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Production and Application of Xylanase by *Fusarium* Spp. Using Wood Shavings as Substrate

F C Adesina[1]*, A A Onilude[2], OA Oluboyede[1], A Olajide[2], B Bamkefa[1] and A Abiola-Olagunju[1]

[1]*Department of Microbiology, Lead City University, Ibadan, Nigeria*

[2]*Department of Microbiology, University of Ibadan, Ibadan, Nigeria*

***Corresponding Author:** F C Adesina, Department of Microbiology, Lead City University, Ibadan, Oyo State Nigeria.

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Abstract

Pulp bleaching potential of purified xylanase from a *Fusarium* spp. was determined. Moistened wood shavings samples after sterilization were inoculated with known inoculum size of fungus in solid state fermentation and incubated for 12 days. Assay of xylanase alongside FPase were carried out at 48 hours interval during incubation. Cell free crude xylanase obtained by separation method was purified step wisely by ammonium sulfate precipitation, gel filtration and anion exchange chromatography. The purified xylanase was characterized and its concentration was varied to bleach brown pulp. Effect of varied concentration of xylanase on kappa number and brightness of pulp was determined. Optimum xylanase activity of 64.6 U/g was obtained on 8*th* day of incubation while FPase activity was negligible throughout. Purification yield of 44% was obtained in final purified xylanase fraction. 25 U/g xylanase concentration on pulp reduced its kappa number from 43.1 to 30.4 after retention time of 2.5 hours at 50°C and brightness increased from 25% to 53%. Xylanase produced has potential of being used as bleaching agent in pulp and paper industry to replace chemical bleachers. Xylanase from fungi could serve as alternative to chemical bleachers in Nigeria Pulp and paper industry because of their high cost and associated environmental issues.

Keywords: *Wood Shavings; Fusarium; Xylanase; Fpase; Solid State Fermentation; Pulp Bleaching*

Introduction

Trees of various type are readily abundant in the Southwestern part of Nigeria because of its predominant forest vegetation. These trees consist of both hard and soft wood types [1]. The hardwood specie include: *Khaya senegalensis* (Mahogany), *Tectona grandis* (Teak), *Terminalia superba* (Afara), *Chlorophora excelsa* (Iroko), *Mansonia altissima* (Masonia), *Entandrophrama candollei* (Omu), *Gossweilerodendron balsamiferum* (Agba) while *Ceiba pentandra* (Araba), *Funtumia africana* (Ire) and *Albizia zygia* (Ayunre) are examples of softwoods [2]. These woody trees are rich lignocellulosic materials consisting majorly of lignin, cellulose and hemicelluloses with the hemicelluloses being the second most abundant of the three [3]. Xylan, a polymer of branched heteropolysaccharide consisting of a backbone of β-1, 4 linked xylopyranosyl units substituted with arabinosyl, glucuronyl and acetyl residues is the most abundant hemicellulose available in tropical woody trees [4]. The trees serve as raw materials for timber, lumbering and furniture industries; milling and processing of these woods generate large quantities of wood shavings representing most of the waste generated from these industries. This waste currently constitutes a large portion of municipal waste in this part of Nigeria and is usually disposed of by burning the biomass. Sometimes they are allowed to persist in the environment until they are carried by erosion to surrounding water bodies or slowly leach into these waters after persisting in dumps for a long period. These disposal methods cause environmental pollution and accumulation of greenhouse gases in the environment which is detrimental to the survival of biological entities. Since this waste is usually abundant in our environment, there is need to seek for an alternative way of disposing them by the waste alternatively could be converted to industrially useful materials if well managed. One of such alternative methods is by utilizing them as substrates for production of microbial enzymes that will be

useful industrially. Xylanase has been reportedly secreted in abundance by fungi in a wide range of lignocellulosic materials especially in solid state fermentation of these materials [4,5]. Xylanases are responsible for the hydrolysis of the xylan component of plant and woody materials [6]. One of the various industrial uses of xylanase is in the pulp and paper industry as biobleaching agent of cellulose pulp and primary treatment of waste generated from the industry [7]. Fungi are known to produce this extracellular enzyme in the process of degrading lignocellulosic substrates [8].

