

# Qualitative assays and quantitative determinations of xylanolytic enzymes of wood rot fungi from Dagaga and Gambo forests, Ethiopia

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Received 8th July, 2019; Accepted 14th August, 2019

**ABSTRACT:** Xylan is a second widely available polysaccharide in nature and can be enzymatically degraded for the production of sugars. The complete biodegradation of xylan needs synergistic action of different xylanolytic enzymes. In this research, potential xylanolytic activities of the wood rot basidiomycete fungi from Dagaga and Gambo forests were screened. Xylanases of the potential fungi were also quantitatively determined. Clear zones round the cultures on xylan supplemented agar media of isolate 011-1D (*Trametes gibbosa*) and 030-1D (*Phellinus tremulae*) were significantly wider than other isolates. The fungal isolates differently responded to the incubation days both in submerged and solid-state fermentations and most isolates gave higher xylanase yield in solid-state fermentation than in submerged fermentation. The enzymes were active and stable in the temperature range of 40 to 55°C and pH range of 4.0 to 6.0. Incubation temperature of 30 to 35°C and pH of 5.0 to 7.0 were found to be suitable for production of the xylanases from the fungal isolates. Among the supplemented carbon sources, carboxymethylcellulose, xylan and sucrose were found suitable for xylanase productions but most isolates differently responded to the nitrogen source supplementations. MgSO<sub>4</sub>, ZnSO<sub>4</sub> and CaCl<sub>2</sub> were also found to be suitable divalent metallic ions for the productions of the enzymes. The isolates could be used for hydrolysis lignocellulosic xylan to 5-carbon sugars by optimizing their growth conditions.

**Keywords:** Ethiopia, lignocellulosic substrates, xylanase, wood rot fungi.

## INTRODUCTION

Lignocellulosic substrates are mainly composed of cellulose, hemicellulose, and lignin (Bibi et al., 2014). Large quantities of lignocellulosic wastes are generated through industrial processes such as paper-pulp, textile and timber industries and agricultural residues annually (Guimaraes et al., 2013) and their disposal is becoming a problem regarding disposal space and environmental pollution. However, these waste plant biomasses can serve as inexpensive substrate for microbial enzymes production (Facchini et al., 2011) and can be converted to reducing sugars (Buzala et al., 2017). Xylan is the second widely available polysaccharide in lignocelluloses (Hettrich et al., 2017) and needs synergistic action of different xylanolytic enzymes (Juturu and Wu, 2012).

Xylanase (EC.3.2.1.8) production has been reported for

bacteria (Sepahy et al., 2011), actinomycetes (Garg et al., 2011) and fungi (Das et al., 2013). Filamentous fungi are attracting greater attention because of their higher secretions of xylanolytic enzymes (Alvarez-Navarrete et al., 2015). These enzymes have wide industrial applications (Kumar et al., 2017). Xylanases are also used for complete saccharification of lignocellulosic biomass for bioethanol production (Malhotra and Chapadgaonkar, 2018). Conversion of lignocellulosic material into its fermentable monomers is a bottleneck step of utilizing the resource for industrial purposes. Screening and obtaining of efficient lignocellulolytic wood rot fungi from the natural environment has been recommended as one best strategy. Thus, the objective of the current study was therefore to assess the xylanolytic activities of wood rotting

fungi collected from Dagaga and Gambo forests of Arsi branch of Oromia forest and wildlife enterprise and optimize their productions.

## MATERIALS AND METHODS

### Qualitative assays of xylanolytic wood rot fungi

To conduct qualitative screening of the fungi for their xylanolytic activities, agar plates containing 2.5 g birch wood xylan, 5.0 g yeast extract, 0.2 g K<sub>2</sub>HPO<sub>4</sub> and 20.0 g agar in a liter of distilled water was prepared (Singh et al., 2012). Fifty-six (56) fungal species were screened. A 5 mm disc from a 5 days old culture was inoculated at the center of the sterile plates and incubated at 28±1°C for 7 days. Then, the plates were flooded with Congo red stain (0.1% w/v) for 15 minutes undisturbed and washed with distilled water and de-stained with 1M NaCl solution for another 15 minutes. A clear zone formation around the fungal colony was used as an indication of the hydrolysis of xylan. The diameter of the clear zone and the diameter of the colony were measured, and enzyme index (EI) was calculated as follow (Florenco et al., 2012).

$$EI = \frac{\text{Diameter of hydrolysis zone}}{\text{Diameter of colony}}$$

The cellulolytic fungi with EI greater than 1.5 were considered efficient and selected for quantitative determination of xylanases.

### Quantitative determinations of xylanolytic enzymes

#### Inoculum preparation

The medium containing 10.0 g glucose, 3.0 g yeast extracts, 3.0 g peptone, 1.0 KH<sub>2</sub>PO<sub>4</sub> and 0.5 g MgSO<sub>4</sub>.7H<sub>2</sub>O per a liter of distilled water was prepared and its pH was adjusted to 6.0 with 2M NaOH and HCl (Altaf et al., 2010). 100 ml of the medium was added 250 ml flasks each and autoclaved at 121°C for 15 minutes. After cooling, four disks (5 mm diameter each) from each isolate (Table 1) were inoculated into the flasks and grown on a rotary shaker at 150 rpm at room temperature. After six days of fungal growth, mycelial pellets were harvested, homogenized and used as inocula for the submerged and solid-state fermentations.

