

Optimizing α -amylase Production from locally Isolated *Aspergillus* species Using Selected Agro Waste as Substrate

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ABSTRACT

The purpose of this research is to determine whether it is possible to produce α -amylase from agricultural waste (groundnut shell). The strain (M1) identified as *Aspergillus* sp. exhibited the largest clearance zone (1.6 cm) and was used in fermentation studies. The activity of α -amylase increased after 24 hours of fermentation, peaked at 72.3 U/mL on day 5, and then began to decline. The effect of optimized environmental conditions studied using OFAT, and it was discovered that pH 6, inoculum size of 1×10^7 spores/mL, incubation period of 120 h, substrate concentration of 3 percent (w/v), and temperature of 35 °C were the best for producing α -amylase from groundnut shell using *Aspergillus* sp. In a single fermentation, these optimum conditions were used, and the experiment yielded an optimum enzyme yield of 121.3 U/mL. This research shows that groundnut shell, a low-cost and commonly available waste, could be an ideal substrate for the manufacture of value-added products.

KEY WORDS: α -AMYLASE, OFAT, FERMENTATION, OPTIMIZATION, *ASPERGILLUS* SPECIES.

INTRODUCTION

Groundnut (*Arachis hypogea*. L) originated in Latin America and was introduced to West Africa by Portuguese traders in the 16th century. Developing nations account for 97% of acreage and 94% of worldwide crop production. (Tela et al. 2021). Nigeria was the third largest producer of groundnuts in the world in 2021, trailing only China and India, with output values of 16,685,915, 6,857,000, and 3,028,571 tonnes respectively (Shanthala et al. 2022). Several researchers have stated that cultivating microorganisms on lignocellulosic materials is a promising strategy for creating enzyme, which will reduce the cost of producing enzyme. Lignocellulosic materials are abundant in the environment, accounting for half of all terrestrial biomass (Sabino et al. 2021) Lignocellulosic wastes are a complex structure made up primarily of cellulose, hemicellulose, and lignin, which are linked by covalent bonds to create a complex network resistant to microbial invasion. Lignocellulosic wastes are a low-cost source of enzyme production (Sabino et al. 2021). To keep lignocellulosic waste from becoming a nuisance in the environment, it has been used to make a

variety of value-added goods, such as enzymes (Santana et al. 2021). Agricultural and industrial operations produce lignocellulosic waste. Sugarcane bark, bagasse and straw, rice straw and rice bran, cassava peel, maize cobs and straw, wheat chaff and bran, banana straw, cassava peel, wood scraps, and groundnut shell are only a few examples of lignocellulosic wastes produced in Nigeria (Igbokwe et al. 2022). It may interest you to learn that these plentiful wastes are underutilized, contributing to pollution problems in the environment. Bioethanol, enzymes, organic acids, biosurfactants, biogas, biohydrogen, and biofertilizers have all been developed using lignocellulosic wastes as a viable substrate due to their high nutrient content (Chen et al. 2022).

Amylases are used to hydrolyze polysaccharides like starch into simple sugar constituents in the starch processing industry. As new opportunities in biotechnology have emerged, the range of amylase applications has expanded to include analytical chemistry, medical and pharmaceutical applications. (Almulaiky, et al. 2021). Amylases are one of the most essential enzymes and are very important in biotechnology; they are a class of industrial enzymes that account for around one quarter of the global enzyme market (Kalia et al. 2021). The key benefit of using microorganisms to produce amylases is the least expensive

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of mass production as well as the ease with which microbes can be influenced to produce desired enzymes. However, the cost of generating α -amylase is so expensive, there is a need to develop more cost-effective methods of producing the enzyme. This can be accomplished by making use of widely available and abundant wastes, such as groundnut shell, which is a common solid waste in underdeveloped countries. Its ability to produce α -amylase will facilitate waste management at a low cost, minimize pollution caused by garbage, and expand the country's economic basis. The goal of this study is to produce α -amylase from cheap and readily available waste (groundnut shell) by optimizing the fermentation conditions by One Factor at a Time (OFAT). The enzyme will be produced by locally isolated *Aspergillus* sp. isolated from soil samples.