In recent years, there has been a phenomenal increase in the use of enzymes as industrial catalysts because they offer advantages over the use of conventional chemical catalysts for numerous reasons [8]. The global market for industrial enzymes was $2.9 billion in 2008, $3.1 billion in 2009 and $3.3 billion in 2010 and has reached over $4.4 billion in 2015, achieving a compound annual growth rate of 6%, according to B.C.C. research. Three quarters of this market is for enzymes involved in hydrolysis of natural polymers of which wood shavings is one [9]. Pulp and paper industry is one of the major polluting industries in the developed and developing countries because of the use of synthetic chemicals for bleaching pulp [10]. Recently, environmental protection agents have risen to minimize and consequently eradicate the release of toxigenic compounds generated into the environment by this industry. One of the ways to achieve this is by replacing the use of bleaching chemicals with microbial enzymes for pulp bleaching [11,12].

The Pulp and paper industry in Nigeria is almost dead mainly because of the escalating price of pulp bleaching chemicals. Development of techniques such as enzymatic bleaching of pulp with fungal xylanase obtained from solid state fermentation (ssf) of wood shavings will contribute to resuscitation of the paper and pulp industry in Nigeria. This is because replacement of chemical bleaching process with enzymatic bleaching using enzymes from ssf of wood shavings by indigenous fungi will reduce cost of production also, the waste would have been well managed and converted into useful material. This work is aimed at using a fungus isolated from degrading wood for the production of xylanase in solid state fermentation of wood shavings, characterizing the xylanase and to determine its efficiency as a potential bleaching agent for brown paper pulp.

Materials and Methods

Collection and processing of samples

Samples of wood shavings were obtained from Bodija plank market in Ibadan North Local Government Area, Ibadan, Oyo State, Nigeria and processed into 5 mm sized particles by milling and sieving. The shaving samples were thermally pretreated at 121°C and 15 psi for one hour to make the wood components more readily available to the fungal enzyme [13].

Isolation and maintenance of microorganisms

Fusarium spp obtained from our previous work [14] was maintained on Potato dextrose Agar, PDA (Oxoid) medium containing 0.1% (w/v) beechwood xylan. The plates were incubated at 30°C for 7 days and fungus was transferred into slants containing the same medium, incubated at same temperature and duration and subsequently kept at 4°C until required for use.

Preparation of fungal inoculum

20 ml of Tween 80 solution (0.1%) was poured into a 5 day old culture of the fungus earlier mentioned on Potato Dextrose Agar slants that had been incubated at 30°C. The suspension was kept under agitation with a magnetic stirrer at 120 revolutions per minute (rpm) to allow fungal spores to form suspension with the liquid. Afterwards the fungal spores were counted and diluted out such that 106 spore was contained in 1ml of the fungal spore suspension.

Production of xylanase by fungus through Solid state fermentation

15g of the pretreated wood shaving sample was supplemented with 5g of destarched wheat bran and placed into 250 ml Erlenmeyer flask. Replicates of each sample was made in different flasks. The samples were moistened with 10 ml of the solution having the following composition K_2HPO_4 – 0.23 g/l, $MgSO_4.7H_2O$ – 0.05 g/l, $NaNO_3$ – 0.05 g/l all dissolved in 700 ml of 0.1 M citrate buffer (pH 6.8) and approximately 300ml of corn steep liquor was added as organic nitrogen source. Sodium citrate was added to adjust the pH to 6.8 after

the addition of corn steep liquor. Pretreated wood shaving samples were autoclaved at 121˚C for 15 min, allowed to cool and were each inoculated with 2 ml of 10^6 spores per ml of the isolate as inoculum. Incubation was at 30˚C under static condition for 12 days. Control flasks were without fungal inoculum and subjected to the same incubation conditions. Crude enzyme within the incubated samples was separated and assayed at 48 hour interval over the period of incubation. Separation of crude enzyme from sporulated mycelia was carried out by first homogenizing the ssf mass in 50 ml of 0.1M citrate buffer (pH 6.8) and using sterile cheese cloth to separate the liquid from the solid substrate. The remaining fungal spore within the liquid was further separated from the liquid by centrifuging at 5000 rev/min at a temperature of 4˚C for 10 minutes, the clear supernatant from each flask was assumed to contain crude enzymes and was used for enzymes and total soluble protein assays. The separation steps was also done for the control flasks.

