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Studies of the Nutritional, Environmental Effects and Repressive Nature of Simple Sugars on the Production of endo-β-mannanase by *Aspergillus flavus* PT7 on Solid State Fermentation

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Authors' contributions

This work was carried out in collaboration among all authors. Author UEA designed the study, wrote the protocol and managed the analyses of the study. Authors NUS and IAI performed the statistical analysis and wrote the first draft of the manuscript. Author AAO managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The importance of nutritional and environmental factors in the production of microbial enzymes cannot be overemphasized. Hence, endo-β-mannanase production was systematically studied in a step-wise approach of building up on the experimentally observed conditions favouring the production of this enzyme in *Aspergillus flavus* PT7.
Place and Duration of Study: Department of Microbiology, University of Ibadan, Nigeria, between January 2018 and December 2018.
Methodology: Thirty-eight (38) fungal isolates obtained were screened for mannolytic ability using standard method. The highest producer of endo-β-mannasase was subjected to various production

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conditions by adjusting the nutritional and environmental factors in view of optimizing the production of this enzyme in the isolate *Aspergillus flavus* PT7.

Results: Copra meal was the highest inducer of mannanase production in the isolate at enzyme activity of 85.86±3.93 U/gds. Production increased to 94.54±0.42 when all forms of extraneous nitrogen sources were excluded from the production medium. pH 5.0, temperature 30°C, moisture content at 100% v/w and inoculum size of 8.0% v/w led to the increase in production by 44% (enzyme activity of 153.24±5.69 U/gds) in 5 days of incubation. Allowing the production set up additional two (2) days led to production increase with a recorded enzyme activity of 170.34±4.35 U/gds. Production of endo-β-mannanase in *A. flavus* PT7 was observed to be inductive as the presence of simple sugars like glucose, galactose, arabinose and xylose led to extended lag period in the production of the enzymes by the isolate.

Conclusion: Production of endo-β-mannanase by *Aspergillus flavus* PT7 was successfully optimized in a step-wise and systematic experimental study of the nutritional and environmental growth conditions of the isolate.

Keywords: Aspergillus flavus PT7; optimization; fermentation; agro waste.

1. INTRODUCTION

Mannans are the second most abundant hemicellulosic polysaccharides in nature. They are usually encountered in the following families: pure mannan, glucomannan, galactoglucomannan and galactomannan, depending on their biological origin [1]. Mannan exists as non-starchy carbohydrates in cell walls of some plants. They are the major polysaccharides of leguminous seeds, coconut and palm kernel seeds, konjac tubers and guar plants. Mannans are found in plants such as the seed endosperm of certain plant species [2] and have been isolated from ivory nut (*Phytelephas macrocarpa*), date (*Phoenix dactylifera*) and green coffee bean (*Coffea arabica*). They are the major structural units in woods and seeds of these plants [3].

The major enzymes required for the hydrolysis of mannan containing plant biomasses into simple sugars are endo-1, 4-β- mannanases (EC 3.2.1.78) and exo-1, 4-β-mannosidases (EC 3.2.1.25). These two enzymes act on the polymeric backbone of the mannan polysaccharide resulting in the release of manno-oligosaccharides, manno-dissacharides and mannose [4]. However, additional enzymes including β-glucosidases (EC 3.2.1.21), α-galactosidases (EC 3.2.1.22) and acetyl mannan esterases are needed to cleave off the side chain sugars attached at different points on the backbone of mannan polysaccharides to allow a more effective hydrolysis of the plant polymer [4].

Microorganisms producing mannanases are ubiquitous in nature as this enzyme is elaborated by compendia of microorganisms largely isolated from natural environments [4]. A vast variety of bacteria, actinomycetes, yeast and fungi are known to be mannanase producers [5]. Various mannanases have been produced from *Streptomyces sp.* [6], *Bacillus subtilis* [7], *Sclerotium* (Athelia) *rolfsii* [8], *Aspergillus awamori* [9] and *Trichoderma harzianum* [10].

Mannanases have found biotechnological applications in several industrial processes such as food, feed, and pulp and paper industries [11]. However, the application of mannanase is still limited due to low yields and high-production costs [12]. This problem can be mitigated by sourcing for efficient mannanase enzyme producer. Haung and Monk [13] asserted that the best way to source for efficient lignocellulytic microorganisms is to isolates them from lignocellulose biomasses as well. Hence, this work was then designed to source for and produce high levels of mannanase from fungal isolates obtained from degrading mannan-substrates.