MATERIAL AND METHODS

Groundnut shells were collected from local farmers in Kano State, Nigeria, then crushed into fine powder in a milling machine. The glassware which include test tubes, MacCartney bottle, beakers, conical flask and measuring cylinder were washed with detergent and rinsed thoroughly with water. They were allowed to dry, wrapped in aluminum foil and then sterilized at temperature of 160 °C for 60 minutes. The inoculating loop and cork-borer were sterilized by dipping in flame until they were red hot. The spatula was sterilized using alcohol and bent glass rod was sterilized with alcohol and flame (Pasin et al. 2020).

Potato Dextrose Agar was prepared following the manufacturer's specification while starch agar was prepared following the method described by (Saha and Mazumdar, 2019). The media were sterilized at 121°C for 15 minutes. Soil samples were used to isolate amylase-producing fungus. The soil samples were taken at a landfill site for cassava garbage. Before plating on Potato Dextrose Agar using the pour plate technique, the samples were serially diluted to a concentration of 10⁻⁴. The fungal plates were incubated for 3-7 days at 30°C. Isolates with unique clusters were carefully selected after incubation and sub-cultured on fresh media to obtain pure culture. At 4 °C, pure cultures were kept in agar slants (Pasin et al. 2020).

All fungi isolates were identified conventionally. Microscopic and macroscopic views of the isolates were used for identification. The colonial morphology of the fungal isolates on the plates was used to identify them. Cultural and morphological factors such as the nature of the hyphae, color of the colonies, appearance of the colonies and growth rates were taken into account for proper characterization of the isolates, as described by (Ani et al. 2021). This involved using a sterile needle to pick a small bit of mycelial mat and placing it on a clean glass slide, staining with lacto-phenol cotton-blue, and covering with a cover slip. There were reproductive and vegetative structures found. During microscopy, the type of spores, sporangia, hyphae branching, and the presence of septa were all considered. A fungal atlas was used to identify the isolates (Ani et al. 2021).

The best isolate used for the production of fungal amylase

was being identified at Centre for Biotechnology Research Kano, Kano, Nigeria. The methods used were the sequencing of ITS1 and ITS2 regions of the genomic DNA, followed by comparison of sequence similarity with other fungi on the National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Screening was performed on the isolated organisms using the method outlined by (Saha and Mazumdar 2019). Peptone, 0.90 g/L; (NH₄)₂HPO₄, 0.40 g/L; KCl, 0.10 g/L; MgSO₄·7H₂O, 0.10 g/L; starch soluble, 10 g/L and 2 percent (w/v) agar-agar were included in the amylase agar. The isolates were streaked on amylase agar and cultured for 7 days at 30 °C. After incubation, the plates were filled with iodine solution and incubated for another 30 minutes. After that, the plates were rinsed with double distilled water and looked for a starch hydrolysis zone of clearance around the colony expansion. As amylase producers, microbial colonies with the highest zone of clearance were chosen.

Using an inoculating loop, spores from 7-day-old fungal cultures were scraped and aseptically transferred to sterile distilled water comprising 0.1 percent Tween-80 as described by El-Ghonemy (2021). To optimize the inoculum size, incubation duration, pH, temperature and substrate concentration, One Factor at a Time (OFAT) was employed. To modify the appropriate moisture level, the fermentation media contained 2 mL of mineral salts solution comprising of 2g potassium dihydrogen phosphate (KH₂PO₄), 5g ammonium nitrate (NH₄NO₃), 1g sodium chloride (NaCl) and 1g magnesium dihydrogen sulfate (MgSO₄·7H₂O) in a liter of distilled water. All the ingredients were combined, autoclaved for 20 minutes at 121°C, then allowed to cool. After that, spore suspensions were placed on a sterile solid substrate and incubated at different temperatures (El-Ghonemy 2021).

