Asian Journal of Biotechnology and Bioresource Technology

7(2): 1-11, 2021; Article no.AJB2T.67643
ISSN: 2457-0125

Activity of α-amylase Produced by *Aspergillus niger* at Different pH, Temperature and Incubation Time Using Solid-state Fermentation Process of Corn and Wheat Wastes

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MSM, MB and SPW designed the study. Authors SPW and MSM performed the statistical analysis. Authors MB, SPW and MSM wrote the protocol and wrote the first draft of the manuscript. Authors MB and SPW managed the analyses of the study. Authors HUA and KVB managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJB2T/2021/v7i230095
Editor(s):
(1) Dr. Fernando José Cebola Lidon, Universidade Nova de Lisboa, Portugal.
Reviewers:
(1) S. V. Bakiya Lakshmi, A Veeriya Vandayar Memorial Sri Pushpam College (Autonomous), India.
(2) S. Muthu Kumar, Pondicherry University, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/67643

Original Research Article

Received 15 February 2021
Accepted 23 April 2021
Published 27 April 2021

ABSTRACT

In Nigeria, agro by-products have not been fully utilized by many and often discarded at the dumping site. This anthropogenic activity is contributing to an increase in pollution and is a threat to public health. Environmental sustainability requires the wise use of resources that include agro by-products. Therefore, there is a need to utilize the agro by-product for the production of enzymes such as α-amylase. α -amylase is one of the important extracellular enzymes with several uses. The development of suitable technology to produce enzymes at a very lower cost is significant. The solid-state fermentation (SSF) process using corn and wheat wastes as a substrate have been utilized. In this study, *Aspergillus niger* from abattoir effluent was identified, isolated and used for

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the production of an enzyme (α-amylase). The study evaluated the effect of temperature, pH and incubation period on the activities of α-amylase produced by *Aspergillus niger*. The activity of α-amylase was found to be higher at pH 6.5 and temperature above 50^{o}C. At 4 days incubation of the solid-state fermentation of corn and wheat wastes, α-amylase activities produced were 90.61 Unit/mL and 87.34 Unit/mL respectively. Also in this study, 3-dimensional presentation of the pH, temperature and incubation time were evaluated. The result presented an optimal condition for amylase activity produced by *Aspergillus niger*.

Keywords: Enzymes; Aspergillus niger; solid-state fermentation (SSF); α-amylase.

1. INTRODUCTION

Enzymes are biocatalyst for a large number of biochemical reactions. α-amylase is one of the most important industrial enzymes use in brewing, textile, and pharmaceutical industries [1]. Amylase can be obtained from plants, animals and microorganisms [2]. Many industries prefer to use amylase from microbial sources [3]. Because is consider organic since is been produce by microorganism. Different types of microorganisms, including fungi, have been utilized in the production of an enzyme, for example, amylase [4]. The use of fungi for the production of enzymes is economical because can be manipulated easily in the residues of agro by-products [5]. Also, fungi are preferred over bacteria for enzyme production because of their filamentous nature, which aids in the penetration of the solid substrate of agro-industrial residues [6]. In Nigeria, staple cereals food such as maize, millet and sorghum are been used as sources of amylase [7]. The complete use of staple food crop as an alternative to microorganism for the production of enzymes such as amylase may have a negative impact on food security. Also, enzymes in commercial quantity from staple crop food when stored for a longer period tend to lose their stability [8]. The use of solid support such as solid-state fermentation (SSF) holds tremendous potential for the production of enzymes [9]. The microorganism uses the carbon source of the crude substrate in the absence or near absence of free water in the interior of a solid matrix [10,11]. The commonly used substrates in SSF are cereal grains waste, for example, corn, wheat and barley [12]. There is also a need to reduced environmental pollution that is emanating from the negative activity of man such as dumping of agro by-product [13]. However, most of the substrates from the agro by-products are characterised by polymeric and insoluble compounds with a high amount of nutrients for microbial growth [12]. In this study, corn and wheat agro by-products were used to build solid-state fermentation and *Aspergillus niger* were incubated in the solid support matrics for the production of amylase. Therefore, the objectives of this study were to isolate and characterize fungi from abattoir wastewater effluent in Kano, Nigeria. And to evaluate the effect of temperature, pH and incubation time on the amylase activities produced using solid-state fermentation of corn and wheat wastes.

