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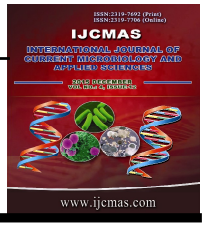

Original Research Article

Production of Xylanases from Fungal Isolates by Solid State Fermentation using Sugar Cane Bagasse

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A B S T R A C T

An investigation was carried out to study the production and optimal activity of exo- xylanase by Solid state fermentation using sugar cane bagasse as the agricultural residue. A total of 31 molds were isolated and thereafter screened for their ability to produce xylanase by using the plate assay technique on potato dextrose agar fortified with 2% (w/v) of xylan. Promising mold isolates for xylanase production were identified as XA, XC_3, XD_2, XM_6 (*Aspergillus niger)*, XC (*Tricoderma harzianum*), XC_1 (*Fusarium oxysporum*), XC_2, XM_2 (*Aspergillus terreus*), XD_3, XJ, XK (*Aspergillus fumigatus*), XM (*Aspergillus tamarii*). Studies on the optimal conditions of performance of temperature and pH of the produced xylanase were in a range of 30^0C - 80^0C and 3.0 - 9.0 respectively. Comparative Studies of the enzyme for its activity as produced by isolate XM, XD and XC were 3000U/ml, 2800U/ml and 3400U/ml respectively. While the specific activity were 246.5U/mg/ml, 97.15U/mg/ml and 123.63U/mg/ml respectively.

Introduction

Xylanases (EC 3.2.1), belongs to the group hemicellulases which are a group of enzymes that are define and classified according to their substrate hemicellulose. The hemicelluloses are polymers of xylose, galactose, mannose, arabinose, other sugars and their uronic acids. These are usually classified according to the sugar residue present.

Xylanase (EC 3.2.1) refers to those enzymes which are capable of hydrolyzing the 1,4-beta-D-xylopyranosyl linkages of the 1,4-beta-D-xylans namely; arabinoxylan, arabinoglucoxylan, arabino-4-0-methyl-D-glucoronoxylan and glucoronoxylan (Gliose and Bisaria, 1987). Xylanase consists of 190 amino acids and has a molecular weight of 21kd. Xylanases belong to the glucanase enzyme family and are characterized by their ability to breakdown various xylans to produce short-chain xylo-oligosaccharides. Xylanases readily crystallizes in ammonium sulphate and sodium/ potassium phosphate across pH 3.5-9.0 and decreases in the temperature range of 10-37^0C.

Haltrich *et al.* (1996), gave an over view of fungal xylanases and showed that the enzyme can be produced by a number of microorganisms including bacteria, yeasts and filamentous fungi such as *Trichoderma sp, Bacillus sp, Aspergillus sp, Fusarium sp, Penicillum sp, Rhizomucor sp,Talaromyces sp* and many more. These fungi produced xylanase extracellularly, with a wide range of activities from 4-400 IU/ml using various substrates both in submerged and solid state fermentation (SSF) processes. Extracellular enzymes are considered important from the industrial point of view as they ease the extraction procedure. Although a number of xylanase productions were performed using submerged system, solid state fermentation was found to be more economical mainly due to the cheap and abundant availability of agricultural wastes which can be used as substrates. Besides that, SSF offers distinct advantages over submerged fermentation including economy of space needed for fermentation; simplicity of the fermentation media; no requirement for complex machinery; equipment and control systems; greater compactness of the fermentation vessel owing to a lower water volume; greater product yield; reduced energy demand; lower capital and low recurring expenditures in industrial operation; easier scale up processes; lesser volume of solvent needed for product recovery; superior yields; absence of foam build up and easier control of contaminants due to the low moisture level in the system (Pandey, 1992).

Xylanases have proven useful in many ways such as in the food industry where it is used in bread making (Mora- Alvarex, 2009); improved animal feeds by improving digestibility, which also improves the quality of the animal litter; Bio-bleaching paper pulp leading to a reduction in organo-chlorine pollutants such as dioxin from paper making processes. In addition, chlorine-free bleaching (such as peroxide or ozone bleaching) can achieve brighter results with the addition of xylanase. Xylanases also improves silage (or chemical fermentative composting) resulting in quality silage and improves subsequent rate of plant cell wall digestion by ruminants.(Great Vista, 2009). Xylanases has been extracted from many different fungi and bacteria (Gliose and Bisaria, 1987). Information on xylanases production along with other several industrial enzymes, are very scarce because they are protected by patent rights.