#### Submerged fermentation (SmF)

Czapek dox liquid medium containing 10.0 g xylan, 3.0 g NaNO<sub>3</sub>, 0.5 g KCl, 1.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MnSO<sub>4</sub>.7H<sub>2</sub>O, 0.01 g FeSO<sub>4</sub>.7H<sub>2</sub>O per liter of distilled water was prepared according to Hussain et al. (2012). Each 250 ml flask containing 100 ml of sterile media was inoculated with 3.0

ml fungal inoculum and incubated at room temperature and 150 rpm. At exactly five, eight, and twelve days of growth, the culture contents were filtered using nylon cloth and then centrifuged at 4000 rpm for 15 minutes. The cell free culture supernatant was used as crude xylanolytic enzyme.

#### Solid state fermentation (SSF)

Ten (10.0 g) gram birch wood xylan was moistened with 12 ml of the czapek dox liquid medium in 250 ml flasks. Each sterile flask was inoculated with 3 ml of mycelial homogenate and incubated at room temperature. Exactly after seven, twelve and fifteen days of incubation, the extracellular enzymes were extracted from the whole biomass twice with 25 ml of distilled water (total volume 50 ml). The solids were separated by filtration through nylon cloth and centrifuged at 4000 rpm for 15 minutes. The cell free culture supernatant was used as crude xylanolytic enzyme.

#### Determination of xylanase activity

Xylanase activity was determined according to Bailey et al. (1992). A test tube containing one ml of the enzyme extract and one ml of 1% w/v birchwood xylan (in 0.5 M sodium acetate buffer, pH 5.0) was incubated at 50°C for 10 minutes. Control tubes were made by adding 1 ml of 1% xylan and 1 ml of distilled water and incubated under the same condition. The resulted reducing sugars were determined according to Miller's modified method of DNS (Jadhav et al., 2013). Absorbance of the mixtures was measured at 540 nm by using spectrophotometer (Jenway Model 6305, UK). Standard curve was constructed using known solutions of xylose calculate the released reducing sugars. Then enzyme activity was calculated using the formula of Firmani et al. (2016) as follow.

$$\text{Enzyme activity} = \frac{[X]}{\text{Mol. Wt. X}} \frac{v}{p \times q} df$$

Where [X] = Xylose concentration (g/l), Mol.Wt.X = Molecular weight of xylose (g/mol), v = total volume of sample in each tube experiment (ml), p = volume of enzyme (ml), q = incubation time (minutes), df = dilution factor. One Xylose unit (U) was defined as the amount of enzyme that released 1 μmol of reducing sugar in one minute under the assay conditions.

#### Characterization of crude xylanase

##### Temperature optima and stabilities

To determine the optimum temperature for xylanase activity, the mixture from 12th day incubated SmF was

**Table 1.** Qualitative assays of xylanolytic of wood rot fungi.

No	Isolate code	Fungal species	Xylanase activity scale <sup>a</sup>
1	011-1D	<i>Trametes gibbosa</i>	+++
2	022(b)-1D	<i>Gymnopus eucalyptorum</i>	+++
3	027-1D	<i>Bjerkandera adusta</i>	++
4	030-1D	<i>Phellinus tremulae</i>	+++
5	026-2D	<i>Pholiota adipose</i>	++
6	029-2D	<i>Lenzites betulina</i>	++
7	033-1G	<i>Armillaria mellea</i>	++++
8	042-1G	<i>Stereum rugosum</i>	++
9	092-1G	<i>Polyporus cinnabarinus</i>	+++
10	004-2G	<i>Lentinellus cochleatus</i>	++

<sup>a</sup> EI calculated on the 7th day of growth: + = EI<1.5, ++ = 1.5<EI<2.5, +++ = 2.5<EI<3.5, ++++ = 3.5<EI<4.5.

incubated for 10 min at 35 to 60°C (at 5 °C intervals) and the reducing sugar released was determined immediately. For temperature stability studies, crude xylanase was pre-incubated in 0.05 M Na-acetate buffer (pH 5.0) at different temperatures (35 to 60°C) for 120 minutes and then xylanase activity was determined.

#### **pH optima and stabilities**

pH of the birch wood xylan and enzyme mixture was adjusted using Na-acetate buffer solution (pH 3.0 to 5.0) and sodium phosphate buffer solution (pH 6.0 to 8.0) and then xylanase activity was determined. For the pH stability experiment, the crude enzyme extract in buffer was pre-incubated under different pH initial conditions (3.0 to 8.0) for 120 minutes at 30°C and xylanase activity was determined.

