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Production, Purification and Characterization of Xylanase from Oyster Mushroom (Pleurotus Sp.)

Fikiru Getachew[1] Melaku Alemu[2] AmehaKebede[3]
1.Lecturer at Dilla University
2.Researcher at Ethiopian National Agricultural Biotechnology Laboratory, Holeta
3.Microbiologists (Prof.) at Haramaya University

Abstract
Back gound: Xylanase is lignocellulolytic enzyme produced by Microorganisms, marine algae, protozoans, crustaceans, insects and snails. Fungal xylanases are attractive to be used in various industrial applications, but xylanase from oyster mushroom collected from Eucalyptus tree bark around Holetta is not screened and characterized. Therefore the objectives of this study were, to evaluate the in vitro production of xylanase by Pleurotus spp. using different lignocellulosic substrates, and to partially purify and characterize the xylanase produced by Pleurotus spp. with respect to changes in pH, temperature, and concentration of different substrates (wheat straw, teff straw, bean straw and Eucalyptus tree bark) to identify the most suitable medium for the production of xylanase. **Results:** The highest enzyme production was obtained on bean straw (2.38U/ml and 1.77 U/ml in solid state fermentation and submerged fermentation, respectively) and the lowest was obtained in media containing Eucalyptus tree bark (0.36 U/ml and 0.58 U/ml in solid state fermentation and submerged fermentation, respectively). Optimal production of xylanase was obtained when Pleurotus ostreatus was grown in solid state fermentation using wheat straw supplemented with 5% birch wood xylan, peptone and KCl salt at pH 4.0 and a temperature of 30°C under stationary conditions for four days. The xylanase from P. ostreatus was partially purified to homogeneity using different concentrations (30 to 80% (wt/vol) of ammonium sulphate. Xylanase having highest specific activity (11.47U/ml) and total protein content of 0.631mg/ml was recovered from the culture supernatant when precipitated with 40% (wt/vol) ammonium sulphate. The optimum activity was observed at 50°C and pH 6.0. The enzyme was very stable at a wide range of temperature and pH. Its apparent K_m and V_{max} were 186.67 µg/ml and 11.58µmole/min, respectively, showing its high affinity towards its substrate. **Conclusions:** Based on these results, the enzyme seems to be very stable at a wide range of temperature and pH in addition to having high affinity toward its substrate. These characteristics hopefully would make this enzyme potentially very attractive for animal feed processing and other industrial applications
Keywords: *Pleurotus spp.* Solid state fermentation, Xylanase, Wheat straw.

1.INTRODUCTION

During the growth of Fungi, various biochemical changes are known to occur, as a result of which enzymes are secreted extra-cellularly to degrade the insoluble materials in to simple and soluble molecules. Extracellular enzymes required to degrade the major components of lignocellulosic biomass into the low molecular weight compounds that can be very easily assimilated by their cells (Leatham, 1985; Cohen *et al.*, 2002; Reddy *et al.*, 2003). In natural environment, xylanases are produced mainly by microorganisms, marine algae, protozoans, crustaceans, insects and snails. Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than those found in yeasts and bacteria (Sunna and Antranikian, 1997). These enzymes affect the food and nutrient value, flavour and shelf life of these fungi (Chandra, 2011).

Hemicellulases are more complex enzymes than other lignocellulolytics and are capable of degrading hemicelluloses. The xylanases are the best characterized and most widely studied of the hemicellulolytic enzymes. Xylanases hydrolyze 1, 4-β-D-xylosidic linkages in xylan to produce xylooligosaccharide. This property makes fungal xylanases attractive to be used in various industrial applications. For instance, in pulp and paper industry, the xylanases are employed in the prebleaching process to reduce the use of the toxic chlorine chemicals. In animal feed industry, xylanases are used to increase the body weight gains of the animals. In bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume and shelf life (Palaniswamy *et al.*, 2008). Several reports indicate that it is practically and economically feasible to apply bioconversion processes to waste lignocellulosic materials, especially in developing countries (Chang and Steinkras, 1982; Martinez *et al.*, 1991).

The use of Xylanase enzymes in the biodegradation of organo pollutants, xenobiotics and industrial contaminants has been studied previously by Cohen *et al.*, (2002). Lignocellulosic wastes contain significant concentrations of soluble carbohydrates that aid in inducing the synthesis of enzymes and ensuring the efficient production of lignocellulolytic enzymes (Reddy *et al.*, 2003). In addition, due to the heterogeneity of xylan, the hydrolysis of hemicelluloses requires the action of a complex enzyme system that is required for a number of main chain and side chain cleaving enzyme activities. The main chain cleaving enzymes are endo-ß-1, 4-

xylanases, ß-1, 4-xylosidases and the more recently discovered enzymes named as exoxylanases (Biely, 1993).

