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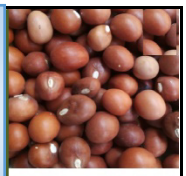

Comparative Analysis of Amylase Enzyme Produced by *Aspergillus niger* Using Rice and Maize Bran as Substrates

A. Bukar[1], U. Musa[2]*, A.B. Yusuf[3], H.U. Puma[2], I.U. Tawfiq[2], I. Mansur[4] and M.M. Ahmad[2]

[1]Department of Microbiology, Faculty of Science, Bayero University Kano, PMB 3011 Gwarzo Road Kano, Nigeria.
[2]Department of Microbiology, Faculty of Science, Gombe State University, PMB 127, Tudun Wada Gombe, Gombe State, Nigeria., Nigeria.
[3]Department of Biochemistry, Faculty of Science, Federal University Gashua, P. M. B 1005 Gashua, Yobe State Nigeria.
[4]Department of Biological Science University of Kashere, P.M.B. 0182 Gombe, Gombe State, Nigeria.

*Corresponding author:
Musa Usman
Department of Microbiology,
Faculty of Science,
Gombe State University,
PMB 127,
Tudun Wada Gombe,
Gombe State,
Nigeria.
Email: musausmanmicro@gmail.com

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ABSTRACT

Amylase is used in the degradation of starch-containing materials into its simpler constituents. This study was aimed at comparing amylase production by *Aspergillus niger* isolated from different sources on different varieties of rice and maize bran. Different strains of *A. niger* were isolated from different sources and were identified using cultural, morphological and molecular characteristics. *A. niger* USGB:1024 isolated from soil obtained from a maize mill and *A. niger* BGSU:17-00030 isolated from spoiled tomato were used for amylase enzyme production as both gave positive results during screening. Amylase enzyme was produced using 15g of substrates, temperature 30 °C, pH 6 and inoculum density of 1 $\times 10^6$. *A. niger* USGB:1024 was found to produce an amylase enzyme with the highest activity (3.15±0.1 IU) on Chinese rice bran at fermentation day 5. *A. niger* BGSU:17-00030 also produced amylase enzyme with the highest activity (1.46±0.01 IU) on Chinese rice bran at 5 days of fermentation. The result has shown that *A. niger* USGB:1024 produced amylase enzyme with the highest activity than *A. niger* BGSU:17-00030.

INTRODUCTION

Amylases are used in many bio-industrial processes and account for a huge fraction of the world's industrial enzyme markets [1]. Amylases catalyse the breakdown of starch to simple sugars which are used as raw materials for various products thereby making the enzymes very important in the food and energy sector [2]. Generally, amylases are classified into α, β and γ subtypes. Fungi, bacteria and yeast are sources of microbial amylase which are mainly used for scientific research and the industrial sector [3]. *Aspergillus niger* group are the fungi we commonly referred to as black mould. The genus is widely distributed from the Arctic region to the tropics. The air everywhere seems to contain the conidia of these organisms, the soil contains the spores of the *Aspergillus* and these organisms are capable of utilizing an enormous variety of substrates for food because of the large number of enzymes they produce, and because of their great enzymatic activities, Amylases are used in several industrial processes and are manufactured commercially by the use of *A. niger* [4]. Agro-based products and wastes were found to be good substrates for the production of many hydrolytic enzymes including amylase, thereby are attracting the attention of researchers [5]. In this regard, brans and flours of different grains and tubers, such as maize, rice, sorghum and wheat, and peels of cassava and potato had been u sed in the development of fermentation medium to increase amy lase production from bacteria and fungi [6].

MATERIAL AND METHODS

Source of Substrate

Two different types of agricultural waste varieties were used as substrates viz. African rice bran (ARB), Chinese rice bran (CRB), Flour maize bran (FMB) and Dent maize bran (DMB) were purchased from Kasuwar Mata within the Gombe

metropolis in a clean container. They were labelled and brought to the laboratory for analysis.

Substrate Processing
The substrates were ground using an electric blender and sieved into a fine powder using a sieve with a 0.5 mm pore size.

