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Optimization of Conditions for Xylanase Production Using *Aspergillus tubingensis* Under Different Carbon Sources

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Abstract

Xylanases are hydrolytic enzymes with wide range of applications in food processing, bleaching of pulp in paper manufacturing industry, bio-conversion of biomass wastes to fermentable sugars and enhancing nutrient digestibility in animal feeds. The optimization of growth conditions and evaluation of an appropriate substrate as carbon source among cassava peels, corn cobs, wheat bran and rice husk on xylanase production by novel strain of *Aspergillus tubingensis* under Solid State Fermentation (SSF) was investigated. The fungal isolate was identified based on ribosomal RNA gene and ITS gene sequencing analysis as *Aspergillus tubingensis*. Results showed that Corn cobs had the highest xylanase production among the four substrates. Corn cobs recorded the highest value of xylanase production at pH of 6.0 (107.97 U/g), after incubation period of 72 hour (111.23 U/g), at temperature of 30°C (44.26 U/g) and at ratio 1:3 (45.68 U/g). The optimum growth conditions for xylanase production by *Aspergillus tubingensis* were: pH 6.0, incubation period of 72 hours, temperature of 30°C and substrate concentration of 1:3 (4g of substrate per 12ml of fermentation medium). Corn cobs showed the highest yield of xylanase activity (111.23 ± 0.31 U/g), followed by Rice husk (101.91 ± 0.72 U/g), Wheat bran (89.30 ± 1.16 U/g) and Cassava peel (87.03 ± 0.57 U/g). In conclusion, among the various agro residues that were used as carbon sources, Corn cobs had maximum xylanase activity. Various culture conditions were optimized by using one factor at a time method and the maximum xylanase production was obtained at pH of 6.0, incubation period of 72 hour, temperature of 30°C and substrate concentration of 1:3 under solid state fermentation. It is therefore suggested that some other notable environmental and fermentation factors that influence metabolism-mediated production yields of xylanase like aeration, agitation, carbon and nitrogen sources, metal ion requirement, inoculum size etc. should be optimized for maximum production of enzyme.

Keywords: Solid State Fermentation, Cassava peels, Corn cobs, Wheat bran, Rice husk

Introduction

The need for continuous use of cheap and locally available crop residues and agro-industrial by-products is a necessity to commercialize agro-wastes, reduction in environmental pollution and

improve employment generation in Africa like Nigeria and Asia. The use of abundantly available low cost agricultural residue as carbon sources

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conditions could be used to decrease cost of xylanase production. Such carbon sources include wheat bran, cassava peel, corn cobs and rice husk. This can be achieved by optimizing the fermentation media (Shah & Madamwar, 2005). Optimization is a process to determine the best cultural and nutritional conditions that significantly influence the yield of xylanases. Apart from the properties of organism, xylanase production is affected by environmental conditions of pH, temperature, initial moisture, inoculum size, period of incubation and the nutritive components of the growth medium (Uday *et al.*, 2016). Optimization of media composition is vital to improve both the productivity and the activity of enzymes like xylanases using one-factor-at-a-time (OFAT) method (Ajijolakewu *et al.*, 2017). This study was therefore conducted to determine the optimum growth conditions for *A. tubingensis* and to select an appropriate substrate as carbon source among cassava peels, corn cobs, wheat bran, and rice husk for efficient production of xylanase using solid state fermentation.

Materials and Methods

Chemicals

All chemicals and reagents used were of analytical grades and are from Sigma (St. Louis, MO USA) or Merck (Darmstadt, Germany)