Temperature, incubation time, pH, inoculum size and substrate concentration are all key factors in enzyme development and have a significant impact on enzyme activity. The optimization experiments were conducted using one factor analysis with three replicates for each determination according to Batista et al. (2021). Fermentation was observed for 7 days at 30 °C. After 24, 48, 72, 96, 120, 144, and 168 hours of incubation, samples were taken to determine amylase activity. The temperature for incubation was set within the range of 30, 35, and 40 °C for the selection of the optimum temperature for amylase production, and the amylase test was performed at the end of the optimum incubation period. Using a pH meter, the pH of the fermenter was modified to several levels ranging from (3.0, 4.0, 5.0, 6.0 and 7.0) using two buffer systems (1N NaOH and 1N HCl). The amylase assay was performed at the previously determined optimum time. Each fermentation medium was fed with varying inoculum sizes (1 × 10⁵ and 1 × 10⁷ spores/mL). Amylase activity was assayed at the end of the optimum incubation period.

Different concentrations of the substrates (groundnut shell) at 1 %, 2 %, 3 %, 4 %, and 5 % (w/v) were used in separate fermentation flask. Amylase activity was assayed at the end of the optimum incubation period. The fermented quantity

was combined with 50 mL distilled water at the end of fermentation and stirred for 1 hour on a rotary shaker at 150 rpm. After filtering through Whatman filter paper No. 1, it was centrifuged for 15 minutes at 6000 rpm. The cell-free supernatant was extracted as crude enzyme for further investigation (Olakusehin et al. 2021).

According to Miller's DNSA assay method, the amount of reducing sugars produced in a mixture containing 1.0 mL soluble starch in phosphate buffer, pH 6.0, and 1.0 mL enzyme extract was measured to determine α -amylase activity (Deshayath et al. 2020). After 10 minutes of incubation in a water bath at 50°C, the reaction was stopped with 1.0 mL of dinitrosalicylic acid (DNSA) reagent and the mixture boiled for 15 minutes. After allowing the test tubes to cool, the absorbance was measured at 540 nm with a UV spectrophotometer. The glucose concentration

discharged was compared to a glucose standard. Under assay conditions, one enzyme activity unit (U) was described as the quantity of enzyme that released one mole of reducing sugar per minute per milliliter.

RESULTS AND DISCUSSION

The results of this work aimed at optimizing alpha amylase production from locally isolated *Aspergillus* sp. using agricultural waste (groundnut shell) as substrate are documented in the subsections below:

Isolation and characterization of fungal isolates: Six fungi were isolated from the soil samples and were given the letters M1, M2, M3, M4, M5, and M6. Table 2 lists the macroscopic and microscopic properties of the isolated fungi, whereas Plates 1-6 depict a microscopic perspective of their vegetative structure.

Table 1. Macroscopic and microscopic characteristics of isolated fungi

Isolate Code	Colonial Characteristics	Morphological Characteristic Under Microscope	Identity
M1	Filamentous with white hyphae, production of black spores was observed on the plate after 72hrs. The reverse of the plate was brown.	Conidiophores were hyaline, erect, simple, thick-walled, enlarged at the apex, forming globose vesicles containing catenulate conidia with conidial heads	<i>Aspergillus niger</i>
M2	Growth was rapid and filled the plate completely within a few days. Colonies were whitish. Dense and cottony which became greyish-brown with age, due to brownish sporangio-spores and brown black sporangia. Mycelia were interwoven Pin like green growth.	Well-developed hyphae, branched freely, coenocytic. Brown colored, smooth walled. Non-septate and erect sporangio-phores developed from the hyphae	<i>Rhizopus stolonifer</i>
M3		Non-Branched conidiophore with bulb end carried conidia.	<i>Aspergillus flavus</i>
M4	Yellow-orange, ochraceous, or buff colonies with restricted growth.	Non-dense colonies, sporulated, amber-colored, flaky texture, white mycelium with yellow to pale orange or gray gold reverse. Strong presence of light brown sclerotia	<i>Aspergillus ochraceus</i>
M5	Colonies showed slow growth, primarily olivaceous-brown to blackish brown, but also brown, grey or buff, suede-like to floccose, and frequently powdery due to abundant conidia production.	Conidiophores were erect, straight or flexuous, unbranched or branched only in the apical region and elongated with geniculate sympodial elongation in some species.	<i>Cladosporium</i> sp.
M6	Colonies were rapidly growing, white, flat, to cream in dry, color and finely suede-like, with no contrary pigment.	The holoarthric fragmentation of undifferentiated hyphae produces chains of hyaline, one-celled, smooth, sub-globose to cylindrical, slimy arthroconidia (ameroconidia).	<i>Geotrichum candidum</i>

Screening of isolated fungi for α -amylase activity:

Three out of the six isolates exhibited obvious zones of clearance on the amylase agar with the highest (greater than 1 cm) occurring in isolates M1 (1.6 cm) and M4 (1.2 cm) which significantly differ from others, as shown in Table 3. The isolate (M1) with the highest zone of clearance was therefore selected for further studies like molecular investigation to confirm the authentication of its identity as shown in Table 4.