This study reports isolation, screening, the production and properties of xylanases produced through solid state fermentation by some species of fungal isolates.

Materials and Methods

Collection of Sample

Decaying woods sample were collected from four (4) locations in Lagos state using sterile polythene nylon and Mc-Cartney bottles. The samples were taken to the laboratory within one hour of sampling for isolation of xylanase producing fungi.

Isolation of Fungal Isolates

One (1) g of each of the samples was accurately and aseptically weighed separately into 10ml of sterile distilled water and mixed vigorously for 10 min. The samples were diluted in ten folds serial dilution in duplicates. From an appropriate dilution of 10^{-2} 10^{-3} 10^{-4} 1.0 ml was taken and dispensed into sterile Petri dish plates respectively and thereafter, cool but molten Potato Dextrose Agar (PDA) supplemented with 0.014g streptomycin to inhibit bacterial growth, was poured onto the Petri dish using pour plate method. The plates were then

incubated at 28+/-2^{0}C for 5-7 days. The potential isolates were sub cultured and preserved on PDA slants.

Screening and Selection for Hyper Producing Fungal Isolates of Xylanases using Xylan Fortified Agar (XFA)

The selection of hyper producing strains of xylanase was carried out on Potato Dextrose Agar (PDA) fortified with 1% w/v of xylan, sterilized at 121^{0}C for 15min. The xylan agar was poured into plates respectively, surfaced dried in an oven set at 45^{0}C. After drying, 5mm size holes were made on the agar surface using sterile cork borers. The plates were aseptically inoculated with the fungal isolates on the holes made with cork borer (spot inoculation) and incubated at room temperature of 25+/- 2^{0}C for three (3) days. The clear zones were observed after the plates were flooded with Gram's Iodine. The area where there was no hydrolysis (clear zone) was stained blue black.

Identification of the Potential Producers of Xylanase

Identification was carried out based on the colony morphology and structural characteristics as observed under the light microscope using wet mount technique. The fungal characteristics were described and identified based on description given by Onions *et al.* (1981); Pitt and Samson (2000). The observed moulds growth were sub-cultured onto a fresh potato dextrose agar (PDA) plates and incubated at 28 +/- 2^{0}C for five (5) days. Pigment production was noted, and wet mount carried out by picking fungal colony onto grease free slide containing two drops of Lactophenol Cotton blue. This wet preparation was covered with cover slip and viewed under X4, and X40 objectives lenses. The methods have been previously reported by Chukwura *et al.* (2007). Preliminary identification of hydrocarbon utilizing fungi (moulds) was based on the keys and details on Smith's Introduction to Industrial Mycology (Onions *et al.,* 1981).

Solid-State Fermentation Cultivation for Xylanase Production

Cultivation was performed in 500ml Erlenmeyer flask containing 100g of solid substrate (sugar cane bagasse with the addition of production medium containing ask containing 50 ml of Horikoshi medium containing (% w/v): sugar cane bagasse, 2.5, peptone, 0.6, beef extract, 0.6, KH_2PO_4, 0.1, $MgSO_4.7H_2O$, 0.0 at pH 8.5. The production medium and the trace elements were autoclaved separately. The flasks were cooled down at room temperature and a known amount of sterilized trace elements was added. The flasks were then inoculated with 1 x 10^6 /ml of the moistening agent and incubated for 5 days at ambient temperature (28 ± ^{0}C). The inoculum was prepared by growing the isolate on Malt Extract Agar (MEA) at 37^{0}C until sporulation**.** The spores were harvested using 0.1% Tween 80, and poured onto the substrate (Smith *et al*., 1996).

Enzyme Extraction

Seven hundred millilitres of cold sterile distilled water (4^{0}C) was added to SSF medium (100g substrate) after cultivation. The mixture was vigorously homogenized for 30 minutes at 200rpm. The solid biomass residues were separated from the suspension by filtration through Whatman's filter paper No.1. The cell free supernatant was used as the crude enzyme source.