#### **Optimization of culture conditions for xylanase production**

Effect of incubation temperatures and incubation pH on xylanase production were determined by SmF using modified czapek dox liquid medium at 5°C intervals (20 to 40°C) and at different pH levels ranging from 3.0 to 8.0. After 12 days of incubation (selected based on qualitative screening results), the cell free extracts from each flask were analyzed for xylanase activity.

Effects of different carbon sources on xylanase productions were determined by SmF of same medium supplemented with 1% of glucose, xylose, sucrose, CMC or xylan substituting the C source. Similarly, effects of nitrogen sources on xylanase productions were determined by supplementing 1% of malt extract, yeast extract, peptone, NaNO<sub>3</sub> or NH<sub>4</sub>NO<sub>3</sub> substituting the N source. For determination of metallic ion effects, 0.05% of CaCl<sub>2</sub>, CuSO<sub>4</sub>, MgSO<sub>4</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub> or ZnSO<sub>4</sub> were supplemented substituting the ion source. The test fungi in

all cases were incubated for 12 days at room temperature and then the cell free extracts from each flask were analyzed for xylanase activity.

#### **Statistical analyses**

All experiments were performed in triplicates. The means of three replicate values for all quantitative data in the experiments obtained were tested in a one-way ANOVA at  $p = 0.05$  using SPSS software and Tukey's test was used to evaluate mean differences between treatments.

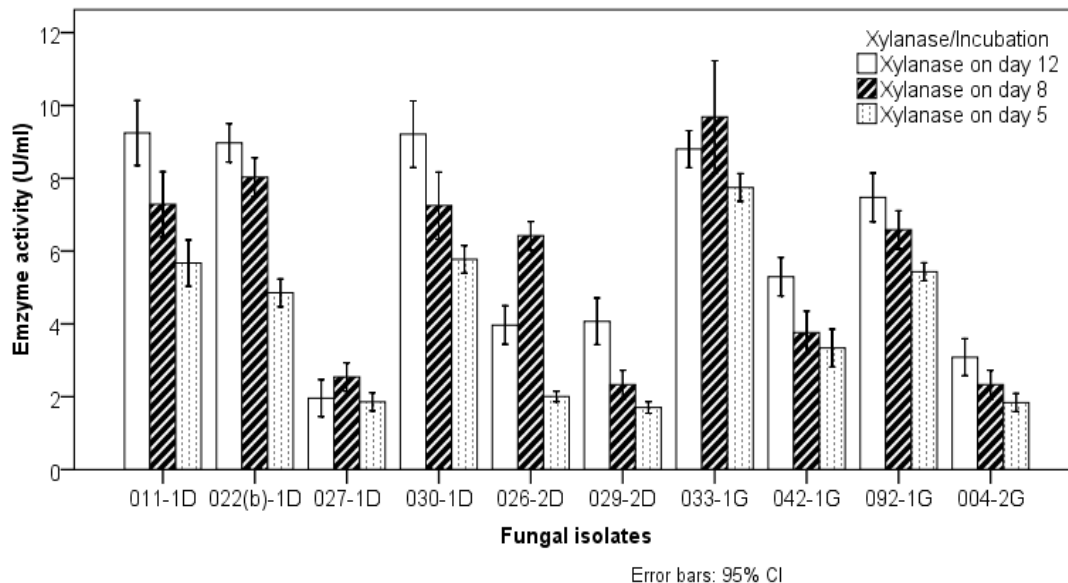
## **RESULTS AND DISCUSSION**

#### **Qualitative assays of the xylanolytic fungal isolates**

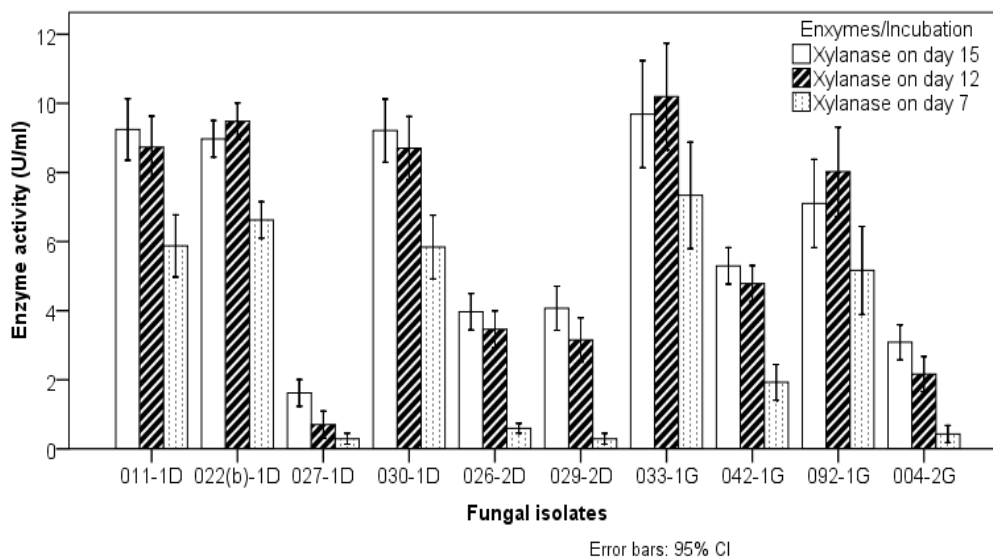
Different EI values were calculated for the tested fungal isolates (Table 1). Isolate 033-1G exhibited the highest EI value range (3.5 to 4.5). Four isolates (092-1G, 022(b)-1D, 030-1D and 011-1D) exhibited EI value range of 2.5 to 3.5 and the remaining isolates displayed less than 2.5 EI values. Damaso et al. (2012) described that isolates showing wider clear zone round their growing mycelia (higher EI values) can be considered as potential xylanolytic. Accordingly, isolates 030-1D, 022(b)-1D, 011-1D, 033-1G, 092-1G were selected for further quantitative xylanolytic assays.