2. MATERIALS AND METHODS

2.1 Sample Procurement

Copra meal, palm kernel cake, potato peels and soybean meal were obtained from Bodija Market within Ibadan metropolis and transported to the Laboratory in clean polythene bags. Locust Bean Gum was purchased from Sigma Chemicals (St. Louis, Mo, USA). All other chemicals were of analytical grade.

2.2 Source of Microorganisms

Mannan degrading fungal species used for this work where isolated from degrading Palm Kernel Cake (PKC) and Potato Peels (PT).

2.3 Isolation of Microbial Isolates

The isolation of mannan degrading fungi was carried out on selective medium ML1 [14] using decaying PKC and PT as supplemented carbon sources at different instances. One (1) gram of each of the samples was suspended in 9 mL of sterile distilled water and agitated vigorously for about 15 minutes. One milliliter of the resulting liquid was transferred to ML1 medium using pour plating technique (Kheng and Ibrahim, 2005). The selective medium ML1 was prepared with the following composition (g/L) PKC or PT, 6.0; yeast extract, 0.5; casein peptone, 1.0; KH_2PO_4, 1.0; $(NH_4)_2HPO_4$, 1.0; $MgSO_4.H_2O$, 0.7; agar, 15.0. All plates were incubated inverted at 30°C for 3 to 5 days. Distinctive fungal colonies were sub-cultured on Malt Extract agar (MEA) several times until pure colonies were obtained. Purified isolates were maintained on MEA slants. The slants were stored at 4°C until use.

2.4 Screening for Production of endo-*β*-mannanase

Selective medium ML1 devoid of agar-agar was used to grow the isolates for the production of endo-β-mannanase. The carbon source was replaced with Locust bean gum (LGB) for the purpose of this screening. Elenmeyer flasks (250 mL) containing 50 mL of the above culture medium was inoculated with a 5 day culture inoculum. The inoculum was a 1 cm (diameter) agar piece cut out with the aid of a cock-borer from a pre-cultured agar plates. After 5 days of incubation at 30°C, the cultures were harvested by cold centrifugation at 10000 rpm, 4°C for 15 minutes and quantitatively assayed for the activity of endo-β-mannanase.

2.4.1 Endo-β-mannanase assay

The assay mixture for endo-β-mannanase activity contained 0.5 mL of 0.5% (w/v) Locust bean gum (LBG) prepared in 50 mM sodium citrate buffer, pH 5.0 and 0.5 mL of the culture broth. The reaction mixture was maintained at 50°C for 30 mins. After incubation, 1 mL of DNS reagent was added. The whole reaction mixture was boiled for 10 mins. The development of red-brown colour was measured with spectrophometer (Lamda 25 UV/Vis Spectrophotometer) at 540 nm. One unit of enzyme activity (U) is defined as the amount of enzyme liberating 1 µmol of mannose per minute under the assay conditions.

2.5 Identification of the Screened Isolate

The Isolate showing remarkable mannanase production ability was selected for identification and further experiments. The cultural, microscopic and molecular characteristic of the selected isolate was examined in other to identify it.

2.5.1 Morphological characterization

Purified fungal isolate was transferred to potato dextrose agar plate and incubated for 3 to 5 days in order to observe its cultural characteristics. The cultural characteristics of the Isolate were observed with respect to their appearance, colour, and shapes on PDA plates. Furthermore, the cellular morphology of the isolates was also observed. Wet mount preparation of the fungal isolate was made using lactophenol blue according to the method of Fawole and Oso [15]. The preparation was then examined with ×40 objective lens.