Assay for Xylanase and FPase

Xylanase activity in the clear supernatant obtained from the separation process in each flask was determined every 48 hours using beech wood xylan as standard substrate by modified method of Bailey (1994) while FPase production was determined using the method of Ghose [15]. For xylanase activity the assay mixture was made to contain 1000 µl 0.5% (w/v) beechwood xylan (Sigma Aldrich) in 0.1M citrate buffer (pH 6.8) and 1000 µl of suitably diluted crude xylanase incubated for 30 min in a water bath at 50˚C. Afterwards the reaction was stopped by adding 3 ml of dinitrosalicylic acid reagent and then boiled for 5 minutes resulting in colour change of the solution. The solution obtained was read in a spectrophotometer at 540 nm [16]. The amount of reducing sugars liberated was quantified using a xylose standard curve for xylanase and glucose standard curve for FPase. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar equivalents per minute under the assay condition. The data presented are averages of triplicate values.

Total soluble protein determination

Total soluble protein in the crude xylanase and purified samples was determined by the method of Lowry., *et al.* [17] using Bovine serum albumin as standard and absorbance was measured at 680 nm in a colorimeter (Jenway 5320E).

Purification of Xylanase

Crude xylanase extracts were purified step wisely by ammonium sulphate precipitation at different saturation levels (20 - 100%) [18]. Each fraction was tested for protein content and xylanase activity and fractions with relatively high xylanase activity were pooled, lyophilized and dissolved in 0.1M citrate buffer (pH 6.8) afterwards. The product was dialyzed against citrate buffer (pH 6.8) for 18 hrs with buffer being replaced with fresh one after every 6hrs during dialysis. 2 ml of the sample was applied to a chromatographic column (Pharmacia 1.5 cm x 20 cm) loaded with DEAE Sephadex A50 for ion exchange chromatography and equilibrated with the buffer at a flow rate of 0.85 ml/min for ion exchange chromatography. Unbound proteins were eluted with the buffer and collected into 10 separate tubes of 5 ml of the eluate in each. Bound proteins were eluted with a linear gradient of NaCl (0.1 - 0.5 M) at a flow rate of 0.85 ml/min into each 5.0 ml tube. Xylanase activities and protein concentrations were examined in the fractions eluted by the buffer and those eluted by linear gradient of NaCl as mentioned earlier. One fraction with relatively high xylanase activity from the unbound proteins was labelled as Fraction X and those eluate with high xylanase activity among the unbound proteins were put together, labelled as Fraction Y and were both characterized separately.

Characterisation of purified fungal xylanase

Effect of temperature on stability of xylanase activity

This was done by varying the incubation temperature of the purified enzyme - substrate mixture from 30 – 70˚C for duration of 1, 2, 3 and 4 hours. Xylanase activity was afterwards determined by the method of Miller, [16]. Relative enzyme activity was deduced from values obtained.

Effect of pH on stability of xylanase activity

Each purified enzyme – substrate mixture was prepared at different pH values of 2, 3, 4, 5, 6, 7, 8, 9 and 10 and then incubated at 50°C for 1, 2, 3 and 4 hours and enzyme activity determined using Miller [16] at the end of each duration at various pH values. Relative enzyme activity was deduced from values obtained.

Application of Purified Xylanase on Brown Paper Pulp

5g of oven dried brown paper pulp sample was weighed and soaked in distilled water for 2 hours. The soaked pulp was transferred into an electric blender (Model NY-306) and blended in wet blender for 10 minutes at low speed and for 15 minutes at medium speed. The pulp was afterwards made into a consistency of 10% (w/v) [19]. Ammonium sulphate precipitated fraction that has been reconstituted into Tris buffer after dialysis was used at different concentrations of 5 U/g, 10 U/g, 15 U/g, 20 U/g and 25 U/g of pulp was each separately applied to samples of the brown paper pulp with pH 8.2. After application, each mixture was incubated in a thermostatic water bath with shaker at 50°C for 2.5 hours, each incubated pulp was kneaded at intervals of 15 minutes for homogenous distribution of the enzyme. Control samples of the pulp were treated under the same condition with distilled water. Xylanase and distilled water treated pulps were filtered individually using Buchner funnel, filtrates were separately collected into Erlenmeyer flasks and kept for further analyses. Pulp treated with xylanase was subsequently washed with 100ml of distilled water to remove residual enzyme [20] and also kept for further use. The colour of the pulp was observed visually for colour change from deep brown to cream colour. The pulp filtrates form xylanase treated pulp was analysed by taking the absorbance at 237 and 465 nm to determine the release of chromophoric and lignin associated materials from it [12]. After treatment of xylanase with pulp, the activity of the enzyme was determined in order to confirm if the activity was retained and stable during the bleaching of pulp.