#### **Quantitative determination of xylanolytic activities**

Isolate 033-1G (*Armillaria mellea*) displayed the highest xylanase amount (9.69±0.62 U/ml) on the 8th day of SmF (Figure 1). The second and third highest xylanase activities were exhibited by isolates 011-1D (9.24±0.36 U/ml) and 030-1D (9.21±0.37 U/ml) on the 12th day of SmF and they did not significantly differ ( $p>0.05$ ). Isolate 022(b)-1D was also exhibited considerable xylanase



**Figure 1.** Xylanase activities of wood rot fungal isolates incubated in SmF.



**Figure 2.** Xylanase activities of the wood rot fungal isolates incubated in SSF.

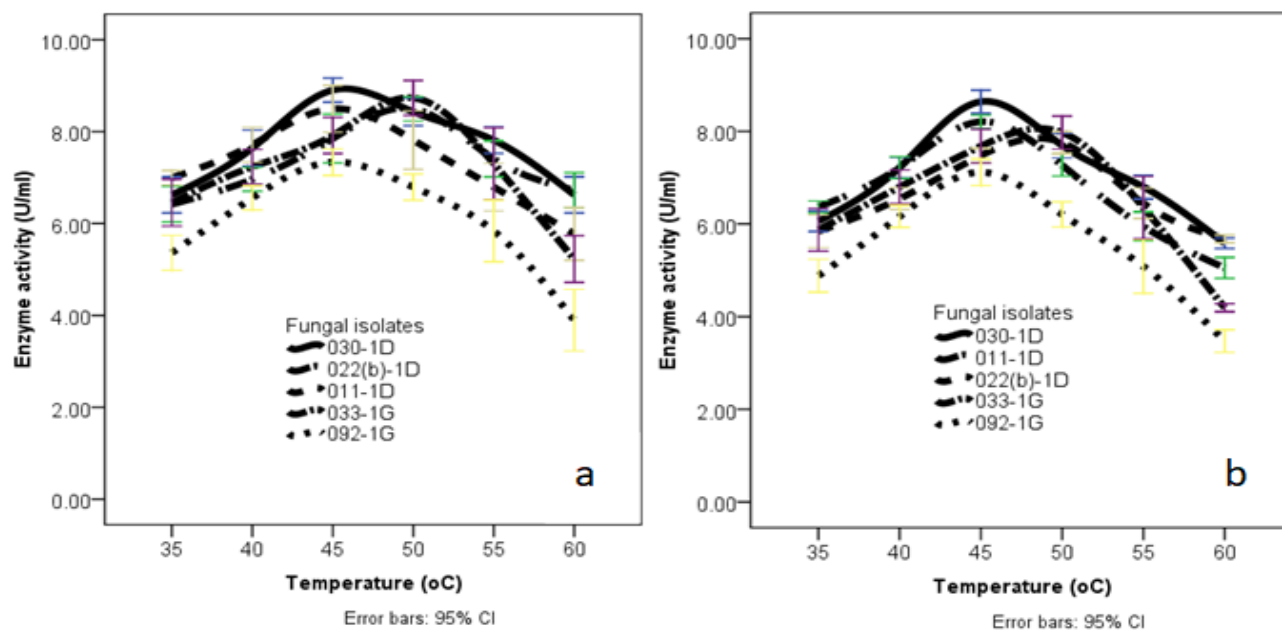
activity. The other isolates secreted significantly lower xylanase activities. On the other hand, all isolates except isolates 033-1G and 092-1G displayed their highest xylanase activities on 15th day SSF. Of all isolates the highest xylanase activity ( $10.20 \pm 0.62$  U/ml) was showed by isolate 033-1G on the 12th day (Figure 2) which was followed by xylanase activities from isolate 033-1G ( $9.69 \pm 0.62$  U/ml) on the 15th day and isolate 022(b)-1D ( $9.48 \pm 0.21$  U/ml) on the 12th day. When the two fermentation conditions compared, all tested fungal isolates showed higher xylanase activities in SSF than in SmF. By cultivating *Pleurotus austreatus* on mandarin peelings in

SmF, Elisashvili et al. (2009) reported xylanase activity of 16.0 U/ml on the 10th day. The authors, unlike findings of this paper, reported lower xylanase activity (4 U/ml) during SSF on 14th day. Similarly, Das et al. (2015) reported that fungal enzymes are better produced in SSF than in SmF.