2.6 Molecular Characterization

2.6.1 DNA extraction

Cells of the selected isolate grown in a broth medium was centrifuged using Thermo scientific Sorvall Lynx 6000 super-speed centrifuge at 10000 rpm for 5 minutes and washed with distilled water. This was then resuspended in a 400 µL solution containing 0.3 mg/mL lyticase and 8 µL/mL β-mercaptoethanol in extraction buffer (1 mol/L sorbitol, 100 mmol/L sodium citrate, 60 mmol/L EDTA, pH 7.0), and incubated in a Clifon waterbath (Model: S/W97719) for 3 hrs at 37°C. Then, 1 volume of lysis buffer (2% SDS in 50 mmol/L Tris, 10 mmol/L EDTA, pH 8.0) was added and the mixture shaken gently and incubated at room temperature for 10 min after which 200 µL of 5 mol/L NaCl was added. The suspension was maintained in ice for 2 hrs. The pellet was harvested by centrifugation at 13,000 rpm for 10 mins, then suspended in 200 µL of Tris–EDTA buffer after which the DNA was then de-proteinated with a phenol–chloroform–isoamylalcohol mixture (25:24:1). The aqueous layer was collected and DNA in it was precipitated with ethanol (2 volumes). It was harvested by centrifugation (13000 rpm for 15 min) and then washed in ice-cold 70% ethanol after which the DNA pellets were dissolved in 60 µL of sterile distilled water.

2.6.2 PCR amplification and sequencing of the rDNA internal transcribed spacer region (ITS)

The primers used to amplify the rDNA ITS were ITS1 (CGG GAT CCG TAG GTG AAC CTG CGG) and ITS4 (CGG GAT CCT CCG CTT ATT GAT ATG C) as described by White et al. [16]. The amplification reaction was done in a 50 µL volume containing 20 pmol of each primer, 300 ng of genomic DNA template, 0.25 mmol/L each dNTP, 1.5 mmol/L MgCl, and 0.5 U of *Taq* polymerase. The reactions were run for 34 cycles with denaturation at 94°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 2 min. An initial denaturation during 4 min at 94°C and a final 5-min extension at 72°C were used. Amplified products from PCRs were sequenced using automated sequencer (Chromus Biotech, Chennai). The sequence Similarity search was done for the rDNA sequences using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast/). The unknown organism was identified using the maximum aligned sequence through the BLAST search.

2.6.3 Storage and maintenance of identified isolates

Identified isolate after screening was cultured in PDA slants using MacCartney bottles. They were then stored at 4°C and sub-cultured every 3 to 4 weeks. This isolate has been deposited in the Gene Bank the KR871216 as the accession number.

2.7 Production Characteristic of Endo-β-mannanase by *Aspergillus flavus PT7*

The various factors that could affect the production of mannanase were varied in order determine the conditions best suited for the production of this enzyme. Factors under consideration included carbon source, nitrogen source, time of fermentation, size of inoculums and initial moisture content of the substrates.

2.7.1 Inoculum preparation

After 5 days of cultivation of the fungal isolate at 30°C, the spores were obtained from the slants by shaking them off from the surface of matured their mycelia using 10 mL of sterile distilled water. Total spore count was calculated. 1 mL of the spore suspension was dispensed into a sterile test tube to which 0.1mL of lactophenol blue was added. A sterile syringe was used to introduce the mixture into the Neubeur counting chamber and was counted under x40 objective lens of the microscope. Once initial spore count was obtained, dilution factors were calculated for fermentation needs. In this experiment, spore concentration in fermentation medium was $1x10^6$ spores/mL [17].

2.7.2 Production of of endo-β-mannanase by *Aspergillus flavus PT7* from different carbon sources

The following carbon sources: Copra meal, palm kernel cake and soy bean meal (agro-waste) in solid state fermentation experiment and birchwood xylan, locust bean gum, carboxymethyl cellulose, glucose, xylose and arabinose (refined carbons) in a submerged fermentation were examined for their ability to induce the production of endo-β-mannanase.

Solid State Fermentation with complex carbon substrates for the production of endo-β-mannanase by *Aspergillus flavus PT7*.

In the solid state fermentation experiment, Ten grams of each of the dried carbon source (agro-waste) (dried to a constant weight by oven drying at 60°C) was wetted with ML1 medium (pH 5.0) at 50% (v/w) [14]. The whole content of the flasks were autoclaved at 121°C for 15min after which they were allowed to cool to room temperature. The cooled substrates were then inoculated with the fungal spores at 4% inoculum level and incubated for 30°C for 72 hrs in a stationary mode. The extraction of the produced enzyme was done by adding 100 mL 50 mM Sodium citrate buffer (pH 5) to the fermented matter. This whole content was then centrifuged at 10000 rpm for 10 minutes. The supernatant was as the crude enzyme preparation. The presence of endo-β-mannanase activities was assayed as previously outlined.