Determination of Xylanase Activity

Xylanase Assay

Xylanase activity was determined by

following the method of Rickard and Laughlin (1980), using the standard curve of known concentration for xylose. Xylanase activity was measured as follows: A suitably diluted solution of the enzyme (1ml) was mixed with 1.0ml of 0.05M citrate buffer (pH 4.8) and (1ml) of xylan. The test sample was placed in a water bath for 60 mins. Then immediately 3 ml of DNSA was added to the reagent mix, the test tubes were transferred and placed in a boiling water bath for 15 mins, absorbance was read at 540nm. One unit enzyme activity was defined as the amount of enzyme that releases 1 µg of xylose per ml per min under the above assay conditions. Specific enzyme activity was expressed as units/mg of protein.

Protein Determination

Protein contents of the enzyme extracts were determined by following the method of Lowry *et al* (1951) with Bovine serum albumin as standard. Protein extract, 0.2ml was measured in two tubes and 0.8ml distilled water was added to it. Distilled water was used as blank while BSA standard curve was equally set up, (200ug/ml), 20-200mg/ml, 5.0ml of alkaline solution was added to all the tubes, mix thoroughly and allowed to stand for 10 mins, 0.5ml of Folin-C solution was added to all the test tubes and left for 30mins after which the optical density was read at 600nm wavelength in a spectrophotometer (T70 PG Instrument UV model). The protein concentration was estimated using values extrapolated from the standard graph of protein.

Calculation:

$$\text{Protein concentration (mg/ml)} = \frac{Absorbance\ value}{Gradient}$$

Determination of Specific Activity

The Specific activity of an enzyme gives the measurement of the activity of the enzyme. This is the activity of an enzyme per milligram of total protein (expressed in units/mg). It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of protein.

Specific activity of the xylanase was determined using the formula below.

Calculation

$$\text{Specific xylanase activity} = \frac{\text{Enzyme activity (units/ml) absorbance value}}{\text{Protein concentration (mg/ml)}}$$

Results and Discussion

A total number of thirty (30) mold strains were isolated from decaying wood samples showing their biological characteristics. On xylan-agar, a total of twelve (12) isolates were capable of exhibiting xylanolytic activity with the diameter of the clear zones ranging from 0.8- 3.6mm (Table 1).

The identities of these xylanase hyper-producing strains are reported using our reference codes in Tables 2 and 3.

The screening assay showed that isolates XM, XD_3, XA, XM_2, XJ, XD_2, XM_6, XT, XK, and XC_3 had zones of clearance of 1.6cm, 1.8cm, 2.5cm, 1.9cm, 1.8cm, 2.5cm, 2.8cm, 1.9cm, 0.8cm, and 2.5cm respectively (Table 1). Observation showed that isolates XC_3 and XM_6 with clearance zones of 2.5cm, and 3.6cm respectively statistically had the highest zones of clearance ($p > 0.05$) (Table 1). This current study could compared with other existing work, thus it tallies with the observations of Kamble and Jadhav (2012) which reported a xylanase clearance zone of 33mm for a novel strain of *Bacillus* in India. The

identification of isolates XC_3, and XM_6 were determined using conventional tools and identified to be *Aspergillus niger* and *Trichoderma harzianum* respectively. These fungi isolated from woods have been adapted to breaking down the xylan in cellulosic materials. Thus they are known to have genes for expression of xylanase. The xylanase usually is extracted under certain industrial and cell physiological conditions.

Fungi are known to produce a lot of hydrolytic enzymes including xylanase. Sharma *et al.* (2015) reported the isolation of 15 fungal species out of a total 90 fungal species including *Aspergillus* sp. S9 with enhanced/ appreciable xylanolytic properties. Dhulappa and Lingappa (2013) reported the isolation of *Aspergillus tamarii* with xylanase hyper-producing potentials. The crude activities of xylanase of *Aspergillus niger* (XC3) and *Trichoderma harzianum* (XM_6) were reported in Table 4. Observation showed that during solid state fermentation, the activities of xylanase of *Aspergillus niger* increased progressively from 42190units/ml by the 24^{th} hour of fermentation. Similarly, the initial crude activity of *Trichoderma harzianum* xylanase was 42250units/ml on the zero hour and this increased progressively to 42410units/ml on the 96^{th} hour of Solid State Fermentation (Table 4). Statistical analyses showed that optimum activities of xylanase of *Aspergillus niger* (XC_3), and *Trichoderma harzianum* (XM_6) respectively (Table 5).