Lignocellulosic wastes contain significant concentrations of soluble carbohydrates that aid in inducing the synthesis of enzymes and ensuring the efficient production of lignocellulolytic enzymes (Reddy *et al*., 2003). In addition, due to the heterogeneity of xylan, the hydrolysis of hemicelluloses requires the action of a complex enzyme system that is required for a number of main chain and side chain cleaving enzyme activities. Biodegradation of organo pollutants, xenobiotics and industrial contaminants has been studied previously by Cohen *et al*. (2002). These enzymes have shown enormous biotechnological potentials as they can be used at a wider level for the degradation of this highly complex plant cell wall material (Ren and Buschle, 2007) and detoxification of agro industrial residues with high phenolic contents (Mata *et al*., 2005).

Industrial enzymes are produced principally by submerged fermentation (SmF) but they are also produced by solid state fermentation (SSF) at a lower level. Although SSF is advantageous in terms of higher volumetric productivity and enzyme concentration, difficulties in scaling up restrict its use as the main technology for the industrial production of enzymes. Hence, this technique supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high water activity, such as bacteria (Babu and Satyanarayana, 1996).

However, in developing countries in general and in Ethiopia particularly, the production, purification and characterization of Xylanase Enzyme from fungi is not reported. Thus, the purpose of this study was to investigate the level of xylanase production by *Pleurotus* spp. in submerged and solid state fermentation using various carbon sources of different substrates with following the specific objectives:

- To evaluate the *in vitro* production of xylanase by *Pleurotus* spp. using different substrates
- To partially purify; and characterize the xylanase produced by *Pleurotus* spp. (in terms of effects of pH, temperature, and concentration of substrates).

2. MATERIALS AND METHODS

2.1. Description of the Study Site

The study was conducted at Holetta Agricultural Research Center, National Biotechnological Research Laboratory which is located at 34 km west of Addis Ababa in the Oromia Special Zone Surrounding Finfinne, Oromia Region. Geographically, HARC is located at latitude, longitude, and an altitude of 9°3’ N, 38° 30’ E and 2391 meters above sea level, respectively. Typically it has a bimodal rainfall pattern with a mean annual precipitation of about 1100 mm. The main rainy season extends from June to September while the short rainy from February to April. Mean annual maximum and minimum temperatures are 21°C (ranging from 20°C and 27°C) and 6°C (ranging from 2°C and 9°C), respectively (Tesfaye *et al*., 2004; EIAR, 2013).

2.2. Screening for Xylanase Producing Pleurotus spp.

Malt extract agar medium (MEA) containing xylan as the sole carbon source were inoculated with *Pleurotus* spp. The plates were incubated for 4 days at 30°C and positive xylanolytic activity was detected by the formation of clear zones of hydrolysis on plate after flooding the plates with 0.1% aqueous Congo red followed by repeated washing with 1 M NaCl. The composition of the MEA plate agar medium was (g/l): birch wood xylan, 1.0; peptone, 5.0; Malt extract, 5.0; K_2HPO_4, 0.2 and agar 20.0 , The final pH of the medium was adjusted to 5.5 prior to sterilization (Teather and Wood, 1987)

2.3. Source and Preparation of Growth Substrates

The growth substrates like teff straw, wheat straw, bean straw, Birch wood xylan and salt minerals were obtained from HARC and from local market. The selection of substrate for enzyme production in a SSF processes depends up on several factors mainly related with cost and availability of the substrates, and thus may involve screening of several agro industrial residues (Pandey *et al*., 1994). The lignocelluloses substrates were oven dried and ground using a manual grinder. All the resulting powder could pass through a 1 mm size mesh, and then the powder passed through 1 mm size mesh used for submerged fermentation and substrate above 1 mm used for solid state fermentation.

2.4. Preparation of Media for Enzyme Production

2.4.1. Media for submerged fermentation

Media used for submerged fermentation were arranged in four separate Erlenmeyer flasks of 250 ml capacity containing each 10 g of a single type of lignocellulosic substrate (eucalyptus tree bark, teff straw, wheat straw or bean straw smaller than 1mm). Experiments were performed in triplicate at room temperature (25±2°C) with shaking at 120rpm. The respective substrates were submerged in 200ml basal medium consisting of (g/l): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2; $CaCl_2$, 0.3; $MgSO_4$, 0.3; $FeSO_4.7H_2O$, 0.5; $MnSO_4.7H_2O$, 0.16; $ZnSO_4.7H_2O$, 0.14; $CoCl_2$, 0.2; and Tween 80, 1 ml; with a final pH of 5.5. The initial pH of the medium was adjusted to 4.0 prior to

sterilization by adding 1N HCl. The media was autoclaved at 121°C (15 lbs) for 20 min and cooled (Papaspyridi *et al*., 2012).