2.7.2.1 Submerged Fermentation of refined carbon substrates for the production of endo-β-mannanase by Aspergillus flavus PT7

In submerged fermentation experiment, the ML1 medium (pH 5.0) was supplemented with each of the refined carbon sources at 1% w/v. The whole content of the flasks were autoclaved at 121°C for 15 min. The flasks and their contents were allowed to cool to room temperature. The cooled substrates were then inoculated with each of the

isolates at 4% (v/w) fungal spores and incubated at 30°C for 7 days in a static or stationary condition. At the end of the fermentation, the whole content of the broth was centrifuged at 10000 rpm for 10 minutes. The supernatant was as the crude enzyme preparation. The presence of endo-β-mannanase activities was assayed as previously outlined.

2.7.3 Effect of Nitrogen sources on the production of endo-β-mannanase by *Aspergillus flavus PT7*

Eight (8) nitrogen sources of which six (6) were organic (Urea, Peptone, Yeast Extract, Casein, Tryptone, and Soy bean Meal) and two (2) inorganic (KNO_3 and NH_4NO_3) were applied into the solid substrate at the concentration of 4% (w/w) to study their effect on the quantity of endo-β-mannanase produced the fungal Isolate. Moisture content was kept at 50%, pH 5.0 and incubation was done at 30°C for 7 days.

2.7.4 Test of Environmental factors on production of endo-β-mannanase by *Aspergillus flavus PT7*

In these series of experiments, the cheap agro-waste supporting the production of Mannanase best was used as the main carbon source in solid state fermentation.

2.7.4.1 Effect of initial pH on the production of endo-β-mannanase by Aspergillus flavus PT7

The effect of different pH levels on the production of endo-β-mannanase was done by adjusting the moisture content of substrate with buffered basal medium of varying pH (3.0, 4.0, 5.0, 6.0 and 7.0). The buffers used included 50mM Sodium citrate buffer (pH 3.5-6.0) and 50mM Sodium phosphate buffer (pH 6.0-7.0). Sterilization was done at 121°C for 15 minutes before inoculating with 2% (v/w) fungal spores. The setup was incubated for 7 days at 30°C.

2.7.4.2 Effect of moisture content on the production of endo-β-mannanase by Aspergillus flavus PT7

The effect of the moisture content of the substrate on the enzyme production was determined at five different moisture levels (50, 75, 100, 125 and 150%; v/w) prepared by the addition of basal medium to the substrates prior to sterilization. Thereafter, the set up was inoculated with 4% (v/w) inoculum and incubated for 7 days at 30°C. The pH of basal medium was kept at 5.0 with sodium citrate buffer.

2.7.4.3 Effect of inoculum size on the production of endo-β-mannanase by Aspergillus flavus PT7

The effects of different sizes of inocula on the production of endo-β-mannanase were studied by inoculating the sterilized buffered substrates (50 mM sodium citrate pH 5.0) with 1%, 2%, 4%, 8% and 10%, (v/w) spore solutions. The inoculum was prepared as described previously. One milli Litre (1 mL) of the inoculum contained $1x10^6$ spores/mL. The set up was incubated at 30°C for 7 days.

2.7.4.4 Effect temperature on the production of endo-β-mannanase by Aspergillus flavus PT7

Four different incubation temperatures- 27°C 30°C, 35°C, and 40°C- were used to cultivate the cultures for endo-β-mannanase synthesis according to the method of Rashid et al. [18] so as to study the effect of temperature on the production of this enzyme by *Aspergillus flavus* PT7.

2.7.4.5 Effect of incubation time on the production of endo-β-mannanase by Aspergillus flavus PT7

The time course of fermentation on endo-β-mannanase production was studied by assaying for the amount of each of the enzymes produced at 3, 5, 7, 10 and 14 days in the crude enzyme filtrate taken at this respective time during fermentation. The pH of the fermentation medium was kept at 5.0 and incubation was done at 30°C.

2.7.5 Regulation of the production of endo-β-mannanase by *Aspergillus flavus PT7*

The effect of the presence of easily utilizable sugars as supplements on the production of the endo-mannanase was studied. The carbohydrates used were glucose, galactose, arabinose and xylose. The fermentation setup was supplemented with 2% (w/w) of these simple sugars singly. It was then incubated at 30°C for 7 days, after which the enzymes produced were harvested, assayed and the results compared to the amount of enyzmes produced in the absence of these simple sugars [19].