Samples Collection
Soil was collected in a container under sterile conditions from a rice mill at Gombe's main market. Spoiled tomato was obtained from Kasuwar mata within the Gombe metropolis.

Isolation of *A. niger* from Different Sources
Different fungi were isolated from soil samples obtained from maize mill and spoiled tomato, by inoculating them onto Potato Dextrose agar plates (containing 0.1 mg/mL Chloramphenicol) using a swab stick and were then incubated at 28 °C for 5 d. Fungi with black colonies with yellow edges obtained from soil samples and tomatoes were observed from the PDA plates and were purified by repeated sub-culturing on separate PDA plates.

Identification of the Isolates
The sub cultured isolates were identified according to the method described by [7].

Screening Fungal Isolates for Amylase Production on Starch Agar Plate
A starch hydrolysis approach on PDA agar plates was used. Clearing zones around the colonies were measured and used to evaluate the level of amylase activity produced by the isolates [7].

Molecular Identification

DNA Extraction
To identify the strains of the isolates producing amylase, the DNA of the isolates was extracted using the protocol of Wizard Genomic DNA purification kit (Promega) following the manufacturer's instructions.

Purification
The purity of the extracted DNA was determined by agarose gel electrophoresis.

PCR amplification and sequencing
For fungi identification, the 18SrDNA and their regions were submitted to PCR amplification using fungus–specific primers, 18D:5'-CCTGGTTGATCCTGCCAGTA, 3',18R:5GCTTGATCCTTCTGCAGGTT 3', ITSI:5' TCCTCCGCTTATTGATATG3'. These generated a DNA fragment of approximately 1.8 and 0.65 kb, respectively. The 18S rDNA and the ITS1 regions were described by White *et al.* [8]. PCR amplification was performed under the following conditions: Initial denaturation at 94 °C for 5 min, denaturation at 94^0C for 50min, annealing at 58 °C for 30 s, extension/ elongation at 72 °C for 30 s. Both denaturation, annealing and extension were done in 35 cycles, 52 °C for 30 s and final extension at 72 °C for 5 min.

PCR products were visualized by electrophoresis in 1% (w/v) agarose gel stained by ethidium bromide using a gel documentation system. The purified PCR product was sequenced in both directions with the IBI Prism 13100-avant Genetic analyser (Applied Biosystems).

Sequences for 16S, 18s rDNA and ITS region were compared with the sequence available in the public database National Centre for Biotechnology Information (NCBI). Based on the BLAST results sequences for other strains were retrieved. All sequences were aligned using Cluster W and the tree was constructed and edited using Molecular Evolutionary Genetics Analysis version 5 [9].

Amylase Production by Solid-State Fermentation
Fifteen grams (15 g) of the different substrates i.e. rice and maize brans were each taken in 250 mL capacity Erlenmeyer flask with 60 mL of mineral medium i.e. 1:4 ratio [10]. The media were adjusted to a pH of 6 and were autoclaved. The flasks were cooled to room temperature and were each inoculated with 1 mL of the spore suspension containing 1 x 10^6 spores/mL and were then incubated at 30 °C for 5 d.

Extraction of Enzyme
The flasks were taken out from the incubator after fermentation and brought to room temperature. The product was recovered from the substrate by shaking it for 30 min in a shaking incubator (250 rpm) with 0.1M citrate buffer at a solid-to-moistening agent ratio of 1:10 of the fermented materials and 0.1 M citrate buffer of pH 6 respectively. The extract was filtered through the Whatman No. 1 filter paper. The filtrate was then centrifuged at 5000 rpm for 20 min. The supernatant obtained was again filtered through Whatman filter paper No. 1 to obtain a cell-free supernatant which was then used as a source of crude enzyme [11].