Microorganism

Isolation and Identification of Xylanolytic Microorganism

The soil samples for the isolation of xylanolytic microorganisms were collected from different areas of agricultural field at the premises of the Nigerian Stored Products Research Institute (NSPRI), km 3, Asa Dam road, Ilorin, Kwara state, Nigeria. The isolation of fungi was carried out on potato dextrose agar (PDA) using the samples obtained from agricultural soils, decaying woods, and agricultural waste materials. One gram of soil sample was suspended in 10 mL of sterile distilled water and shake vigorously for 10 min. Thereafter, 1.0 mL of the resulting liquid was spread on the surface of PDA using an L-shaped glass rod and incubated at 37°C for 6 days. The fungal isolate formed was subcultured to purify it and examined for xylanolytic

activities. Screening for xylanolytic activities was performed on malt extract agar (MEA) containing 0.1% (w/v) of xylan from beech wood. Positive xylanolytic isolate was detected according to the clear zones of hydrolysis on the xylan. The potential isolates were subcultured and maintained on MEA slants. The slants were stored at 4°C prior to use. The selected isolate was identified by cultural techniques and verified by molecular identification using partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene sequencing at IITA, Ibadan, Nigeria. The potential isolate, *Aspergillus tubingensis*, was grown (subculture) on potato dextrose agar (PDA) medium at 30°C for 4 days and stored at 4°C on PDA slant.

Inoculum Development

Ten milliliters (10 mL) of 1% Tween-80-sterile distilled water was added to 4-day old pure culture on agar plate to obtain spore suspensions. Suspensions were sieved through 0.5mm filter to remove mycelia. With appropriate dilutions, inoculum sizes (spores/mL) were determined by direct microscopic observation using haemocytometer (Aberkane *et al.*, 2002; Rodriguez-Tudela *et al.*, 2003).

Substrates

Four different agro-industrial by-products and crop residues namely corncob, wheat bran, cassava peel and rice husk were used as carbon sources for the optimization of growth condition for enzyme production. Rice husk was procured locally from rice milling industry at Sango, Ilorin, Kwara State. Corn cobs were collected after harvesting the corn on the field at Teaching and Research Farm, University of Ilorin, Nigeria. Wheat bran was obtained at T and K Agro-Allied Enterprises, Fate-Tanke road, Ilorin, Kwara State. Cassava peel was sourced locally at Gari processing industry, Sango, Ilorin, Kwara State. The four substrates were dried in the oven at 80°C for 2 h, ground, and passed through a sieve (0.5 mm pore size) to obtain the fine powder used for the study.

Optimization of growth conditions

Physicochemical parameters such as substrate concentration, inoculum size, pH, incubation

period, and temperature may affect the enzyme production. Hence, there is need to find the optimum fermentation conditions for maximum production of xylanase. One-factor-at-a-time (OFAT) approach was employed to evaluate the optimal fermentation conditions for xylanase production by solid-state fermentation using *Aspergillus tubingensis*. This was carried out by varying the four (4) physiochemical parameters like pH, substrate concentration, temperature and incubation period.

i. Effect of pH: The effect of initial hydrogen ion concentration (pH) on enzyme production was assessed for optimization by adjusting the initial pH of production medium at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with 1M NaOH and 1% sulphuric acid.

ii. Effect of Incubation Period: To optimize incubation period for xylanase production, Erlenmeyer flasks (250 mL) containing four gram (4g) of each of the four substrates were prepared. Samples were harvested for a period of 24, 48, 72, 96 and 120 hours while keeping the other fermentation conditions constant.

iii. Effect of Temperature: Effect of incubation temperature on xylanase production was studied by incubating the inoculated flasks at different temperatures ranging from 25, to 50°C at intervals of 5 °C while keeping the other fermentation conditions constant.

iv. Effect of Substrate concentration: Substrate concentration is an important factor at SSF. It has a lot of influences on other culture fermentation factors. Substrate concentration was assessed for optimum xylanase production by varying the ratio of substrate (g) to fermentation medium (mL) at 1:1, 1:2, 1:3, 1:4 and 1:5 (g/mL). The effect of different substrates (Rice husk, Cassava peel, Corncob and Wheat bran) were studied at different concentrations at ratio 1:1, 1:2, 1:3, 1:4 and 1:5 (4g: 4mL, 4g: 8mL, 4g: 12mL, 4g:16mL and 4g: 20mL) respectively during fermentation process for a period of 72 hours.