Using locally isolated *Aspergillus* sp., the current study attempts to optimize α -amylase synthesis from groundnut

shell. The findings of this study demonstrated that *Aspergillus* sp. could produce α -amylase from the substrate (groundnut shell). Six (6) distinct fungal isolates were obtained from various samples (Table 2). The isolates' morphological and microscopic properties were investigated and reported (Table 2). After that, the isolates were tested for α -amylase activity to see if they could generate the enzyme. The zone of clearance displayed by the different isolates was evaluated using amylase agar, as described in the screening of amylase producing microorganisms' section above. During the hydrolysis test, the observed zone of clearance revealed that isolate M1 had the largest

diameter of 1.6 cm, which was larger than the other isolates (Table 3). The clearance zones created around the colonies suggest that the fungal isolates can produce extracellular amylase. The findings of this investigation corroborate those of Olakusehin et al. (2021), who claimed that *Aspergillus flavus* S2-OY has amyolytic characteristics. According to Sahnoun et al. (2015), *Aspergillus oryzae* S2 has amyolytic characteristics.

Figure 1: Microscopic view of the vegetative structure of *Aspergillus niger* (x 40)



Figure 2: Microscopic view of the structure of *Rhizopus stolonifer* (x 40)

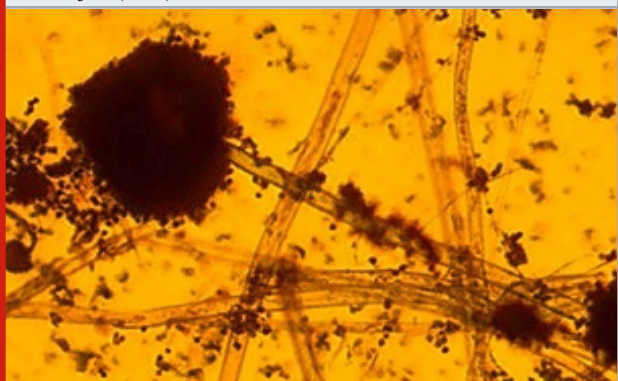


Figure 3: Microscopic view of the vegetative structure of *Aspergillus ochraceus* (x 40)



Figure 4: Microscopic view of the vegetative structure of *Aspergillus flavus* (x 40)

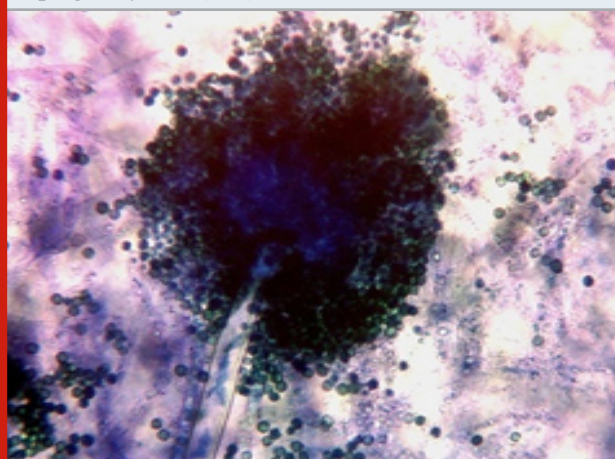
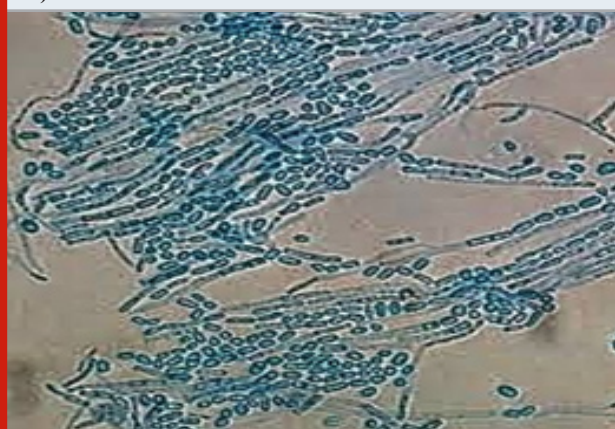