3. RESULTS AND DISCUSSION

A total of 38 moulds were isolated from degraded palm kernel cake (PKC) and potato peels (PT). The isolate with remarkable ability to produce mannanase enzymes was identified. The identification was done after cultural, morphological and molecular examination of the selected isolates.

The Isolate designated as PT7 was the best producer of mannanase with enzyme activity of 2.11±0.21 U/mL on submerged fermentation. It appears greenish on culture medium; has septate hypha with long conidiosphores. The conidial heads are radiate in shape. It was molecularly confirmed to be *Aspergillus flavus*. As illustrated in an un-rooted dendogram constructed using a neighbour joining tree model (Fig. 1) *Aspergillus flavus* PT7 is closely related to *Aspergillus* sp BAB-3401. This isolate has been deposited in the Gene Bank the KR871216 as the accession number.

3.1 Effect of Different Carbon Sources on the Production of endo-β-mannanase by *Aspergillus flavus* PT7

Mannanase was best produced in the presence of locust bean gum with enzyme activity of 97.35±1.18 U/gds. Copra meal was the second best inducer of this enzyme with an activity of 85.86±3.93 U/gds. This was followed by xylan and CMC with 45.07±4.12 U/gds and 45.97±0.08 U/gds enzyme activity respectively. Production in the presence of soybean meal, PKC, xylose, arabinose and glucose was generally low with the least value of 10.41±1.14 U/gds recorded for glucose (Table 1).

The isolate was able to grow and produce mannanase on all cheap agro-waste (carbon sources) tried out although in varying quantities. The variations in the ability of these carbon sources to support mannanase production in *A. niger* PT4 can be attributed to the composition of carbohydrates in these substrates as well as their nutritional contents [20,19]. Copra meal, of all the cheap agro-waste used as carbon source was the best inducer of mannanase production in the isolate. The proximate composition analysis of CM in the cause of this work revealed that CM has higher protein and moisture content than PKC, thus its ability to support the growth of the isolate and allowed higher enzymes production.

3.2 Effect of Different Nitrogen Sources on the Production of endo-β-mannanase by *Aspergillus flavus* PT7

The effect of different nitrogen sources on the production of mannan-degrading enzymes using the best supporting complex carbon source for each enzyme in a solid state fermentation was experimented. The nitrogen sources tried out included peptone, yeast extract, urea, casein, soybean meal, and tryptone (organic nitrogen sources); others included KNO_3 and $(NH_4)_2SO_4$ (inorganic nitrogen sources) (Table 2). Fermentation where the additional nitrogen was

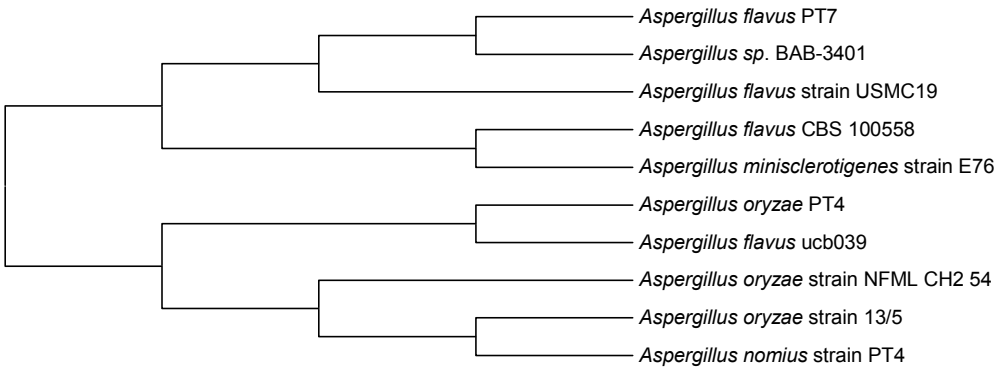

Fig. 1. The phylogenetic tree of *Aspergillus flavus* PT7 based on the 18S rRNA sequence comparism of related isolates. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA6

not added served as the control. Highest activity of 94.54±0.42 U/gds was reached in absence of any supplementary nitrogen source during the production of mannanase by *A. flavus* PT7. All other nitrogen sources gave value lower than that of the control. Mannanase production in the presence of tryptone, KNO_3 and peptone were not significantly different with average activity of 70.00 U/gds recorded. Urea, soybean meal and ammonium sulphate had least support for the production of mannanase.