Production of Xylanase by Solid State Fermentation

Xylanase biosynthesis was carried out in 250mL conical flasks using SSF in a production medium containing 12mL mineral salt medium. Four gram (4g) of each of the four (4) substrates (corncob, wheat bran, rice husk and cassava peel) were added to each flask and kept. Substrates were moistened with 12mL of mineral salt medium. Composition of the growth medium includes (g/l): yeast extract (2.5), K₂HPO₄ (2), CaCl₂ (0.4), CaCO₃ (0.001), CuSO₄ (0.002), Tween-80 (2), MgSO₄ (0.3), FeSO₄ (0.005), MnSO₄ (0.002), ZnSO₄·7H₂O (0.0014), CoCl₂ (0.002), NaCl₂ (0.002). They were autoclaved at 121°C for 30 min, cooled and inoculated with 1mL of standardized inoculums (2 x 10⁶ spores/ mL) into the medium and the fermentation was allowed to proceed at 30°C for 5 days. Each Experiment was carried out in triplicate.

Xylanase production on various lignocellulosic substrates

The fungal isolate *Aspergillus tubingensis* was grown in 250 mL conical flasks containing 4 g each of various lignocellulosic substrates (corn cob, wheat bran, rice husk, and cassava peel) and moistened with MS medium. The enzyme was extracted and assayed.

Crude Enzyme Extraction

The enzyme from the substrates was extracted separately using 0.05M citrate buffer (pH 4.8). Following the addition of buffer, the whole flask was kept in a rotary incubator at 180 rpm (30°C) for 15 minutes and squeezed through muslin cloth to extract the crude enzyme. The enzyme extract obtained was centrifuged at 10,000 rpm at 4°C for 15 min. The clear supernatant obtained was used as the enzyme sample for assay.

Enzyme Assay

Xylanase activity is most commonly determined by measuring the release of reducing sugars from prepared xylan, such as oat spelt, Birchwood or Beachwood xylan. Xylanase activity (IU/g) was determined quantitatively by DNSA Method (Bailey *et al.*, 1992) using 1% xylan (w/v) from beechwood (Sigma, USA) as substrate. One unit of xylanase is defined as the amount of enzyme that liberates 1 μmol reducing sugar as

xylose/mL/min under the assay condition. The reducing sugars produced were quantified by the dinitrosalicylic acid method using D-xylose as standard. 0.2 mL of suitably diluted crude enzyme (culture filtrate) was added to 1.8 mL of xylan solution 1% beech wood xylan in test tube placed in water bath at 50°C for 5 minute as described by (Bailey *et al.*, 1992). The reaction was stopped by adding 3mL DNS (3, 5 dinitrosalicylic acid) reagent and kept the tubes in boiling water (100°C) for 5 min. The tubes were allowed to cool and the reducing sugars liberated were measured (absorbance) at 540nm wavelength with spectrophotometer (Spectronic Genesys TMS, USA) against appropriate blank. The amount of xylose liberated was calculated with the standard curve of xylose as described by (Ajijolakewu *et al.*, 2016).

Xylan Substrate Solution (1%)

One gram of beech wood xylan was weighed and dissolved in 100 mL of distilled water. It was stored in an air-tight container and preserved in the refrigerator prior to usage.

Xylose Standard

This was prepared by accurately weighing 0.15g of xylose and dissolved in 100mL of citrate buffer. Dilutions were made from the stock solution prepared (Bailey *et al.*, 1992).

Standard Curve for Xylose

Different concentrations (2.0, 3.3, 5.0, 6.7, 10 $\mu\text{mol}/\text{mL}$) of Xylose were prepared in distilled water. 0.5 mL of each concentration was added in test tubes along with 0.5mL of DNS solutions.

These test tubes were boiled for 5 minutes in boiling water and then cooled. A blank was also prepared by dissolving 1mL each of DNS and distilled water. Absorbance of these solutions was determined by using a spectrophotometer at 540 nm. A graph was drawn by plotting concentration against absorbance.