Figure 5: Microscopic view of the structure *Cladosporium* sp. (x 40)



Figure 6: Microscopic view of *Geotrichum candidum* (x 40)



Further research was conducted using isolate M1 (*Aspergillus* sp.). The isolate was used to perform time course fermentation under non-optimized conditions of pH 7.0, 2 % substrate concentration, and 1×10^7 spore/mL inoculum size. The results showed that α -amylase activity peaked at 72.3 U/mL on day 5, with a maximum value of 72.3 U/mL. Using *Aspergillus* sp., a time course

fermentation was used to determine and monitor the trend in α -amylase synthesis from groundnut shell. The recorded enzyme yield is quite similar to that published by (Ahmed et al. 2019), who reported amylase activity of 72.4 U/mL after optimizing with OFAT (Ahmed et al. 2019).

Table 3. Zone of clearance (cm) of different fungal isolates

Isolate code	Tentative name	Zone of clearance (cm)
M1	<i>Aspergillus niger</i>	1.6
M2	<i>Rhizopus stolonifera</i>	0.5
M3	<i>Aspergillus ochraceus</i>	1.0
M4	<i>Aspergillus flavus</i>	1.2
M5	<i>Cladosporium spp.</i>	0.7
M6	<i>Geotrichum candidum</i>	

Table 3. Molecular confirmation of M1

Isolate	Organism	Number of Bases	Identity	Accession number
M1	<i>Aspergillus niger</i> ATCC 16888	576	100.0 %	NR_111348.1

Figure 2: Shows the influence of varied incubation periods (days 1-7) on α -amylase production in *Aspergillus* sp. On day 5, the maximum enzyme activity (72.3 U/mL) was detected.

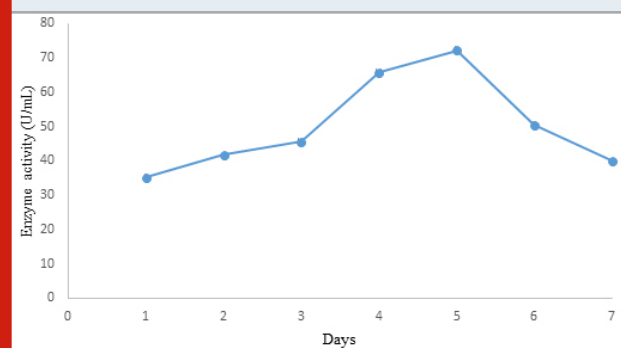


Figure 2: Trend in α -amylase production from groundnut shell using *Aspergillus* sp. Values are means \pm SD; n=3

Table 4. Effect of varying temperature (30, 35, 40 °C) on α -amylase production

Temperature (°C)	Enzyme activity (U/mL)
30	65.2
35	88.2
40	71.3

The impact of numerous parameters was explored using OFAT, which was used to optimize a wide variety of factors and values. The impact of several parameters several parameters were investigated including pH, incubation time, temperature, substrate concentration and inoculum size. The best variables for α -amylase synthesis were pH 6, inoculum size of 1×10^7 spores/mL, incubation length of 120 h, substrate concentration of 3 percent (w/v), and temperature of 35 °C, as determined by an optimization experiment. In a single fermentation, these optimum conditions were used, and the experiment yielded an optimum enzyme yield of 121.3 U/mL. When compared to non-optimized settings, the enzyme activity obtained after optimization was 40 % higher. This is consistent with the findings of (Ahmed et al. 2019), who found amylase activity of 145.4 U/mL after a Response Surface Methodology optimization trial (RSM). The findings of this study shows that groundnut shell can be used to produce value-added products like α -amylase (El-Ghony 2021).