#### Partial characterization of crude xylanolytic extracts

##### *Temperature optima and stabilities*

The highest xylanase activity was displayed by isolate



**Figure 3.** Temperature optima (a) and stabilities (b) of xylanases of five efficient fungal isolates.

030-1D (*Phellinus tremulae*) ( $8.90 \pm 0.11$  U/ml) at 45°C (Figure 3a). Significantly lower than the above xylanase activity, but not significantly differ from each other, were exhibited by isolate 011-1D at 45°C and by isolate 022(b)-1D at 50°C. Isolate 092-1G, displaying its own highest xylanase activity at 45°C showed the least xylanase activity. Temperature stability results also showed similar pattern of xylanase activities. But the activity values were significantly lower than the values in temperature optima (Figure 3b). Bilal et al. (2015) reported the highest xylanase activity of 11.0 U/ml at 28°C from *Trichoderma viride*. Though this is slightly higher than activity values reported by this research, its incubation temperature is much lower. Silva et al. (2015) assayed activity profiles of two xylanases Xyl I and Xyl II from *Trichoderma inhamatum* in SmF of xylan and reported stable activities of Xyl I at 50°C and Xyl II at 45 to 55°C.

#### **pH optima and stabilities**

The most pH optima xylanase activity value was exhibited by isolate 030-1D (*Phellinus tremulae*) ( $9.38 \pm 0.16$  U/ml) at pH 5.0 and followed by activity value from isolate 030-1D ( $8.60 \pm 0.18$  U/ml) at pH 4.0 (Figure 4a). The most stable xylanase activity was displayed by isolate 030-1D ( $8.63 \pm 0.11$  U/ml) at pH 5.0 (Figure 4b). Bilal et al. (2015) reported the highest pH stable xylanase activity of 14.73 U/ml at pH 5.5 by growing *Trichoderma reesei* on wheat bran. According to the authors further increase in incubation pH results in a declined xylanase activity. Similarly, Silva et al. (2015) assayed xylanase activity profiles from *Trichoderma inhamatum* in SmF and reported

both optimum and stable xylanase activity values in the pH range of 5.0 to 5.5. Compared to these reported optimum and stable xylanase activities, the isolates in the present research need lower pH incubation medium.

#### **Optimization of culture conditions for xylanase production**

##### **Effect of temperature on xylanase production**

Isolate 033-1G (*Armilleria mellea*) was able to produce the highest xylanase amount ( $10.88 \pm 0.12$  U/ml) in the temperature range of 30 to 35°C (Figure 5). This range was also optimum for xylanase production from isolate 011-1D ( $10.47 \pm 0.12$  U/ml). Isolate 022(b)-1D (*Gymnopus eucalyptorum*) secreted xylanase amount of  $9.79 \pm 0.21$  U/ml at incubation temperature of 35°C. In all, isolates incubations at temperatures below 25°C and above 40°C resulted in lower xylanase productions. Soliman et al. (2012) reported optimum temperature range of 30 to 40°C for xylanase production from *A. niger* and *T. viride*. Similarly, Suleman et al. (2016) observed maximum xylanase production at 35°C on wheat bran from *A. niger*. Temperature optima for xylanase production reported in this research were found to be similar to reports of other authors.

##### **Effect of initial pH on xylanase production**

The highest xylanase amount ( $8.29 \pm 0.24$  U/ml) was produced by isolate 033-1G at pH 7.0 and the second highest xylanase amount ( $7.78 \pm 0.15$  U/ml) was produced