Analyses of Pulp

The pulp at a consistency of 3% was chlorinated for 45 minutes at 30°C. Pulp was further treated with Hydrogen peroxide at 10% consistency after several wash, incubated for 90 minutes at 78°C and washed again with distilled water. Kappa number and brightness of the pulp was finally determined using the Technical Association of Pulp and Paper Industry (TAPPI) test methods T236 cm-85 (Tasman and Berzins, 1957). The Kappa number was defined as the amount in ml of 0.1 N $KMnO_4$ solution required by 1g moisture free-pulp under the experimental condition. Degree of brightness (%), which describes how close the tested paper is to whiteness, was done using a laboratory scale colour difference meter at a wavelength of 457 nm.

Data Analyses

The experiments were done in triplicates independent of one another and the data presented are means of the triplicate values obtained and standard deviation. Statistical significance was analxyzed at $p < 0.05$ by one-way analysis of variance (ANOVA) and paired t-test using Statistical Package for Social Science (SPSS) software v18 (IBM, USA).

Results and Discussion

Figure 1 shows the time course of xylanase and FPase production by *Fusarium* spp with the enzyme production increasing from the 2nd day of incubation to the maximum for FPase on the 6th day with differences in FPase activity being only significant between the 4th and 8th day of incubation ($p < 0.05$). The peak for xylanase was on the 8th day and the differences in xylanase activity was significantly different at different days ($p < 0.05$). Production of the enzyme did not seize after the peak but decreased gradually until the 12th day of incubation ($p < 0.05$). However there was no enzyme production in all the control flasks. This gradual decrease may be as a result of the production of components and enzymes such as proteases that inhibit the enzyme activity Takahashi., *et al* [21]. Accumulation of end products that are inhibitory to the activity of the enzymes may have also contributed to the decrease in enzyme activity [22] observed after the 8th day of incubation. Different microorganisms have been reported to produce xylanase using different substrates and each of them had different duration of peak activity. *Penicillium canescens* showed maximum xylanase production after 192h of incubation [23] while *Fusarium solani* F7 [24] and *Aspergillus sydowii* SBS 45 [25] did so after 6 and 9 days of fermentation, respectively. Duration of maximum enzyme

activity was reported by Bajaj and Singh [26] to depend on substrate type and environmental condition, strain of microorganism and its genotypic characteristics.

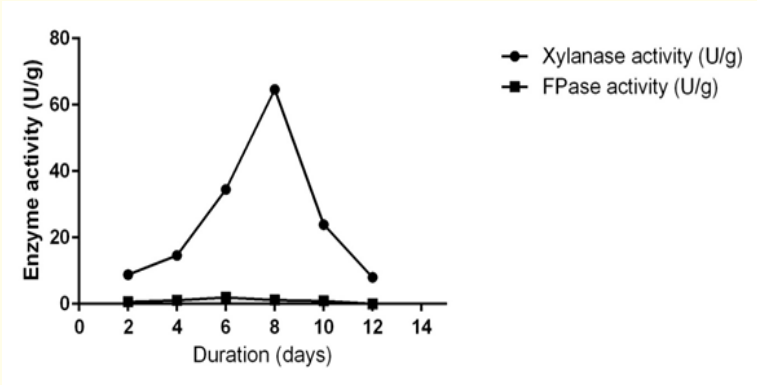

Figure 1: Xylanase and FPase activity of Fusarium spp.

Wheat bran was selected as adjuvant to the main substrate in this work because its cell-wall polysaccharides has been reported to contain 40% xylan, and that it does not aggregate under high moisture conditions, thus providing a large surface area for xylanase activity [27].

While there had been several reports of isolating cellulase free xylanases from fungi [28], the cellulase (FPase) activity of the fungus is highly negligible when compared to its xylanase activity. Also Ruckmanl and Rajendran [29]; Sudan and Bajaj [30] reportedly isolated xylanase with considerably negligible cellulose activity. This is quite useful for this experiment since the xylanase is intended for bleaching of pulp and it is an indication that the cellulose fibre will not be considerably attacked by the xylanase since it is virtually FPase free while it is being used for bleaching pulp. This was also the reason the FPase was also characterized along with the xylanase to determine the temperature and pH at which its activity is stable. The exclusion of mineral components containing Fe^{3+}, Zn^{2+} and Ca^{2+} ions from the moistening medium was deliberate since Nair., *et al.* [11] observed and reported that these ions affect the efficiency of the xylanase in bleaching pulp process and since this may affect the enzyme activity also thus their exclusion.