Xylanolytic Activity

Xylanase activity is measured by determining the release of reducing sugars from prepared beechwood xylan. Measurement of enzyme activity was carried out under conditions closely defined with respect to temperature, pH, substrate concentration and substrate type, since all these factors affect the activity of enzyme (Beauchemin *et al.*, 1999). Effects of enzyme on sugar correspond to the level of xylanolytic activities on the degradability of substrate (lignocelluloses) and it is measured by absorbance using spectrophotometer.

Statistical Analysis

All the experiments were carried in triplicates and the data represents the mean value of three replicates. Statistical differences of generated data was determined by one-way Analysis of Variance ($\alpha = 0.05$) using the General Linear Model (GLM) procedure of (SAS, 2008). Significant differences between means were compared using the Duncan's Multiple Range Test of the same GLM.

Result and Discussion

The result of the molecular analysis of fungi isolate carried out at Bioscience Research Laboratory at the International Institute of Tropical Agriculture (IITA), Ibadan is as shown in Table 1.

Table 1: Isolate Annotation

Isolate Annotation	Putative Organism Identity	Number of bases/Query Cover	Probable Identity (%)	Accession Number
Fungal Isolate	<i>Aspergillus tubingensis</i>	580 (85%)	98.44%	MT312764.1

Aspergillus tubingensis strain G36 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

Results of optimization of xylanase production using various agro-waste residues as substrates/carbon sources for the xylanase production by *Aspergillus tubingensis* are shown in Fig.1 to Fig. 4. Notable environmental and fermentation factors that influence metabolism-mediated production yields include pH, temperature, aeration, agitation, carbon and nitrogen sources, metal ion requirement, incubation time, initial inoculum size, Ch *et al.* (2008). Among the lignocellulosic materials tested as carbon source, Corn cobs showed the highest xylanase activity (111.23 ± 0.31 U/g) among the four substrates followed by rice husk (101.19 ± 0.72 U/g). The cost availability and physicochemical characteristics of substrates were the main factors for the selection of substrates for enzyme production (Delabona *et al.*, 2012).

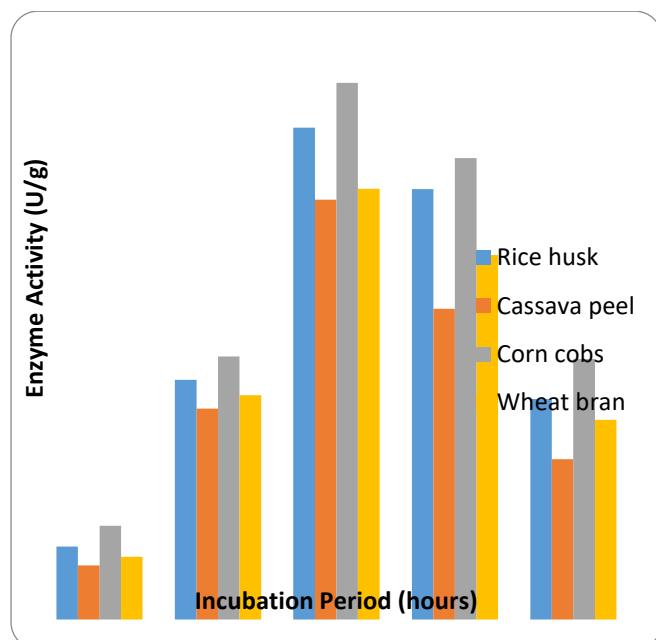


Figure 1: Effect of pH on xylanase production by *Aspergillus tubingensis*

Effect of pH on xylanase production by *Aspergillus tubingensis*

The pH is an important cultural condition that determines growth rate and influences the level of enzyme production by microorganisms. The best xylanase activity (107.97 ± 0.90 U/g) was obtained when the pH value was at 6.0 on Corn cobs as shown in Fig. 1. The highest yield of xylanase for other substrates was obtained at pH value of 6.0 for Rice husk (95.61 ± 0.98 U/g), Wheat bran