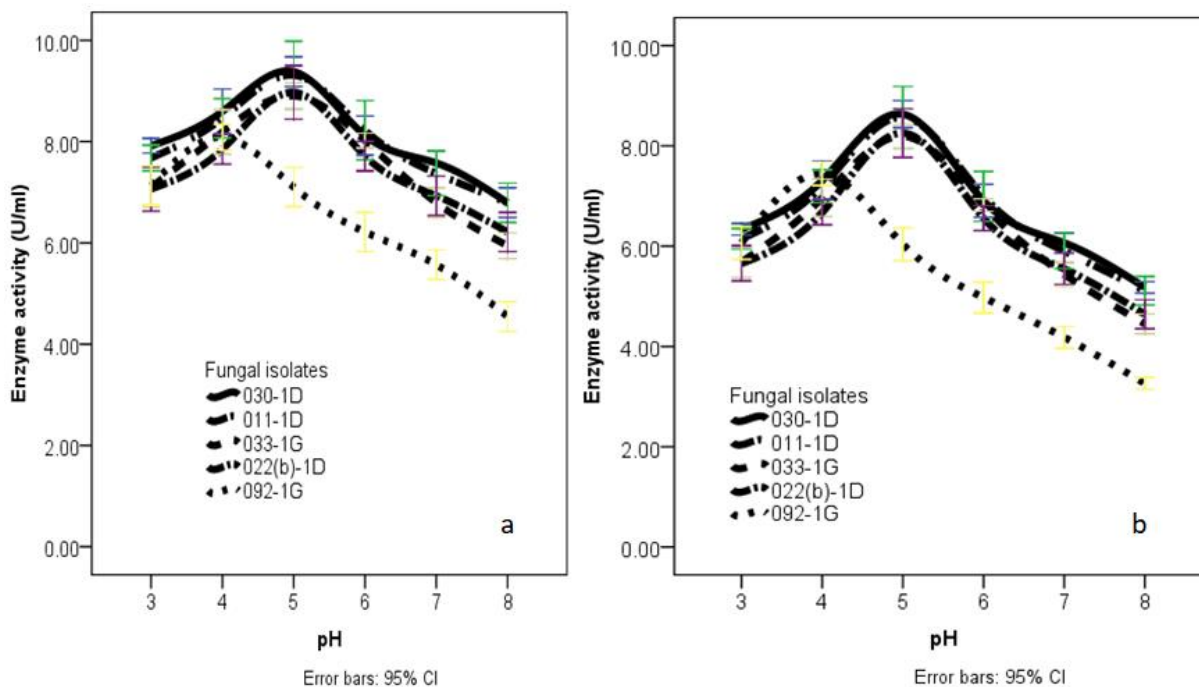


Figure 4. pH optima (a) and stabilities of xylanases of five efficient fungal isolates.

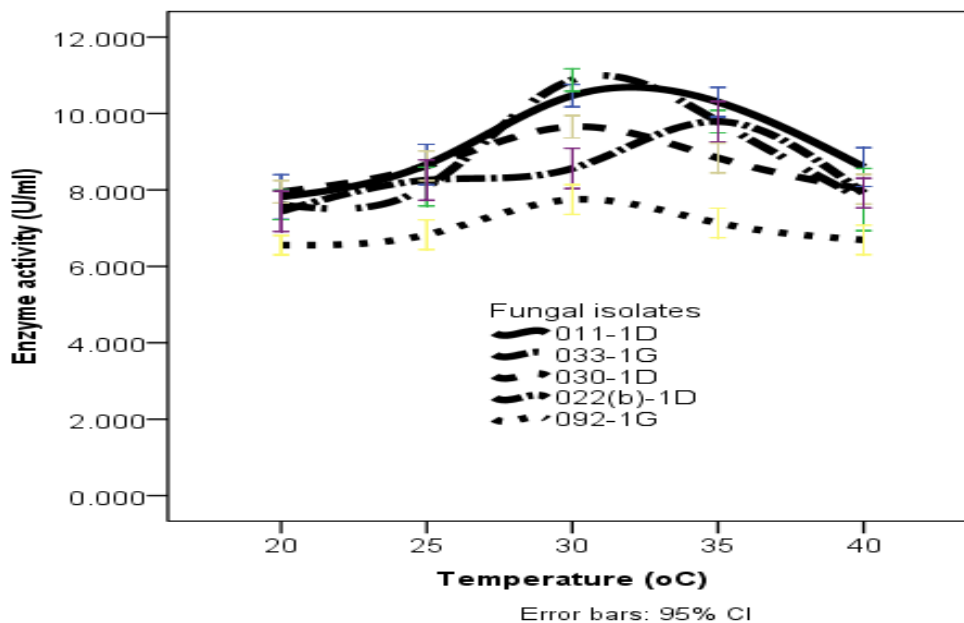


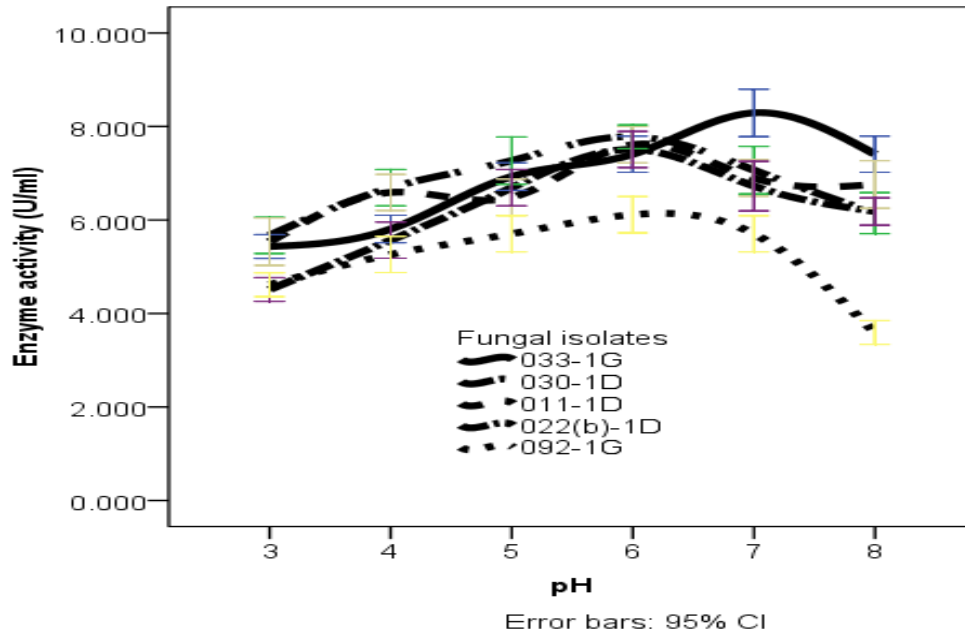
Figure 5. Effect of temperature on xylanase productions.