Table 1. Production the production of endo-β-mannanase by *Aspergillus flavus* PT7 using different carbon sources in a solid state fermentation at 50% moisture level

Substrates	Mannanase activity (U/gds)
Soy-Bean meal	33.44±0.48^{d}
Copra meal	85.86±3.93^{b}
PKC	35.86±0.40^{d}
Xylan	45.07±4.12^{c}
CMC	45.97±0.08^{c}
LBG	97.35±1.18^{a}
Xylose	31.31±4.16^{de}
Arabinose	24.86±0.34^{e}
Glucose	10.41±1.14^{f}

Key: PKC-Palm Kernel Cake; CMC- Caboxymethyl Cellulose; LGB- Locust Bean Gum

The carbon source used in the production of the enzyme (copra meal), just like PKC has high protein content; about 15-20% crude protein (Sundu and Dingle 2003). This is enough to support growth and enzyme production on from isolate. This is similar to observation of Marouk and El Ahwany [19] that omitting nitrogen source from the growth medium of *Bacillus amyloliquefaciens* when potato peel was the sole carbon source resulted in the production of high quantities of mannanase. This indicates that some lingo-cellulosic material can serve as carbon and nitrogen source.

3.3 Effect of pH on the Production of endo-β-mannanase by *Aspergillus flavus* PT7

The influence of initial pH of the culture medium on the endo-β-mannanase production was investigated within the pH range of 3.0 to 7.0 (Fig. 2). The pH of the medium used was regulated by wetting the substrates with a basal medium prepared in appropriate buffer. Mannanase production from *A. flavus* PT7 was highest at pH 5.0 with enzyme activity of 85.31±2.43 U/gds.

There was a gradual decrease in mannanase activity at pH 6.0 and 7.0 with enzyme activities of 62.11±0.59 and 55.97±0.83 U/gds respectively. The optimum pH 5.0 for mannanase production is similar to that reported by [21,22]. Kote et al. [21] and Chantorn et al. [22] reported maximum production of mannanase from *Aspergillus flavus* and *Penicillium oxalicum* KUB-SN2-1 grown in a fermentation medium having an intial pH at 5.0 respectively.

Table 2. Effect of different nitrogen sources on production of endo-β-mannanase using the best supporting complex carbon source for individual enzyme in a solid state fermentation at 50% moisture level

Nitrogen sources (0.4 g/g)	Mannanase activity (U/gds)
Peptone	71.02±1.17^{c}
Yeast Extract	63.71±0.61^{d}
Urea	43.30±1.58^{f}
KNO_3	77.96±0.87^{c}
Casein	63.06±3.31^{d}
Soybean Meal	41.91±0.20^{f}
Tryptone	69.88±0.53^{c}
Ammonium Sulphate	49.43±0.87^{e}
Control	94.54±0.42^{a}

3.4 Effect of Different Moisture Levels on the Production of endo-β-mannanase by *Aspergillus flavus* PT7

Table 3 shows the effect of moisture content on the production of mannanase by the fungi isolate. The moisture content of the substrates was adjusted at different ratios between the substrates and the basal medium added. The basal medium was prepared by dissolving appropriate salts and nitrogen sources in buffers.

Production of mannanase by *A. flavus* PT7 was not significantly different at 50 and 75% (v/w) moisture with 87.28±0.42 and 82.62±14.10 U/gds. At 100% (v/w), highest production of cellulase was reached (124.32±10.79 U/gds). There was a sharp decrease in enzyme production at higher moisture levels of 125 and 150% (v/w).

In solid state fermentation for the production of microbial metabolites, moisture plays a very vital role and drastically influences the fermentation process [23]. Depending on the substrate used and the organism too, moisture demand could be as low as 50% (v/w) and as high as 150% (v/w). This variation could be as a result of difference in

the rate of water absorption by different substrates [23]. Water causes the swelling of substrate thus enhancing good utilization of substrates by microorganisms.