2. MATERIALS AND METHODS

2.1 Sample Collection and Processing of Corn and Wheat Residue

Corn and wheat were purchased from Fagge local market in the Fagge Local Government Council, Kano State, Nigeria. And were ground, sieved and the residue was stored at room temperature. The residue was used in this study.

2.2 Sample Collect and Culturing

Abattoir wastewater effluent was aseptically collected from the gutter of Fagge abattoir, Kano state, Nigeria. The abattoir wastewater effluent sample collected was transported to the microbiology laboratory of Bayero University, Kano, Nigeria. Exactly 10 mL of the wastewater was diluted in 90 mL of sterile distilled water, followed by serial dilution. Then, the serial diluent was aseptically inoculated onto a different plate of sterile Potato Dextrose Agar (PDA). Subculturing was carried out until the pure culture of *Aspergillus niger* was obtained.

2.3 Microscopic Observation and Isolation of *Aspergillus niger*

A small portion of the mycelia growth was carefully picked using a sterilized inoculating needle and placed in a drop of lactophenol cotton blue on a microscope slide and covered with a coverslip. The slide was examined under the microscope, first closer view under (x10) and then with (x40) magnification of the objective lens. The isolates were characterized based on the detailed morphology of aerial and substrate

hyphae, type of hyphae, and type of asexual spores. The morphological examination was compared with the description of Cheesbrough [14]; Oyeleke et al. [15]; Domsch and Gams [16].

2.4 Preparation of Inoculum

Spore suspension of selected *Aspergillus niger* isolates was prepared by scraping off fungal spores within a 1cm cork borer with 40 mL of distilled water. This was made up to a 60 mL mark. Two (2) mL of this suspension was used to inoculate the substrate.

2.5 Solid-State Fermentation for α-amylase Activity

The medium was prepared as described by (Sethi and Gupta, 2015). Five grams each of corn and wheat bran were separately weighed into a 250 ml Erlenmeyer flask each and moistened with 5 ml of the following fermentation medium composition (1.25g of KCl, 0.35g of KH_2PO_4, 0.025 g of $MgSO_4.7H_2O$, 2.5g of NH4NO3, 0.0025g of FeSO4.7H2O, 5 soluble starch, 100 ml of distilled water at pH 6.5). The substrate and fermentation medium were mixed thoroughly, heated on a hot plate to homogenized and then sterilized in an autoclave at 121^{o}C for 15 min. Each Erlenmeyer flask contained substrate and was inoculated with 2 ml of the spore suspension of *A. niger*.

2.6 Enzyme Extraction

Fifty (50) mL of 0.1 M phosphate buffer and 0.1 M and acetate buffer for (pH 6 above) and (pH 5 below) respectively were poured on each substrate bed and agitated for 30 min at 250 rpm using a rotary shaker. The solution was filtered using a cheesecloth and the filtrate was centrifuged at 2000 rpm for 5 min. The decanted supernatant was used as the crude enzyme extract [17,18].

2.7 Screening for Amylolytic Activity

The amylolytic activity of the test isolates was determined by using the starch agar plate method as described by Fossi et al. [19], by inoculating the test organism individually into Potatoes Dextrose Agar medium which was supplemented with 1 g (1%) of starch. The agar plates were then incubated at 30^{o}C for 5 days. After the incubation period, Lugol's iodine solution was added to the culture plate to identify the zones around the cultures. The diameter formed after the addition of iodine solution was measured to represent the amylolytic activity.

2.8 Determination of α-amylase Activity

The α-amylase activity was determined by measuring the reducing sugars released as a result of the action of crude enzymes on starch. Amylase activity was determined using the method described by Sindiri et al. [20]. The reaction mixture consists of 0.5 mL of the crude enzyme, 0.5 mL of 1% soluble starch in 0.02 M citrate phosphate buffer with 0.06M NaCl, pH 6.5. The mixture was incubated for 3 min at room temperature, the reducing sugars liberated were estimated using the 3, 5-dinitrosalicylic acid (DNS) method [21]. Colour development was read at 540 nm with a UV – mini spectrophotometer against a blank, prepared by substituting the hydrolyze sample with distilled water. The reducing sugar content was subsequently determined by referring to a standard curve of known glucose concentration.