Table 5. Effect of varying pH (3.0 - 7.0) on α -amylase production

PH	Enzyme activity (U/mL)
3	63.9
4	66.0
5	72.7
6	87.3
7	79.6

Table 6. Effect of varying inoculum size (1×10^5 and 1×10^7 spores/mL) on the total α -amylase production

Inoculum size (spores/mL)	Enzyme activity (U/mL)
1×10^5	65.6
1×10^7	73.9

Table 7. Effect of varying substrate concentration (1 % - 5 % w/v) on α -amylase production

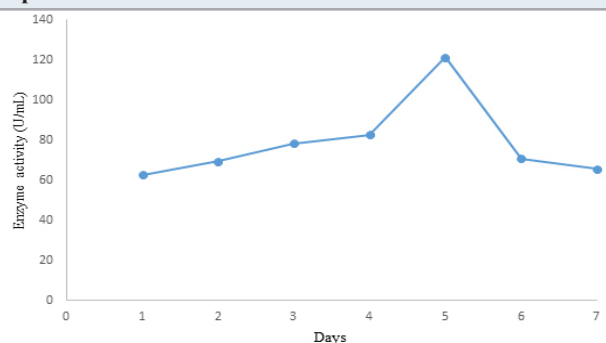
Substrate concentration (%)	Enzyme activity (U/mL)
1	57.2
2	59.3
3	65.1
4	54.9
5	52.3

α -amylase production by *Aspergillus* sp.: Time course fermentation: The result of the pre-optimization experiment under the initial conditions of growth (pH 7.0, 2 % substrate concentration and inoculum size of 1×10^7 spores/mL) is shown in Figure 2. It was observed that α -amylase activity

picked after 24 h of fermentation reaching its peak (72.3 U/mL) at day 5 before declining (Figure 2).

Effect of optimized environmental condition, substrate, and inoculum size on α -amylase production by *Aspergillus* sp.: Figure 2 shows the influence of varied incubation periods (days 1-7) on α -amylase production in *Aspergillus* sp. On day 5, the maximum enzyme activity (72.3 U/mL) was detected.

Figure 3: Trend in α -amylase production using set of optimum conditions



Glucose standard curve

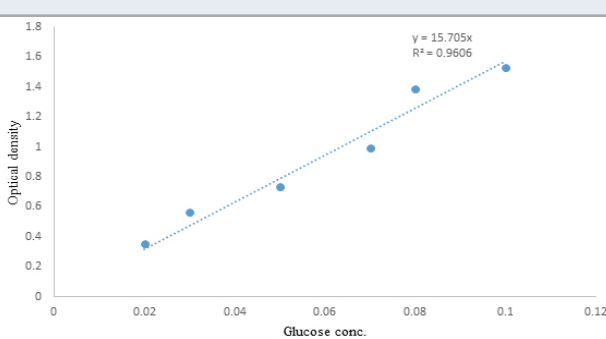


Table 4 shows the effect of temperature (30, 35, and 40 degrees Celsius) on α -amylase production. At 35 degrees Celsius, the greatest enzyme activity was (88.2 U/mL).

Table 5 shows the effect of changing pH (3.0 - 7.0) on α -amylase production. At pH 6, the maximum enzyme production was found (87.3 U/mL).

Table 6 shows the effect of different inoculum sizes (1×10^5 and 1×10^7 spores/mL) on total α -amylase production. Using a 1×10^7 spores/mL inoculum size, the greatest enzyme production was found at (73.9 U/mL).

Table 7 shows the effect of different substrate concentrations (1 % - 5 % w/v) on α -amylase production. At a substrate concentration of 3 % w/v, the greatest enzyme production was obtained (65.1 U/mL).

α -amylase production using optimum conditions: The set of optimum conditions obtained from this study were combined in a single fermentation and the result is shown in Figure 3.

CONCLUSION

The findings of the present study have shown that in non-optimized experiments, the maximum α -amylase concentration was 72.3 U/mL, while the optimum concentration was 121.3 U/mL after optimization with the optimum conditions of pH 6, inoculum size of 1×10^7 spores/mL, incubation period of 120 h, substrate concentration of 3% (w/v), and temperature of 35 °C. The use of groundnut shell as an enzyme substrate proved to be highly promising, with reasonable yields. As a result, these findings imply that groundnut shell, which is typically regarded as a waste, might be exploited as a cheap agro-industrial substrate for enzyme production.

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