3.5 Effect of Different Inoculum Sizes on the Production of endo-β-mannanase by *Aspergillus flavus* PT7

Inoculum sizes based on standardized number of spores having $1x10^6$ spores/ml were examined using spore concentration of 1, 2, 4 8 and 10% (v/w) of dry substrates. It was found out that increase in inoculum size led to increase in enzyme production (Table 4). Production of mannanase was optimum at 8% (v/w) inoculum size with an activity of 128.56±0.42 U/gds, however this activity was not significantly different from the 120.54 ±10.77 recorded at 4% (v/w) inoculum size. At 10% (v/w) inoculum size, production dropped drastically to 56.96±8.20 U/gds. Mannanase production at 2% (v/w) and 10% (v/w) inoculum sizes though not significantly different, but they were the lowest.

Determining the optimum density of microorganism for enzyme production in solid state fermentation is very important if best production conditions must be met. Inoculum size below optimum will affect the time needed for cells to proliferate, colonize and utilize the substrate and produce the desired products [24]. At the other extreme, higher inoculum density above optimum has been generally observed to affect enzyme production adversely [25]. This can be correlated to the fact that too much microbial biomass is produced as a result of higher inoculum thus leading to depletion of nutrient in shorter time without adequate metabolite production [26].

3.6 Effect of Different Incubation Temperature on the Production of endo-β-mannanase by *Aspergillus flavus* PT7

The effect of incubation temperature on mannanase production by the fungal isolate was examined. Mannanase production was highest at 30°C. Production at 27°C and 35°C were not significantly different. The highest activities recorded for this enzyme was 158.52±10.99. There was a gradually reduction in the quantities of enzymes produced from 35°C to 40°C (Table 5).

This is in line with the observations of other authors [27-29]. Enzymes production at this temperature is applauded because it is still within room temperature thus energy cost can be saved. According to Manpreet et al. [30], solid states fermentation is better operated within the mesophilic range because most of the microoganism used in the solid state fermentation are mesophilic having their optimum growth between 20°C and 40°C. Furthermore, a higher temperature above this could lead to dying up of the available water thus reducing the moisture concentration in the substrates that would have been used by microorganisms for growth and metabolite production.

Table 3. Effect of moisture content on the production of endo-β-mannanase in solid state fermentation incubated at 30°C for 7days

Moisture (%, v/w)	Mannanase activity (U/gds)
50	$87.28±0.42^{b}$
75	$82.62±14.10^{bc}$
100	$124.32±10.79^{a}$
125	$65.55±0.58^{bc}$
150	$57.98±4.38^{c}$

3.7 Effect of Incubation Time on the Production of endo-β-mannanase by *Aspergillus flavus* PT7

Table 6 shows the production of mannanase enzyme between 4 to 18 days of cultivation. Samples were taken at 3 and 4 days intervals and assayed for the quantity of enzyme produced. Production of mannanase increased from 130.23±0.021 U/gds on day 4 to reach a peak on day 7 with 170.34±4.35 U/gds. In subsequent days, there was a gradual reduction in the amount of enzyme produced. Production at day 10 and 14 were not significantly different. The least value of 62.62±6.16 U/gds was recorded on day 18. Wanderly et al. [31] asserted that the incubation time for enzyme production by microorganisms depends on the nutrients present in their growth medium as well as other cultural conditions. According to Jahangeer et al. [32], the kinetics of enzyme production by most fungal isolates is observed to be highest after 7 days and production declines after 10 days of incubation. This decline in enzyme production is usually as a result of decline in the available nutrient for growth and enzyme production.