Quantitative Enzyme Assays
Amylase activity was determined according to the method described by Miller [12]. The enzyme was assayed by using one mL of crude enzyme (culture filtrate), one mL of buffered solution (0.1 M Citrate buffer of pH 5) and one mL of 1% soluble starch. This mixture was incubated for 30 min at 45 °C. The reaction was terminated by the addition of 3 mL of 3, 5-dinitrosalicylic acid reagent containing 10 g of 3, 5 DNSA and 300 g of potassium sodium tartrate. Two mL of buffer (0.1 M citrate buffer pH 6) and one mL of 1% soluble starch solution were used as reference blanks. All the test tubes containing 3, 5-DNS treated reaction products were heated in a boiling water bath for 15 min. The final volume in each case was made to 7 mL by adding 1 ml of distilled water. Absorbance was measured at 575 nm using a UV-Visible spectrophotometer and compared with a standard curve using 0.10 to 1.0 mg of glucose/mL. The experiments were conducted in triplicates and standard error was determined. Enzyme activity was expressed in International Units (IU). One IU is defined as one µmol of glucose (for amylase activity) equivalents released per minute per mL under the standard assay conditions by using a glucose standard curve [13].

Enzyme (α-amylase) activity= $\frac{Product\ concentration \times 1000 \times dilution\ factor}{Molecular\ weight\ of\ glucose \times incubation\ time}$

RESULTS

Isolation and Identification of Fungi
Table 1 shows the result of isolation and identification of fungi from different sources. Two different species of fungi were isolated from different sources and were confirmed as *A. niger* using morphological characteristics and microscopically using lactophenol-cotton blue stain.

Table 1. Cultural and morphological characteristics of fungi isolated from different soil and tomato.

S/N	Sources	Cultural characteristics	Morphological characteristics	Inference
1	SM	Black and with yellow edges	Hyphae are septate, conidiospores terminate in a swollen vesicle	*A. niger*
2	T	Black and with yellow edges	Hyphae are septate, conidiospores terminate in a swollen vesicle	*A. niger*

Keys: T – Tomato
SM – Soil from maize mill

Screening of *A. niger* isolates for amylase production

Fig. 1. *A. niger* on PDA plate (black colony with yellow edges) after 5 d of incubation.

Table 2 shows the result of the screening of *A. niger* for amylase production on starch agar. The result of the screening showed that *A. niger* isolated from soil had the biggest zone of hydrolysis (78 mm), while *A. niger* isolated from tomato had the smallest (38 mm). Both organisms were used for amylase enzyme production.

Table 2. Screening of *A. niger* isolates for amylase production on starch agar.

S/N	Source	Isolates	Zones of Hydrolysis (mm)
1	Soil	*A. niger*	78
2	Tomato	*A. niger*	38

Fig. 2. Zones of hydrolysis (yellow or reddish) produced by *A. niger* BGSU:17-00030 *and A. niger* BGSU:17-00030 on starch agar respectively.

Molecular Identification

A. niger USGB:1024 isolated from soil and *A. niger* BGSU:17-00030 isolated from tomato were characterized using molecular technique and were found to be similar to *A. niger* DTO:129-E9 and *A. niger* IMI 050566 respectively as shown in **Fig. 3**.

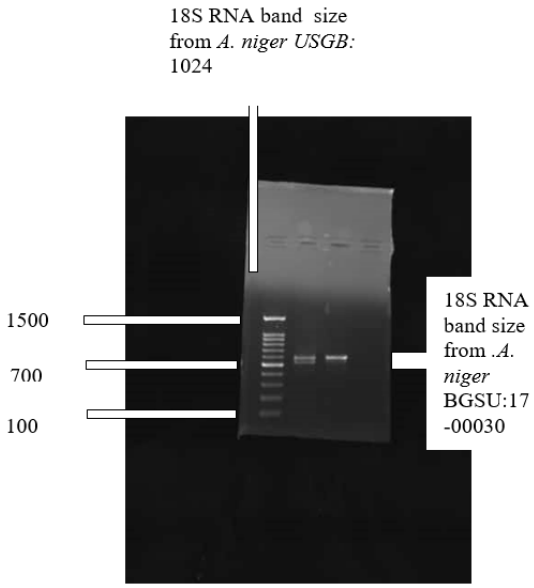

Fig. 3. Agarose (1.5%) 16s RNA amplified fragments for isolates of *A. niger USGB:1024* and *A. niger* BGSU:17-00030.