2.9 Protein Determination

The protein concentration of the enzyme extracts was determined following the method of Lowry et al. (1970) with Bovine Serum Albumin (BSA) as standard and 0.2ml of protein extract was measured into tubes and 0.8 ml distilled water was added to it. Distilled water was used as blank while BSA standard curve was equally set up (5, 10, 15, 20, 25, 30 mg/ml), 5.0ml of the alkaline solution was added into 10 ml of all the tubes, mixed thoroughly and allowed to stand for 10 mins, The absorbance was read at 540nm in a spectrophotometer.

2.10 Effect of pH, Temperature and Incubation Time on α-amylase Activity

Using the solid-state fermentation (SSF) the effect of pH on α-amylase activity in the different substrates (corn and wheat wasters) was investigated by adjusting the pH of basal salt solutions to 3.5, 4.0, 5.0, 6.0, 6.5, and 8.0. The substrates were then incubated for 5 days at room temperature. In another different experiment, the effect of temperature on α-amylase activity was examined using SSF in different substrates and incubated at 30, 40, 50, 60 and 70^{0}C at pH 5.5 for 4 days. Also, the effect of the incubation period on α-amylase activity

was studied by evaluating the enzyme activity on 24, 48, 72, 96 and 120hrs of incubation period in the different solid substrates at pH 5.5 and room temperature.

2.11 Determination of Specific Activity

The specific activity of an enzyme gives the measurement of the activity of the enzyme (expressed in units/mg).

$$\text{Specific activity} = \frac{\text{Enzyme activity (Unit/ml)}}{\text{Protein Concentrat ion (mg/ml)}} = \text{unit/mg}$$

2.12 Optimization of pH, Temperature and Incubation Period on Amylase Activity

The optimum pH value, temperature and incubation period for α- amylase activity on the corn and wheat wastes by isolated *Aspergillus niger* under solid-state fermentation were studied. For the pH value, 0.1 M acetate buffer was used for the pH range of 3.0-5.5 while 0.1 M phosphate buffer was used for the pH range of 6.0-8.0. For the determination of optimum temperature, the reaction mixtures were incubated at the various temperatures of 30-70^{o}C at constant pH and incubation time [17].

2.13 Statistical Analysis

The experiment was carried out with at least triplicate where necessary. The data obtained were analyzed using Design expert (6.0.6 software). The analysis of variance (ANOVA) and regression analysis was performed on the data obtained. The results obtained from the central composite design (CCD) were used to fit a second-order polynomial equation.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

Where Y = Predicted α-amylase response

β_0 = intercept; β_1, β_2, β_3 = coefficient of linear effect; β_{11}, β_{22}, β_{33} = coefficient of variable squared effects; β_{12}, β_{13}, β_{23} = coefficient interaction effect of variables and A,B, C,A^2,B^2,C^2 AB, AC, BC = independent variables for initial pH, temperature, and incubation period respectively. Fischer's test was used to determine the significance variable, and the coefficient of determining of the R^2 value was used to explain the proportion of variance by the model.

3. RESULTS

3.1 Isolation, Identification and Screening

The culture of *Aspergillus* sp. was isolated from abattoir wastewater effluent. Lactophenol cotton blue stain of the culture was observed. And the culture-confirmed as *Aspergillus niger*. The culture was tested for starch hydrolysis. When starch agar medium was inoculated with the organism and subsequently flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of α-amylase. *Aspergillus niger* a higher greater area of clearance was selected for further studies on amylase activity (data not shown).

3.2 Effect of pH

The result in Fig. 1 shows the effect of pH on α-amylase activity produced by *Aspergillus niger*. The activity of α-amylase produced at pH 6.5 was the highest in both the corn and wheat SSF with 82.45 Unit/mL and 119.36 Unit/mL, respectively.

3.3 Effect of Temperature

Fig. 2 shows the effect of different temperature on α-amylase activity in corn and wheat residue using *Aspergillus niger*. α-amylase produced shows to be thermostable. The optimum temperature for α-amylase activity in both the substrate was 50 ^{o}C with the corresponding amylase activity of 163.71 unit/mL and 144.48 unit/mL of corn and wheat residues.