The activity of the xylanase produced by the fungus increased significantly ($p < 0.05$) to 76.8U after ammonium sulphate precipitation and lyophilisation. During ion exchange purification, a fraction of the unbound protein eluted by the equilibrating buffer was observed to have xylanase activity of 21.3U and this was kept as Fraction X. Two active peaks were also eluted at 0.2 and 0.3 M sodium chloride while eluting the bound proteins using a linear gradient of 0.1 - 0.5M NaCl, this was shown on Figure 2. These two peaks had xylanase activities of 21.9 and 6.3U so in addition to the activity of the xylanase from unbound proteins, a total xylanase activity of 49.5U representing 76.6% yield after ion exchange chromatography is obtained. Fractions of the unbound and bound proteins were characterized separately and activity of the xylanase from the unbound protein was found to be inhibited at pH higher than 7 and temperature higher than 35^0C (data not shown) while xylanase obtained from bound proteins still maintained its activity up to 50^0C and pH 8.5. The latter xylanase was thus used for stability tests (data shown) and its purification was presented on the purification table. This was because enzymatic bleaching condition of temperature 40 - 55^0C and pH 8 will render the xylanase from unbound proteins inactive while that from bound proteins will remain active.

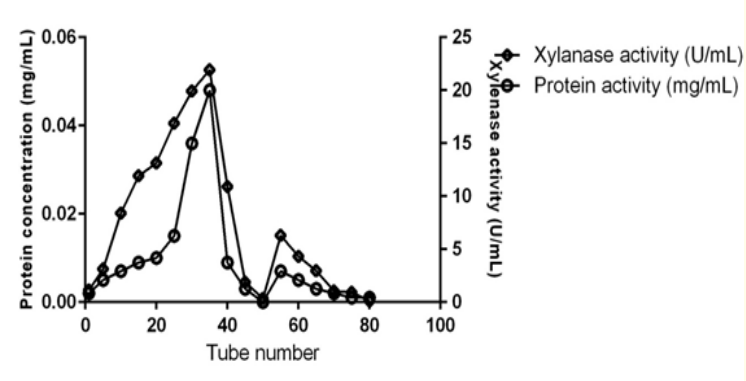

Figure 2: Ion Exchange Chromatography Profile of Xylanase by Fusarium spp.

Purification step	Total Soluble Protein (mg)	Xylanase activity (U)	Specific xylanase activity (U/mg)	Total Yield (%)	Purification Fold
Crude xylanase	6.9	64.6	9.36	100	1.0
Dialysed xylanase	4.1	76.8	18.73	103	2.0
Anion Exchange	2.0	28.2	14.1	44	

Table 1: Purification of the Xylanase Total Soluble Protein (mg).

Temperature and pH stability profile of the xylanase fraction Y showed that it is highly stable over a wide range of temperature and pH range. The xylanase was stable up to 55°C retaining up to 75% activity after I hour exposure to such temperature and pH range of 4 to 8. It was able to retain up to 80% activity after exposure for I hour to pH 9.

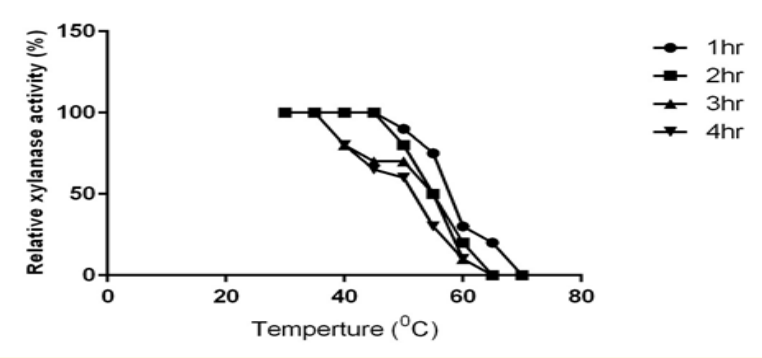

Figure 3: Temperature Stability of xylanase by Fusarium spp.