(89.53 ± 0.72 U/g) and Cassava peel (85.40 ± 0.50 U/g). The lowest xylanase activity was obtained when the pH value was 10.0 for all substrates; Corn cobs (23.68 ± 0.58 U/g), Rice husk (20.80 ± 0.67 U/g), Wheat bran (18.72 ± 0.88 U/g) and Cassava peel (15.27 ± 1.47 U/g). Hence, the optimal pH value observed was pH 6.0. The result obtained was in accordance with the result of Bhushan *et al.* (2012), who reported a pH of 6.0 for maximum xylanase production by *Aspergillus flavus* MTCC-9390 using wheat straw as substrate and 25 mL of Mandels and Sternburg's (MS) medium. Production of enzyme at higher or lower pH decreases due to inactivation of enzymatic system. Ionic characters of amino and carboxylic group are also influenced by difference in pH which affects the catalytic activity of enzymes (Agnihotri *et al.*, 2010). Microorganism holds a range of pH for its growth and activity with an optimum values and between these ranges, the initial pH influences enzymatic system and the transport of enzyme across cell membrane (Poorna *et al.*, 2007; Mohana *et al.*, 2008).

Effect of Incubation Period on Xylanase Production by *Aspergillus tubingensis*

The results in Fig. 2 showed that xylanase activities commenced after the first day (24 h) of incubation at 30°C. A significant decrease of xylanase activities were noticed after day 3 (72 h). The highest xylanase activity was obtained at 72 hours, for all substrates. Corn cobs showed the highest yield of xylanase activity (111.23 ± 0.31 U/g), followed by Rice husk (101.91 ± 0.72 U/g), Wheat bran (89.30 ± 1.16 U/g) and Cassava peel (87.03 ± 0.57 U/g). The lowest yield was obtained at 24 hours for all substrates; Corn cobs (19.44 ± 0.27 U/g), Rice husk (15.17 ± 0.39 U/g), Wheat bran (13.01 ± 1.87 U/g) and Cassava peel (11.24 ± 0.71 U/g). The middle incubation period for optimum production of xylanase tend to reduce the risk of contamination and decrease the cost of xylanase production. This is in accordance with the report of Fadel *et al.* (2001). A crude endo-xylanase produced by BCC14405 was optimized by cultivating on medium containing wheat bran, rice bran and soy meal supplement was reported to have produced 89.5 IU/ml Reis *et al.* (2003).

Optimization of culture condition for xylanase production from *Aspergillus fumigatus* strain MA28 was carried out by growing them on different substrates like wheat bran, rice bran, sawdust, rice straw, powdered corncob, maize bran, wheat flour mill waste or cotton seed cake as the carbon source (Bajaj and Abbass, 2011).

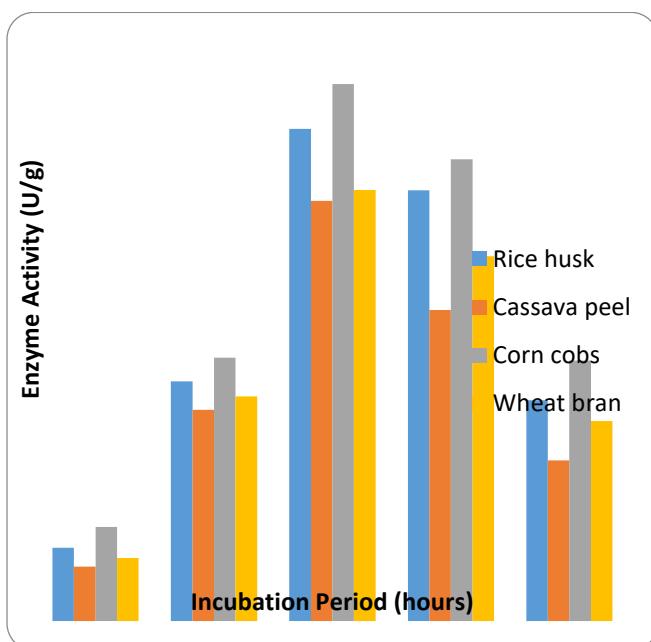


Figure 2: Effect of Incubation period on xylanase production by *Aspergillus tubingensis*