3.4 Effect of Incubation Period

Fig. 3 shows the effect of incubation time on α-amylase activity in corn and wheat residue using *Aspergillus niger*. A high yield of α-amylase activity in corn and wheat wastes were noticed after 4 days of incubation (amylase activities from corn and wheat wastes are 90.61 Unit/mL and 87.34 Unit/mL, respectively). The production of amylase by *Aspergillus niger* under solid-state fermentation in both the residues of corn and wheat increases with time while the highest activity was recorded after 4 days of incubation.

3.5 Interaction Effect of pH and Temperatures (AB); pH and Incubation Period (AC), and Temperature and Incubation period (BC)

In Fig. 4 the relationship effects between temperature and pH while the incubation time

was kept at the centre level of 10 ml solution of the fermentation media. Low and high-temperature conditions did not produce higher α-amylase activity. While the higher amount of α-amylase activity was recorded between 45-50.50°C. The pH 5-7 showed a slight positive effect in the production of α-amylase activity. Fig. 5 shows the interaction between an incubation time and initial pH while the temperature was held at the centre level 10 ml solution of the fermentation media. The highest activity was recorded in the middle levels (90-108 h) of the incubation period at pH (5.8-6.5). Fig. 6 shows the interaction between an incubation time and temperature, while pH was kept at the centre level 10 ml solution of the fermentation media. The highest activity was recorded in the middle levels of both factors, that is, 90-108 h and 45-50.50°C for incubation time and temperature, respectively.

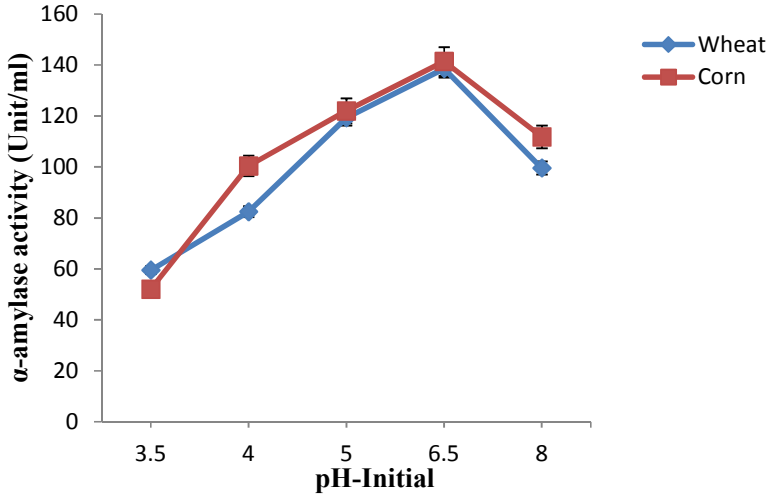

Fig. 1. Effect of pH on α-amylase activity produced by *Aspergillus niger* using solid-state fermentation of corn and wheat wastes

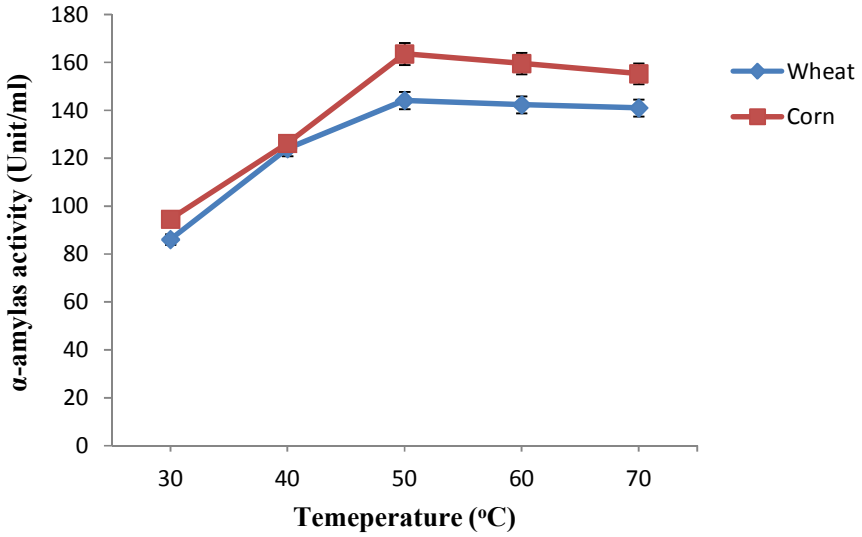

Fig. 2. Effect of Temperature on α-amylase activity produced by *Aspergillus niger* using solid-state fermentation of corn and wheat wastes