Optimization Parameters on Enzyme Production

Table 3 shows the result of amylase production using amount of substrate of 15 g, temperature of 300C, pH6, and inoculum density of 106 using *A. niger* USGB:1024 on different varieties of rice (Chinese and Africa) and maize bran (flour and dent) as substrates. The result has shown that the highest amylase activity was obtained on Chinese rice bran as substrate (3.15± 0.06 IU) at day 5 followed by dent maize bran (2.4±0.01 IU) and flour maize bran (2.36±0.010 IU) while African rice bran has the lowest activity (2.21±0.01IU).

Table 3. Amylase Production using *A. niger* USGB: 1024. Medium – 20 mL mineral medium. The amount of substrate was 15 g. The incubation time was 5 d. The incubation temperature and pH were 30 °C and pH 6, respectively. The inoculum concentration was 1×10^6 spores/mL. The data represents the mean values of the experiment performed in triplicate. ± represents standard error.

SC	Amylase activity (IU)		
CRB	2.57±0.08	3.00±0.02	3.15± 0.1
ARB	1.69±0.02	2.01±0.01	2.21±0.02
FMB	1.84±0.02	2.10± 0.01	2.36±0.01
DMB	1.95±0.01	2.18±0.01	2.44±0.01

Keys:
SC- Amount of substrate
DCB - Dent maize bran
FCB - Flour maize bran
CRB - Chinese rice bran
ARB-African rice bran

Table 4 shows the result of amylase production using an amount of substrate of 15 g, temperature of 30 °C, pH 6, and inoculum density of 10^6 using *A. niger* BGSU:17-00030 on different varieties of rice (Chinese and Africa) and maize bran (flour and dent) as substrates.

The result has shown that the highest amylase activity was produced by Chinese rice bran as substrate (1.46± 0.06) at day 5 followed by dent maize bran (1.35±0.02 IU) and flour maize bran (1.21±0.01 IU) while African rice bran has the lowest amylase activities (2.36±0.01 IU). *A. niger* USGB:1024 which has the biggest zone of hydrolysis during screening for amylase production on starch agar produced amylase with the highest activity on all the varieties of rice and maize bran while *A. niger* BGSU:17-00030 which has the lowest amylase activity on all varieties of substrate.

Table 4. Amylase production using *A. niger* BGSU:17-00030. The medium consists of 20 mL of mineral medium. The amount of substrate was 15 g. Incubation time-5 d. The incubation temperature was at 30 °C, incubation pH was pH- 6 and Inoculum concentration was at 1 × 10^6 spores/mL. The data represents mean values of experiments performed in triplicates ± represents standard error.

SC	Amylase activity (IU) *A. niger* BGSU:17-00030		
	3rd day	4th day	5th day
CRB	0.87±0.03	1.08±0.02	1.46±0.01
ARB	0.60±0.01	0.90± 0.02	1.10±0.02
FMB	0.72±0.01	0.89 ±0.02	1.21±0.0
DMB	0.76±0.01	0.96 ±0.01	1.35±0.02

Keys:
SC- Amount of substrate DCB - Dent maize bran
FCB - Flour maize bran CRB - Chinese rice bran

DISCUSSION

Two different isolates of *A. niger* were isolated from different environments. Isolation of *A. niger* from soil obtained from maize mill can be because many different species of fungi are found in the soil, especially near the soil surface where aerobic conditions prevail. Such fungi are active in breaking down different variety of biological materials found in the soil [14,15]. The result of the isolation of *A. niger* from soil has shown that soil especially from the starch processing site is a good source of *A. niger* because it can grow easily in the soil [14].

The isolation of *A. niger* from tomato has shown that fruits such as tomato are a good source of *A. niger* capable of synthesizing cell wall degrading enzymes such as amylase that breaks down large polysaccharides such as starch into simple reducing sugar which is used for growth and multiplication [16]. Isolation of *A. niger* from soil obtained from a mill for amylase enzyme production agrees with previous studies [16,17].