Effect of Temperature on xylanase production by *Aspergillus tubingensis*

The highest xylanase activity was obtained when the temperature was at 30°C for all substrates as shown in Figure 3. At 30°C, Corn cobs showed the highest yield of xylanase activity (44.26 ± 0.93 U/g), followed by Rice husk (39.16 ± 1.20 U/g), Wheat bran (36.83 ± 1.19 U/g) and Cassava peel (33.52 ± 0.29 U/g). The lowest yield was obtained at higher temperature of 50°C for all substrates; Corn cobs (14.85 ± 0.70 U/g), Rice husk (12.78 ± 0.10 U/g), Wheat bran (11.34 ± 0.85 U/g) and Cassava peel (10.37 ± 0.84 U/g). According to Seyis & Aksoz (2005), the fermentation temperature has vital effect on the level of xylanase production because of its important role in the metabolic activities of microorganisms. The optimum temperature was recorded for xylanase production at 30°C by *Aspergillus tubingensis*. Increased or decreased temperature from 30°C resulted to

decrease of xylanase production. At lower temperature, xylanase production decrease because of lower transport of substrates through the cell membrane (Pal & Khanum, 2010). Higher temperature results to poor growth because of thermal denaturation of enzymes for metabolic pathways, that shows the higher maintenance energy for cellular growth and lower the production of metabolites (Shah & Madamwar, 2005). The enzyme production gradually decreased with increase in temperature. This result was similar to the previous report that optimum temperature was recorded at 30°C from the strains of *Trichoderma* sp. (Pang *et al.*, 2006) and *Aspergillus niger* (Simoes *et al.*, 2009). It was reported that optimum temperature for enzyme production also depends on strain variation of the microorganisms (Gautam *et al.*, 2010). This result is comparable to earlier report that maximum xylanase production (2701 U/gds) was observed by *Aspergillus foetidus* at 30°C under SSF conditions after 4 days of incubation (Shah & Madamwar, 2005).

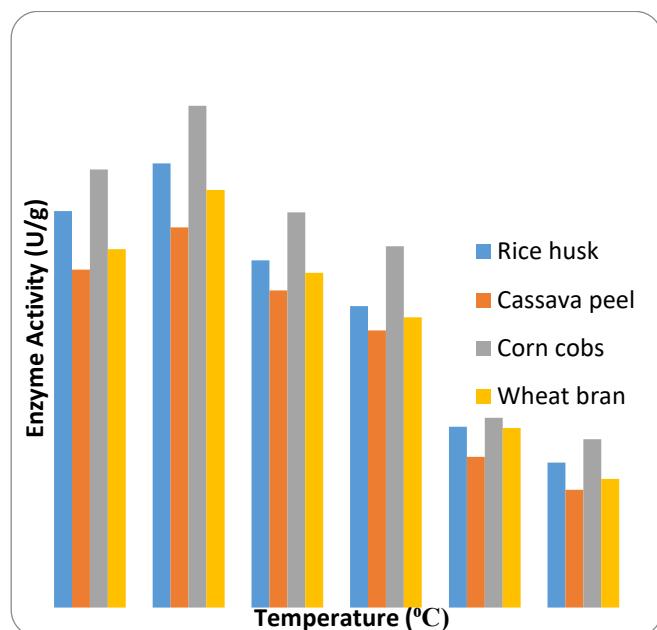


Figure 3: Effect of Temperature on xylanase production by *Aspergillus tubingensis*

Effect of Substrate Concentration on Xylanase Production by *Aspergillus tubingensis*

The results in Fig. 4 showed that Corncob produced the highest yield of xylanase activity at ratio 1:3 (45.68 ± 0.31 U/g), followed by Rice husk