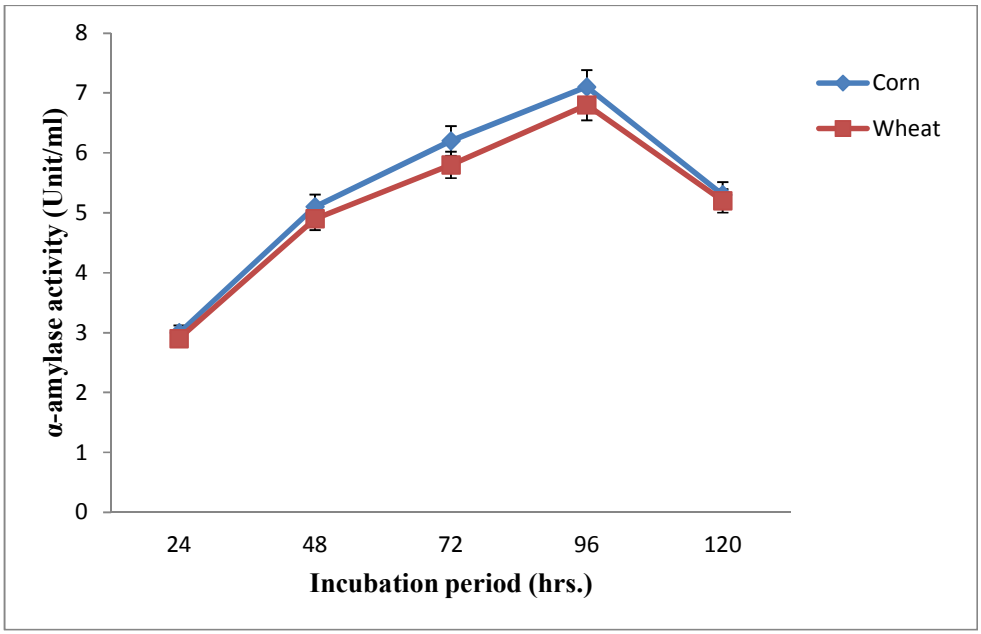

Fig. 3. Effect of pH on α-amylase activity produced by *Aspergillus niger* using solid-state fermentation of corn and wheat wastes

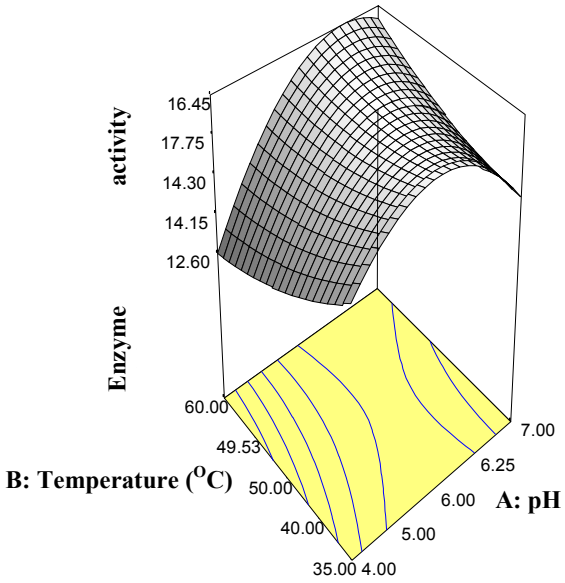

Fig. 4. Three dimensional (3D) presentation of the effect of temperature and pH on α- amylase activity produced by *Aspergillus niger* using solid-state fermentation of corn and wheat wastes