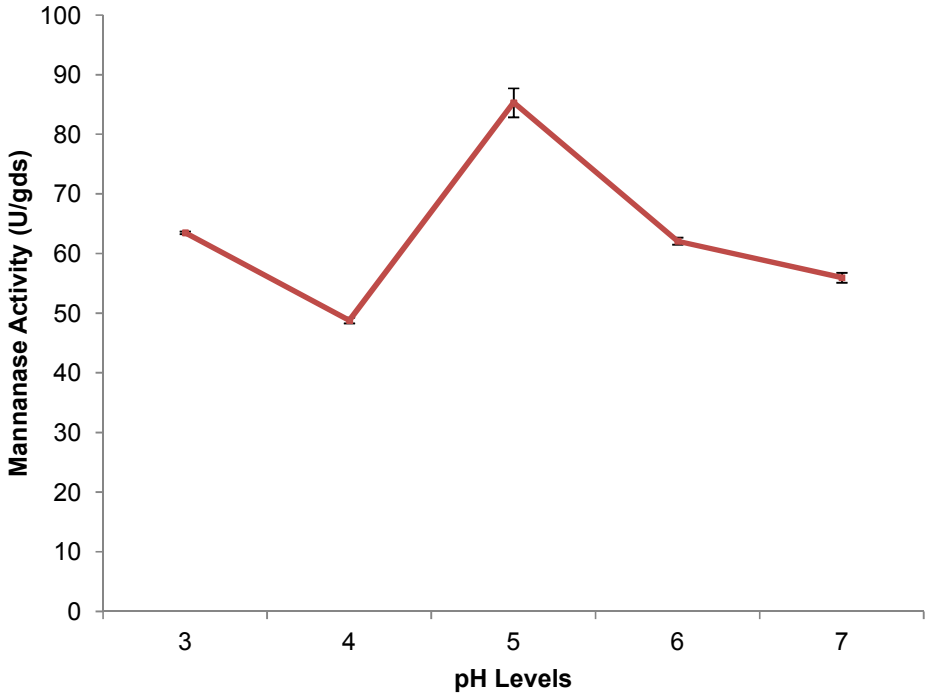

Fig. 2. Effect of initial pH on the production of endo-β-mannanase using the best carbon and nitrogen sources in a solid state fermentation at 50% moisture level

Table 4. Effect of inoculum size (% v/w) on production of endo-β-mannanase in solid state fermentation incubated at 30°C for 7 days using the best moisture level of 100% (v/w)

Inoculum size (%)	Mannanase activity (U/gds)
1	12.39±0.40[c]
2	46.30±0.64[b]
4	120.54±10.77[a]
8	128.56±0.42[a]
10	56.96±8.20[b]

Table 5. Effect of incubation temperature on production of endo-β-mannanase in solid state fermentation incubated for 7days using the best moisture levels and inoculum sizes (8% (v/w)

Temperature (°C)	Mannanase activity (U/gds)
27	100.43±0.19[b]
30	153.24±5.69[a]
35	104.23±0.34[b]
40	40.06±3.70[c]

3.8 Repressive and Inductive Effects of Simple Sugars on the Production of Endo-β-mannanase by *Aspergillus flavus* PT7

Addition of various carbohydrates at 0.2 g/g in the production medium containing CM as substrates in order to evaluate their inductive/repressive effect on the production of mannanase enzymes was tested (Table 7). The sugars used included glucose, galactose, xylose and arabinose. There was a severe inhibitory effect associated with the addition of simpler and easily utilizable carbohydrates on the production of mannanase. Highest repressive effect on mannanase production was in the presence of glucose (92.0.17+0.06 U/gds). Production of mannanase (of which mannanase is one) by microorganisms has largely been found to be inducible [33]. Similar trend was observed by Sachslehner et al. [34] and Mabrouk and El-Ahwany [19] in the production of mannan-degrading enzymes from *Sclerotium rolsfii* and *Bacillus amyloliquefaciens* respectively. They both reported inhibition in the production of mannan-degrading enzymes in these organisms

in the presence of easily utilizable glucose monomers.

Table 6. Effect of incubation time on the production of endo-β-mannanase in solid state fermentation incubated for 7days using the best moisture levels and inoculum sizes (8% (v/w)

Incubation time (days)	Mannanase activity (U/gds)
4	130.23±0.21[b]
7	170.34±4.35[a]
10	117.71±0.10[c]
14	113.63±0.21[c]
18	62.62±6.16[d]

Table 7. Repressive and Inductive effects of simple sugars (0.2 g/g) on the production of endo-β-mannanase in a solid state fermentation

Sugars (0.2 g/g)	Mannanase activity (U/gds)
control	170.01±0.14[a]
Glucose	92.17±0.06[c]
Galactose	95.21±0.10[b]
Arabinose	91.01±1.22[c]
Xylose	95.37±0.30[b]

4. CONCLUSION

In this study, endo-β-mannosidase was successfully produced using *Aspergillus flavus* PT7. The production was systematically optimized by building on previously established conditions favouring the production of this enzyme by the isolate. Using the optimum conditions of Copra meal as carbon source, no nitrogen source, pH 5.0, temperature 30°C, moisture content of 100% v/w. inoculum size 8.0% and 7 days of incubation; production was increased from 85.86±3.99 to 170.34±4.35 U/gds. The production of endo-β-mannanase by *Aspergillus flavus* PT7 was established to be indicative.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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