The isolation of *A. niger* from fruits especially tomato is in line with the work of Obafemi *et al.* [16] who reported Aspergillus sp. as fungi associated with spoilage of tomato fruits. Sujeeta *et al.* [14] reported the isolation of different fungi from different sources such as fruits, flour, soil, vegetables, baked food and flour. The isolates screened were found to be positive for amylase production. The widest zone of hydrolysis (78 mm) was produced by *A. niger* USGB:1024. The bigger the size of the clearance produced on the starch agar, the higher the activity produced. According to Thebti et al. [18] the activity of enzymes is revealed in the size of the clearing zone produced around the organisms.

The biggest and the smallest zones of hydrolysis produced by *A. niger* USGB:1024 and *A. niger* BGSU:17-00030 showed that *A. niger* USGB:1024 can produce amylase enzyme in substantial quantity on the starch agar [19] and thereby having a higher potential for the production of amylase enzyme on agro-waste while *A. niger* BGSU:17-00030 has the least potential for the production of amylase enzyme since it produced smaller amount of amylase enzyme on the starch agar. The use of starch nutrient agar and iodine for detecting amylase-producing microorganisms has been reported by Batista et al. [7] that starch hydrolysis can be detected on plates as a clear zone surrounding a colony. This is in line with the result obtained during screening of the different species of *A. niger* isolated from different sources for amylase production. Chinese rice bran was identified as the substrate that produced amylase enzyme with the highest activity using *A. niger* USGB:1024 (3.15±0.1 IU) and BGSU:17 00030 (1.46±0.01IU) respectively. This is because it contains more lignocellulosic substances and is high in dietary content (starch, gum and pectin) compared to maize bran [20].

The result of this research has shown that Chinese rice bran is the best substrate for amylase enzyme production and, thus is a suitable nutrient source for amylase production by microorganisms. The result of this research is not in agreement with the work of Ire *et al.* who reported a maximum amylase activity of 305.26±0.00UI/mL using wheat bran as substrate [21]. It is also not in line with the work of Andi et al., [22] who used *A. niger* with different variety of waste substrates, i.e. corn cob, rice husk and sugarcane bagasse to produce α-amylase and β-glucosidase. The optimum fermentation time for each substrate was found to be 6 d. The highest activity units for α-amylase and β-glucosidase were 81.86 U/mL and 95.02 U/mL using corn cob as substrate respectively.

The result has shown that the highest amylase activity was produced by Chinese rice bran as substrate (1.46± 0.06) at day 5 followed by dent maize bran (1.35±0.02 IU) and flour maize bran (1.21±0.01 IU) while African rice bran has the lowest amylase activities (2.36±0.01 IU). *A. niger* USGB:1024 which has the biggest zone of hydrolysis during screening for amylase production on starch agar produced amylase with the highest activity on all the varieties of rice and maize bran while *A. niger* BGSU:17-00030 which has the lowest amylase activity on all varieties of substrate.

A. niger USGB:1024 produced the highest amylase activity on all the varieties of rice and maize bran using optimization parameters. Chinese rice bran (3.15± 0.1 IU) followed by Dent maize bran (2.44±0.01 IU) and African rice bran produced the amylase enzyme with the least activity (2.36±0.01 IU). This may be due to the place where it was isolated which was known to be a good source of amylase production [23]. The result of this study has shown that *A. niger* USGB:1024 can be used for the commercial production of amylase enzyme since it is capable of producing amylase enzyme with highest activity [19,24]. This result indicates variation in amylase production among different strains of *A. niger* [21,25,26]. It was reported that *A. niger* isolated from soil have higher amylase production on different agricultural residues [8,15,24,27–29]

CONCLUSION

Two different species of *A. niger* were isolated from different sources and identified using molecular techniques as *A. niger* USGB:1024 and *A. niger* BGSU:17 00030 isolated from soil and tomato respectively. *A. niger* USGB:1024 and *A. niger* BGSU:17 00030 were observed to have the biggest and smallest zones of hydrolysis of 78 and 38 mm on starch agar respectively. *A. niger* USGB:1024 was observed to perform better on Chinese rice bran as substrate (3.15± IU) using 15g amount of substrate, pH of 6, temperature of 30 °C, and inoculum density of $10^{6.}$ when compared with *A. niger* BGSU:17-00030 (1.46±0.01 IU) at day 5.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest between them.

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