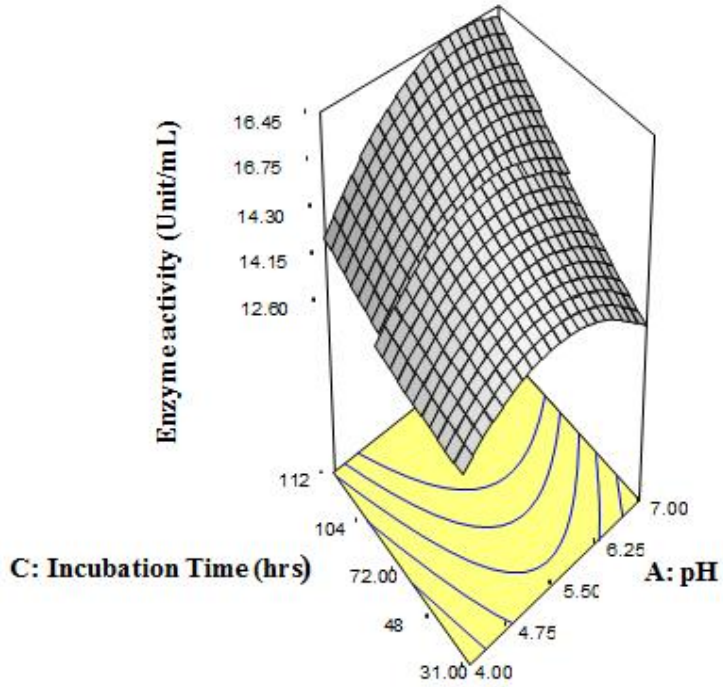

Fig. 5. Three dimensional (3D) presentation of the pH and incubation period (AC) on α-amylase activity produced by *Aspergillus niger* using solid-state fermentation of corn and wheat wastes when Temperature was kept at the centre level.

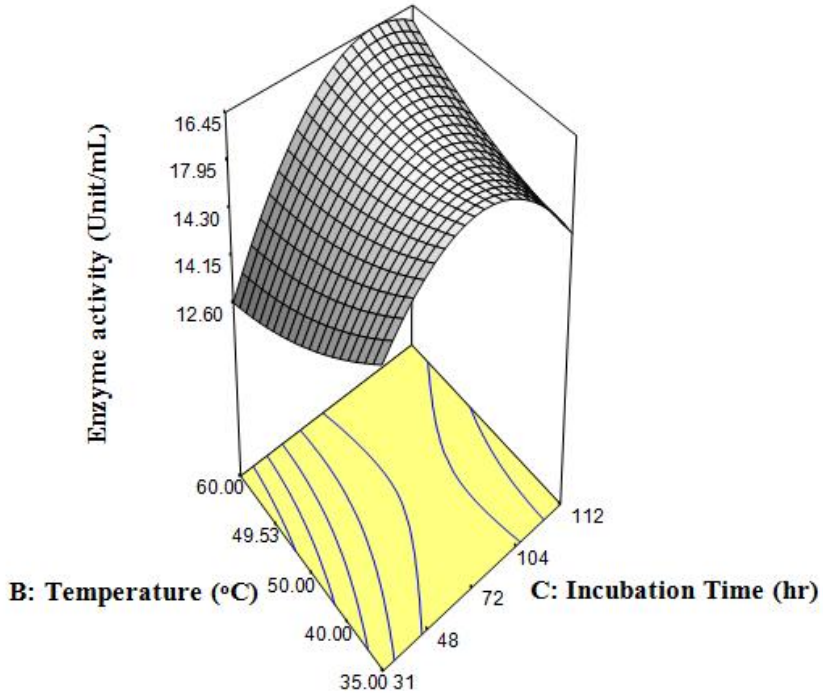

Fig. 6. Three dimensional (3D) presentation of the effect of temperature and incubation (BC) on α- amylase activity produced by *Aspergillus niger* using solid-state fermentation of corn and wheat wastes when pH was kept at the centre level

4. DISCUSSION

In this study, solid-state fermentation (SSF) as a solid support system for the production of an important biomolecule such as α-amylase by *Aspergillus niger* has been examined. Studies revealed that *Aspergillus japonicus* [22,23,4] *Aspergillus penicillioides* [24] and *Aspergillus oryzae* [25] have shown potential in the production of α-amylase. In Nigeria, the use of isolates of fungi, for example, *Aspergillus niger* from local abattoir wastewater or amylase activity has not been fully investigated.