Findings from this current study did not tally with the reports of Khurana *et al.* (2007) which documented an optimum xylanase activity of 1500units/ml in *Streptomyces violaceorubes* using wheat bran as substrate. In this current study, the substrate used was sugar cane bagasse (previously reported to be of high xylan (content). This difference in substrates used can justify the significant difference in xylanase activities in both studies.

Also, on the use of agro-based agricultural wastes, Irfan *et al.* (2012) reported in line with this present study the use of sugar bagasse as substrate to produce xylanase of crude activity of 45units/ml. The xylanase of *Simplicillium obclaratum* MTCC 9604 produced through Solid State fermentation of wheat bran as sole carbon source was 236.4units/ml within 5days of fermentation (Roy *et al.,* 2013). Rajesh and Rajesh (2012) observed crude activities of 11.4 and 13.58units/ml during Solid State fermentation of sugar cane bagasse by *Bacillus polymyxa* and *Cellulomonas uda.*

The specific xylanase activities of XC_3, and XM_6 xylanase were 7287 and 7354 unit/mg respectively (Table 6). This shows that specific activities of *Trichoderma harzianum* xylanase were statistically more significant than the activity of *Aspergillus niger* ($p < 0.05$). This implies that both *Trichoderma harzianum* XM_6 and *Aspergillus niger* XC_3 are good strains for production of industrial xylanase to tackle wide range of industrial operations.

Roy and Rowshanul (2009) in a related investigation encountered *Bacillus cereus* strain from Bangladesh soil with specific activities between 1.2mmol/min/mg to19.8 mmol/min/mg at various levels of purification. Goyal *et al.* (2005) reported the Solid State fermentation of maize straw, xylan powder and carboxymethyl cellulose (CMC) by *Trichoderma viridi* to produce xylanases with crude activities of 5002units, 3990units, and 1523.3 units respectively.

The use of cost effective substrate for industrial enzymes production is the only practised way of producing cost effective enzymes for industrial applications.

Table 1 Zones of Clearance (Mm) of Molds Isolates on the Xylan Substrate

Isolates code	Zones of clearance (cm)		
	R1	R 2	AVERAGE ± SD
XM	1.7	1.5	1.6 ± 0.141[b]
XD_3	2.3	1.3	1.8 ± 0.707[b]
XA	2.6	2.3	2.5 ± 0.212[c]
XM_2	2.5	2.7	1.9 ± 0.141[b]
XJ	1.8	1.8	1.8 ± 0.000[b]
XD_2	2.2	2.8	2.5 ± 0.424[c]
XM_6	2.0	3.6	2.8 ± 1.131[cd]
XT	2.3	1.6	1.9 ± 0.919[a]
XK	0.8	0.8	0.8 ± 0.000[a]
XC_3	2.6	2.5	2.5 ± 0.070[c]

Means followed by the same letter along the rows are not significantly different at p 0.05 (Duncan multiple range tests). S.D;standard deviation**.** R1 and R2 are replicates 1 and 2 respectively. S.D; Standard deviation

Table.2 Morphological Characteristics of Fungal Isolates

S/no	Isolate code	Pigmentation (colony color)	Color of reverse	Color of medium	Texture of surface	Probable organisms
1	XA	Black	Cream	White	Fluffy and aerial mycelial	*Aspergillus niger*
2	XC	Verdigris green with irregular shape and patches	White	Cream	Highly fluffy and velvety	*Tricoderma harzianum*
3	XC_1	White peach to salmon pink	Pink	Cream	Velvety and fluffy	*Fusarium oxysporium*
4	XC_2	Sand brown	Brownish	Cream	Velvety to floccose	*Aspergillus terreus*
5	XC3	Black	Cream	White	Fluffy and aerial mycelia	*Aspergillus niger*
6	XD_2	Black	Cream	White	Fluffy and aerial mycelia	*Aspergillus niger*
7	XD_3	Green	White	White	Slightly velvety to floccose	*Aspergillus fumigatus*
8	XJ	Green	White	White	Velvety to floccose	*Aspergillus fumigatus*
9	XK	Green	White	White	Velvety and floccose	*Aspergillus fumigatus*
10	XM	Brown	Brownish	White	Velvety	*Aspergillus tamarii*
11	XM_2	Sand brown	Brownish	Cream	Velvety to floccose	*Aspergillus terreus*
12	XM_6	Black	Cream	White	Fluffy and velvety with aerial mycelial	*Aspergillus niger*