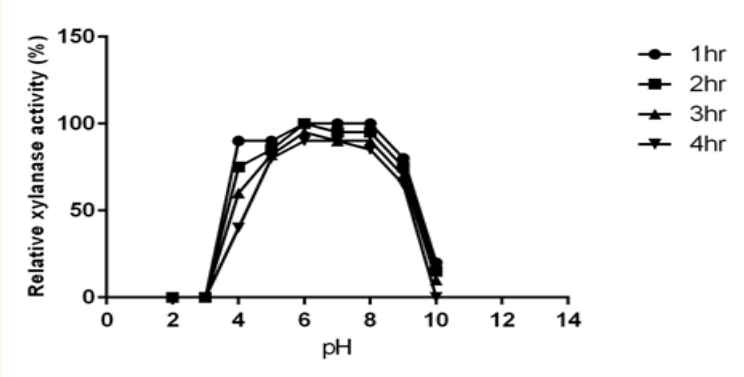

Figure 4: pH Stability of xylanase by Fusarium spp.

Table 2 shows the result of effect of xylanase treatment on brown paper pulp. The kappa number was observed to reduce gradually at an inverse proportion with xylanase concentration while the release of chromophoric compounds into the hydrolysate or filtrate was directly proportional to enzyme concentration. The differences in reduction was significantly different ($p < 0.05$). Kappa number reduced up to 29.5% at xylanase concentration of 25 U/g and this represents the highest decrease in Kappa number among the xylanase doses tested in this work. Chromophoric compounds in the filtrate was also highest at 25 U/g treatment of pulp. Efficiency in bleaching of pulp has been associated with increase in release of chromophoric compounds into pulp filtrate [31]. The results obtained for reduction in Kappa number and release of chromophoric compounds show that the highest bleaching efficiency was with the use of 25 U/g xylanase. Other workers also reported the efficient bleaching of different grades of pulp by different concentration of xylanase. The xylanase produced by *Aspergillus caespitosus* was reported by Sandrim., *et al.* [32] to reduce kappa number by 12.6% after treatment of pulp with 10 U/g dry pulp for 2 hours reduced, while xylanase of *Aspergillus flavus* and *Aspergillus niger* kappa efficiency corresponded to 36.32 and 25.93%, respectively on the pulp they were treated with. Medeiros., *et al.* [31] reported that xylanases from *Trichoderma longibrachiatum, Penicillium corylophilum* and *A. niger* reduced kappa number by only 1.1, 0.5 and 0.6 points when used to treat pulp at a concentration of 5 U/g dry pulp for 4 hours while *Aspergillus fumigatus* ABK9 xylanase (100 U/g) used by Das., *et al.* (2013) reduced kappa number by 4 points after 6 hours treatment of pulp. Xylanase of *Thermomyces lanuginosus* MC134 was also able to reduce kappa number by 3.2 points using a concentration of 50 U/g for 3 hours [33].

Treatment of pulp	Kappa number	Amount of xylose (mg/g) at 237nm	Amount of xylose (mg/g) at 465nm	Pulp brightness (%)
Initial pulp	4.3 ± 0.013	0.01 ± 0.007	0.02	5
5 U/g	41.3 ± 0.041	2.98 ± 0.003	0.13 ± 0.0072	9
10 U/g	39.4 ± 0.032	3.95 ± 0.001	0.24 ± 0.0043	5
15 U/g	36.3 ± 0.013	5.16 ± 0.003	0.39 ± 0.174	1
20 U/g	33.1 ± 0.61	6.89 ± 0.003	0.48 ± 0.0034	8
25 U/g	30.4 ± 0.003	9.03 ± 0.013	0.61 ± 0.035	3

Table 2: Effect of Xylanase on treated pulp.

A significant reduction of about 9.8% of H_2O_2 and 10.2% of chlorine was achieved when 25 U/g xylanase was used for treatment of pulp ($p < 0.05$). It was observed that there was a gradual reduction of the amount of bleaching chemicals used as the xylanase dose for pulp increased. Nair., *et al.* [11] achieved a result of 14.3% and 28.6% in elemental chlorine and H_2O_2 when 25 U/g xylanase *A. niger* SBC was used. Several researchers had also reported different percentage reduction in chlorine and H_2O_2 with different dose of xylanases from different sources [34-37].

Conclusion

In this work we succeeded in isolating fungal xylanases from wood shaving waste, purifying and characterising it and using the purified xylanases to bleach a brown pulp thus reducing the requirement for synthetic pulp bleachers that are unfriendly to the environment. This work is a good start to achieving replacement of synthetic pulp bleachers with fungal xylanases which are more environmentally friendly and the fact that waste is used as part of the raw material for the xylanases production may eventually cause a reduction in cost of bleaching brown pulp. Optimisation and scale up of this work will contribute to achieving production of microbial enzyme in Nigeria using this waste as substrate and subsequently reducing the presence of the waste around us, coupled with the fact that its disposal will be less environmentally challenging.

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