(40.13 ± 0.23 U/g), Wheat bran (39.66 ± 0.36 U/g) and Cassava peel (37.09 ± 1.57 U/g). The lowest yield was obtained at ratio 1:5 (18.97 ± 1.28 U/g) for Corn cobs, Rice husk (15.17 ± 0.39 U/g) at ratio 1:1, Wheat bran (13.01 ± 1.87 U/g) at ratio 1:1 and Cassava peel (11.24 ± 0.71 U/g) at ratio 1:1. It was noted that when the concentration of each carbon source was increased, the enzyme activity level in the culture medium decreased. This could be as a result of the higher levels of substrates that eventually reduced the culture agitation resulting in a reduced accessibility of the fungus to the substrates, and hence, a lower observed enzymatic activity (Nichawee *et al.*, 2011). This result was in accordance with the report of Pal and Khanum, (2010) that Corncob, wheat bran and xylan are known to be good microorganism xylanase inducers. It was reported that optimum level of substrate concentration depends on substrate type, substrate size, organism and test enzyme (Fadel, 2000). The decline of xylanase activity at lower substrate levels (1:1 and 1:2) may also be associated with shallow depth, high moisture content and high inoculum size. The findings of this study are in accordance with the findings of (Kumar and Satyanarayana, 2004) and (Fadel *et al.*, 2001).

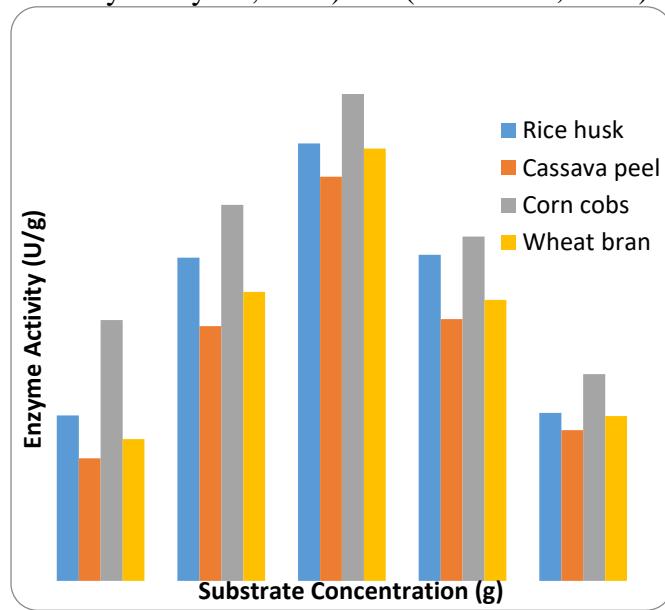


Figure 4: Effect of substrate concentration on xylanase production by *Aspergillus tubingensis*

However, the decrease in xylanase activity at higher level (1:5) may be associated with high substrate depth, low moisture content, lower inoculums size, reduction of substrate swelling and

nutrient diffusion. These results are comparable to those obtained by (Lonsane *et al.*, 1985) and (Fadel *et al.*, 2001).

Conclusion

Fungi isolate was isolated and identified as *Aspergillus tubingensis* following the culture technique and molecular identification.

Agricultural wastes such as cassava peels, corn cobs, rice husk and wheat bran could be used as substrates for the production of xylanase. Result showed that substrate with the highest yield of xylanase activity is Corn cobs.

The optimum growth conditions for the production of xylanase by *Aspergillus tubingensis* were: pH 6.0, incubation period of 72 hours, temperature of 30°C and substrate concentration of 1:3 (4g of substrate per 12ml of fermentation medium). The optimization of cultural conditions has shown to increase the growth rate of organism and xylanase activity.