In this study, isolates of *Aspergillus niger* from the abattoir wastewater tested for the production of hydrolytic enzymes showed the highest zone of clearance of 23 mm. The selected isolates were inoculated into the substrate matrix of corn and wheat wastes for the production of α-amylase. Ohimain et al. [26] demonstrated the existence of amylase activity by Pseudomonas, Bacillus, Micrococcus, Candida, Aspergillus, Fumigatus, Penicillium, Mucur and Fusarium isolated from palm oil mill effluent. Also, amylase was produced by *Fusarium* species isolated from fermented mineral salt supplemented with 25% corn starch [27]. In another different study, cassava peels inoculated with bacterial isolates have shown to produce amylase [28]. Amylase production by microorganisms is caused by several factors among which are the availability of carbohydrates, nitrogen compounds and other minerals Day et al. [29]. Also, favourable surrounding conditions is important for proper growth of microbes and production of enzymes.

In this study, the pH, temperature, and incubation time were important factors that determine the production of amylase activity by *Aspergillus niger* using corn and wheat wastes substrates. Amylase was produced from both corn and wheat substrates, although more amylase activity was recorded (49%) in corn substrates than that of a wheat substrate (44%). The difference in α-amylase activity produced from the two substrates could be due to the degree of complexity of the chemical substrate structure utilized by *Aspergillus niger* [30]. Substrate composition has been reported to significantly influence enzyme production and activity [31]. In this study, the pH with a higher amount of amylase activity produced was found to be 6.5 for corn and wheat substrates. Alli et al. [17], recorded a comparable result of pH 6.5 for the highest amylase activity from fungi. Okolo et al. [32] also observed similar values of pH 6.0 to 6.5 produced by *Aspergillus niger.*

In this study, the gradual increase in amylase activity correlates with the temperature regime of 30-50^{o}C for corn and wheat wastes substrates. This suggests that enzymes production can attend a thermostable state [33]. In another similar study, higher enzyme activity corresponds to a temperature of 45^{o}C [34]. The findings by Oyeleke et al. [15] was different where the lower temperature of 30^{o}C produced high amylase activity by *A. flavus* and *A. fumigatus* strains. Although, it has been postulated that at lower temperature or more extreme temperature low enzyme activity may be recorded due to the inactivation or thermal denaturation of enzyme protein [35]. Oyeleke et al. [15] also reported that an increase in temperature led to a decrease in amylase activity.

Here in this study, a high yield of α-amylase activity produced by *Aspergillus niger* was observed in both corn and wheat waste after 4 days of incubation. But after 4 days of incubation, there was an instant decrease in the activity of α-amylase which might be due to the reduction of nutrient or buildup of the toxic end product or loss of total moisture or even change might have occurred due to temperature and pH of the fermentation medium. In this present study, pH 6.5 contributed to the highest α-amylase activity produced. While temperature 50^{o}C showed higher produced activity of α-amylase for both corn and wheat waste.

Also in this study, the interaction effect of environmental conditions of the media such as pH, temperature and incubation time were evaluated and results presented using 3-dimensional graphs. The result shows that pH of 5-6.5, temperature of 45-52.50^{o}C and incubation time 90-108 h produced a higher amount of amylase activities in both corn and wheat wastes. Furthermore, the values of these factors analyzed shows the optimum of each predicted optimized values of pH (6.25), temperature (49.53^{o}C) and incubation period 104 h with a and predicted amylase activity of 17.95 U/mL. Among the different factors studied, temperature was the most significant parameter that influenced the production of amylase activity.

5. CONCLUSION

Aspergillus niger was isolated and characterized from an abattoir effluent, and use for the

production of the α-amylase activity in a solid-state fermentation process of corn and wheat wastes. Isolates of *Aspergillus niger* showed a zone clearance of 23 mm in diameter. While pH 6.5 has the highest α-amylase activity in corn and wheat wastes. Changed in the temperature to 50ºC produced a higher amount of α-amylase activity while 4 days of incubation time also produced higher α-amylase activity.

The relationship of the interaction effect of the factors (pH, temperature and incubation time) on the amylase production activity by *Aspergillus niger* in solid-state fermentation media, were evaluated and the result showed the optimal environmental conditions on 3-dimensional effect.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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