by isolate 030-1D at pH 6.0 (Figure 6). Slightly acidic pH (5.0-7.0) was found suitable for productions of xylanases from the fungal isolates. Similarly, Soliman et al. (2012) reported maximum xylanase productions at pH range from 4.5 to 6.5 for *A.niger* and *T. viride*. The authors also explained that xylanase production was decreased at under pH 4.0 and pH above 6.5. Pandey et al. (2014b) also

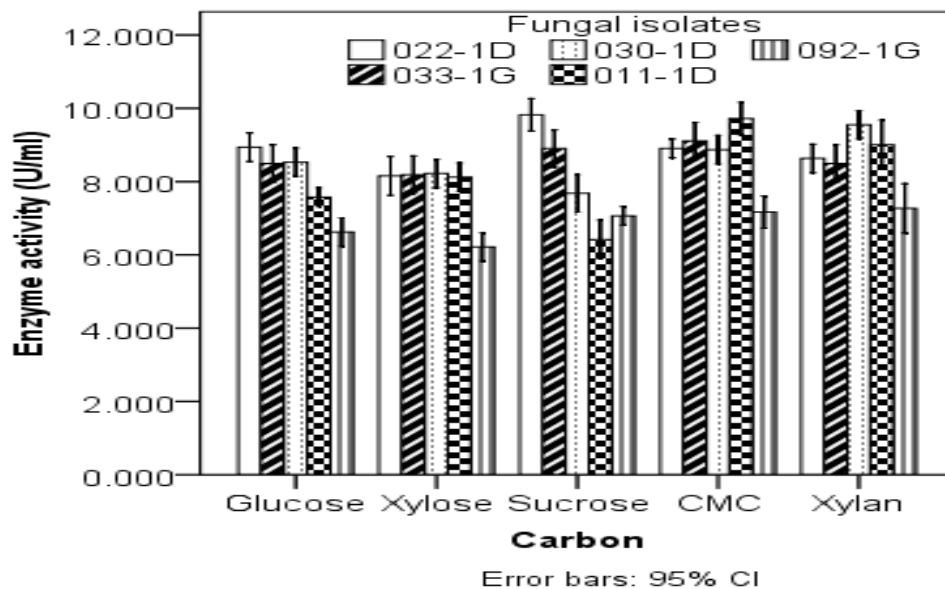
reported maximum xylanase production of 12.06 U/ml at pH 5.5 for *T.viride* grown on wheat bran.

