibition as a result of the presence of Cu^{2+} ions reflects the competition between the exogenous cations and the protein associated cations (Singh *et al.*, 2014). The presence of Mg^{2+} , Na^+ , Fe^{2+} and Ca^{2+} increased the activity of the enzyme with Ca^{2+} having the highest activity. In a similar manner, Adejuwon (2011) and Sexena and Singh (2011) reported that the presence of Ca^{2+} , Na^+ and Mg^{2+} ions enhanced the activity of amylase from *Penicillium* species and *Bacillus* species respectively. This result is also supported by reports of Burhan *et al.*, (2003) where the presence of Ca^{2+} ion enhanced the activity of amylase from *Bacillus sp.* ANT-6. Ca^{2+} ion is known for its stabilizing effects on the thermo stability of amylases gotten from various microorganisms (Sivaramakrishnan *et al.*, 2006).

In conclusion the four agricultural waste products (wheat bran, cassava peels, groundnut husk and rice bran) studied in this work showed potentials for amylase production. The study revealed that wheat bran gave the highest amylase production followed by cassava peels, groundnut and rice bran. These substrates are readily available in large quantities in Nigeria and can be of benefit in low cost industrial production of amylase. This study also revealed that amylase producing fungi can be gotten from decayed agricultural wastes. The fungal isolate used for this work was identified as *Aspergillus niger* DTO: 133-H5 (KX786646) using its 18S rRNA sequence analysis.

Wheat bran served as an inexpensive and under-utilized agricultural waste product,

supported the growth of *Aspergillus niger* DTO: 133-H5 (KX786646) and the production of amylase under the fermentation parameters investigated. Optimal SSF conditions which favoured the production of amylase were; fermentation period of 48 h, initial pH 6.0, initial temperature of 30°C, substrate to moisture content ratio of 1:5 and inoculum size of 1.30×10^{10} cells/ml.

The partially purified amylase obtained was found to be thermo-stable and acid stable with highest activity observed at 50°C and pH 4.0. The enzyme was active in the presence of Ca^{2+} , Na^+ , Mg^{2+} and Fe^{2+} ions while its activity was inhibited in the presence of Hg^{2+} , Cu^{2+} and Zn^{2+} ions. This study is therefore, relevant with regard to re-utilization of agricultural waste products and conversion of waste to wealth as well as reducing the level of pollution caused by these agricultural wastes.

The results obtained are significant as they have shown that the enzyme is thermo-active and active under slightly acidic conditions thus suitable for industrial processes which require high temperature and low pH such as textile wet processing, pretreatment of non-gelatinized starch to gelatinized starch, soap and detergent production.

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How to cite this article:

Ire, F.S., O.C. Eruteya and Amaechi, V. 2017. Optimization of Culture Conditions Using One-Factor-at-Time Methodology and Partial Purification of Amylase from *Aspergillus niger* of DTO: H5 Under Solid State Fermentation. *Int.J.Curr.Microbiol.App.Sci.* 6(5): 307-325.
doi: <http://dx.doi.org/10.20546/ijcmas.2017.605.035>