Table.3 Microscopic Characteristics of Fungal Isolates

S/NO	ISOLATE CODE	MICROSCOPIC CHARACTERISTICS	PROBABLE ORGANISMS
1	XA	Conidial heads round and radiates. Conidiopores colourless to brown and smooth. Vesicles globose while phialides borne directly on the vesicle. Metulae and foot cells are usually present.	*Aspergillus niger*
2	XC	Conidiopores bearing branches or phiali irregularly or in verticals at a right angle to slide. Conigenous cells hyaline. Phialic conidia D-septate, hyaline, glubose elongated with truncate base and usually gre	*Tricoderma harzianum*
3	XC_1	Conidiogenous cells hyaline, polyphialides, conidia, microconidia hyaline are oval to cylindrical even curved. Macroconidia 3-5 septate. Chlamydopores are produced.	*Fusarium oxyspotrum*
4	XC_2	Conidial heads strongly, columnar, conidiophores smooth and colourless. Vesicle hemispherical and domeshaped. Metulae closely packed. Metulae and phialides are usually present. Conidia small, smooth globose to sub-globose.	*Aspergillus terreus*
5	XC3	Conidia heads, round or globose large and radiate. Conidiopores colourless to brown and smooth. Vesicles globose while phialides borne directly on the vesicle. Metulae and frot cells are usually present.	*Aspergillus niger*
6	XD_2	Same as above	*Aspergillus niger*
7	XD_3	Conidial heads columnar of varying length. Canidiophores smooth, short and often greenish, vesicles flask-shaped and greenish, phialides borne directly on the vesicles. Conidial small, globose rough. Cleiothecia white to off white producing 8-spored axi.	*Aspergillus fumigatus*
8	XJ	Same as above	*Aspergillus fumigatus*
9	XK	Same as above	*Aspergillus fumigatus*
10	XM	Conidial heads large, radiate, vesicle thin walled, Metulae and phialides present. Conidial dark cylindrical to pyriform and closely roughened.	*Aspergillus tamarii*
11	XM_2	Same as XC_2	*Aspergillus terreus*
12	XM_6	Same as XD_2	*Aspergillus niger*

Table.4 Changes in the Activities of Xylanase Produced by Fungal Isolates During the 120hrs Study

	Enzyme Activity (unit/ml)	
Time	**XC**	**XM_6**
0h	32870.00[a]	40700.00[b]
24h	42190.00[b]	42250.00[b]
48h	42280.00[b]	42440.00[c]
72h	42300.00	42470.00
96h	42400.00[c]	42410.00[c]
120h	42410.00[c]	42350.00[b]

XC; *Trichoderma harzianum*, XM_6; *Aspergillus niger*

Means followed by the same letter along the rows are not significantly different at p 0.05 (Duncan multiple range tests). S.D; standard deviation

Table.6 Specific Activities Of *Tricoderma Harzianum* And *Apergillus niger* Xylanases Harvested At 120hrs

Time (120h)	Enzyme Activity (unit/ml)	Protein Content. (mg/ml)	Specific activity (unit/mg)
XC	42410.00[a]	5.83[a]	7287.0[a]
XM_6	42350.00[a]	5.76[b]	7352.0[b]

Means followed by the same letter along the rows are not significantly different at p 0.05 (Duncan multiple range tests). S.D; standard deviation

Depending on the abundance, other wastes which have been utilized for xylanase production include: soya oil cake, wheat bran, tomato pomace, avicel, wheat straw, rice bran, brewers spent grains (Motta *et al.*, 2014; Orji et *al.*, 2014).

Research findings from this current investigation have established great potentials of two fungal strains sourced from the local environment for production of xylanase. Also, this study showed that sugar cane bagasse which is waste lacks value and can be utilized extensively for xylanase production.

It is recommended that the industries using xylanase and FIIRO as research institute should collaborate and advance the study to commercial scale. This is the only practical way to stop importation of xylanase, create wealth from the waste (sugar cane bagasse), and create employment in various industries for the teaming population of youths.

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