#### Effect of Carbon sources xylanase production

Isolate 022-1D produced the highest xylanase amount of



**Figure 6.** Effect of initial pH on xylanase productions.

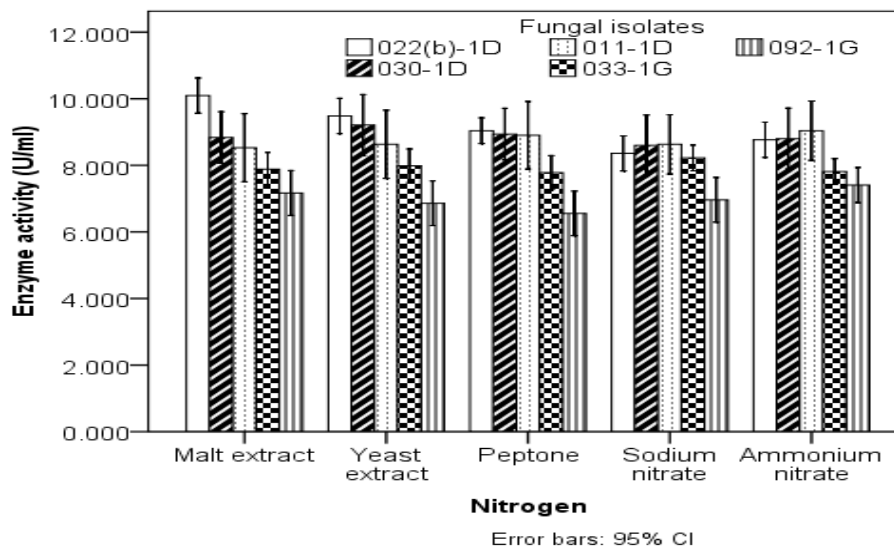


**Figure 7.** Effect of carbon source on xylanase productions.

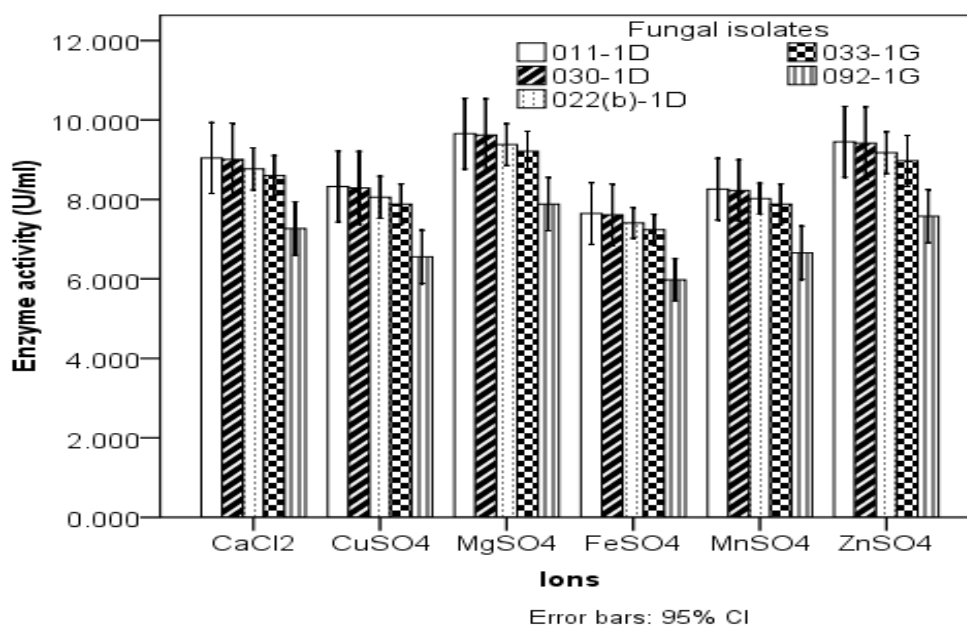
9.82±0.18 U/ml when sucrose was supplemented as carbon source (Figure 7). The next two highest xylanase productions were obtained from isolate 011-1D when CMC supplemented and from isolate 030-1D when xylan supplemented. Unlike the report made in this paper, Pandey et al. (2014a) reported the highest xylanase amount when xylan was supplemented at 1% concentration. Pandey et al. (2014b) reported the highest xylanase production of 15.96 U/ml for *T. harzianum* Thazad by supplementing wheat bran as a carbon source.

#### **Effect of Nitrogen sources on xylanase production**

Isolate 022(b)-1D secreted the highest xylanase amount when malt extract (10.10±0.21 U/ml) was supplemented and this amount was followed by xylanase yield by the same isolate when yeast extract supplemented (9.48±0.24 U/ml) (Figure 8). On the other hand, isolate 030-1D secreted xylanase productions of 8.80±0.37 U/ml and 8.60±0.37 U/ml in ammonium nitrate and sodium nitrate supplemented media, respectively. Pandey et al. (2014b)



**Figure 8.** Effect of nitrogen source on xylanase productions.



**Figure 9.** Effect of divalent metallic ions xylanase productions.

studied effect of different nitrogen sources (yeast extract, beef extract, peptone, soybean residue and corn powder) on xylanase production by replacing yeast extract in growth media (pH 6.0) and reported corn powder as the best nitrogen source since it gave the maximum xylanase activity for *T. harzianum* Th-azad.

#### **Effect of metallic ions on xylanase production**

Isolate 011-1D and 030-1D secreted the two top highest

xylanase amounts, respectively, which did not show significance difference in MgSO<sub>4</sub> supplementation (Figure 9). Supplementation of FeSO<sub>4</sub> in all isolates resulted in least xylanase productions. ZnSO<sub>4</sub> and CaCl<sub>2</sub> had also considerable xylanase productions for the test fungal isolates. In line to this report, Guan et al. (2016) reported Mg<sup>2+</sup> at 5.0 mM as the most suitable supplementation by comparing the effects of Zn<sup>2+</sup>, Fe<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, or Ca<sup>2+</sup> supplementations on xylanase productions from different wood rot fungi. But Cu<sup>2+</sup>, according to the authors' report, completely inhibited xylanase production. Similarly, Reis et



al. (2015) reported 17% of xylanase production improvement after addition of MgSO<sub>4</sub> during their research on wood rot fungi.

## Conclusion

Xylanases are nowadays being utilized in wider area of applications and their prices getting high. Screening fungal isolates from natural habitat is among the recommended strategies of getting efficient xylanase secreting fungi. High xylanase secreting wood rot fungi were obtained by screening wood rot fungi from Dagaga and Gambo forests owned by the Oromia forest and wildlife enterprise (OFWE). Amount of xylanase secretion was found to be highly depended on growth conditions, incubation temperatures and pH, and media supplementations. It was realized that by supplementing different media ingredients and adjusting optimum growth conditions, xylanase production from the fungal isolates could be maximized. Using this result as a baseline data, further screening works could bring more efficient fungal isolates for higher xylanase productions.

## ACKNOWLEDGEMENT

The authors would like thank the Ethiopian environment and forest research institute (EEFRI) and Wondo Genet College of forestry and natural resources (WGCF-NR) for the financial support.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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