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Appendix

Table 2: Xylose Standard

CONCENTRATION μmol/ml	ABS 1	ABS 2	ABS 3	AVERAGE ABSORBANCE
1:1 (10)	1.857	1.868	1.863	1.863
1:1.5 (6.7)	1.242	1.239	1.235	1.239
1:2 (5)	0.938	0.925	0.934	0.932
1:3 (3.3)	0.514	0.531	0.509	0.518
1:5 (2)	0.372	0.366	0.389	0.376

Key: ABS-absorbance in triplicate

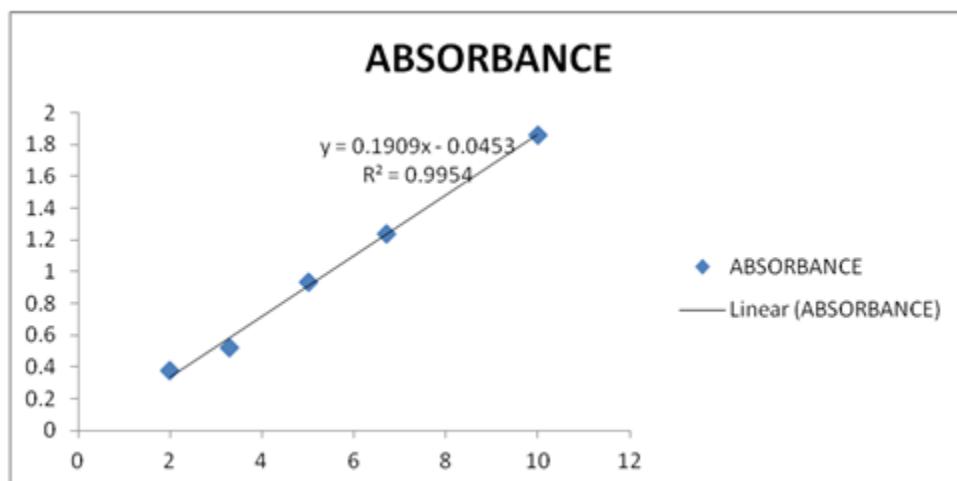


Figure 1: Graph of Absorbance Vs Concentration

Table 3: Xylose Standard

CONCENTRATION μmol/ml	ABS 1	ABS 2	ABS 3	AVERAGE ABSORBANCE
10	1.584	1.572	1.578	1.578
6.7	1.319	1.322	1.325	1.322
5	1.217	1.203	1.208	1.209
3.3	0.966	0.978	0.969	0.971
2	0.771	0.722	0.785	0.759

ABS-absorbance in triplicate

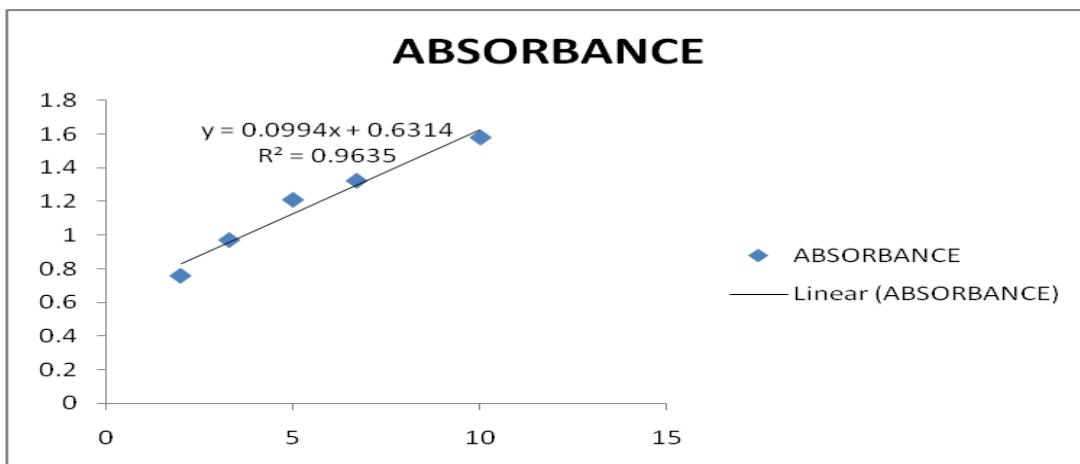


Figure 2: Graph of Absorbance Vs Concentration