2.4.2. Media for solid state fermentation

Media used for solid state fermentation were arranged in four separate flasks of 250 ml capacity containing each 10 g of a single type of lignocellulosic substrate (eucalyptus tree bark or teff straw or wheat straw or bean straw). Experiments were performed in triplicate using Erlenmeyer flasks at 30°C. The respective substrates were moistened with 24 ml of salt solution (KH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 0.3 g, NH_4NO_3 0.1 g, $CaCl_2.2H_2O$ 0.03 g and 1 ml of 1% $FeCl_3$), with a final pH of 5.5. The media was then autoclaved at 121°C (15 lbs) for 20 min and cooled (Jemaneh, 2007).

2.5. Preparation of Inoculum

PDA inocula were prepared on PDA plates, where the fungal stock stored at 4°C was transferred onto PDA plates with a sterilized needle and then allowed to grow at 30°C in the dark for 4 days. The growing edges of the spore were cut with a sterilized 4 mm core borer and used as inocula. The inoculum size was $3.6x10^6$ spores/ml as determined by microscopic enumeration with a cell counting hematocytometer chamber (Neubauer chamber, Marienfeld, Germany). After it was inoculated to agar plates, the myceli of the inoculum piece were placed face down so that they had good contact with the agar surface (Jemaneh, 2007).

2.6. Growth of Pleurotus Sp. and Extraction of Xylanase

2.6.1. Submerged fermentation

Three mm^2 square of spore containing PDA was used to inoculate the flasks containing submerged media. After 4 days of mushroom cultivation, biomass was filtered through cotton gauze and the solids separated by centrifugation 10000rpm for10 min at 4°C. The supernatant was filtered through Whatman no. 1 filter paper and the clear filtrate was used as crude enzyme extract.

2.6.2. Solid state fermentation

The solid state media were inoculated with three mm square of spore containing PDA. After mixing, the flasks were incubated at 28 ± 2°C temperatures for 4 days under static conditions. After 4 days of incubation the solid state fermentation 100ml citrate buffer (0.05M at pH 5.3) rotated on rotary shaker at 120 rpm for at least 2 h at room temperature for maximum enzyme extraction. Liquid homogenate was then filtered through cotton gauze and centrifuged at 10000rpm for 10 minute at 4°C to remove solid particulate matter. Supernatant was filtered through Whatman no. 1 filter paper and the clear filtrate was used as crude xylanase preparation.

2.7. Enzyme Assay

2.7.1. Quantitative assay

The.xylanolytic activity was quantitatively assayed based on enzymatic hydrolysis of birch wood xylan and the reaction of the liberated reducing sugar with 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Xylose was used as the reference reducing sugar. One unit of xylanase activity was defined as the amount of enzyme that liberated reducing sugar at the rate of 1 µmole/min. The reaction mixture contained 900 µl xylan solution (1% birch wood xylan in 0.05 M citrate buffer pH 5.3) and 100 µl crude enzyme. After 10 minutes of incubation at 50°C, the reaction was stopped by adding 2ml DNS reagent followed by boiling for 5minutes and cooling (Bailey *et al*., 1992).

Absorbance was measured on spectrophotometer (Novaspec III) at 540 nm against a reagent blank. One unit (U) is defined as the amount of enzyme that releases1µ mol of reducing sugar equivalent to xylose per minute. The amount of xylanase produced in SSF and SmF was expressed as U/ml.

$$\text{Units/ml enzyme} = \frac{(\text{�booles of xylose liberated})(\text{df})}{(10)\,(0.1)}$$

df = Dilution factor
10 = Time of assay (in minutes) as per unit definition
0.1 = Volume (in milliliters) of enzyme used

2.7.2. Protein assay of the filtrate

Protein content of the culture supernatants was assayed by the folin ciocalteau method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as standard.

2.8. Optimization of Growth Conditions and Characterization of Xylanase

2.8.1. Time course of xylanase production

P*leurotus* isolates were grown on the basal liquid medium containing xylan and incubated at 30°C for 2-14 days and samples were harvested at 48 hour time intervals. The amount of xylanase was measured as described in

section 2.7.1.

2.8.2. Effect of pH on the production of xylanase

After preparation of the basal liquid medium containing xylan, suitable aliquots were adjusted at pH 2, 4, 6, 8, 10 and 12 using 0.1M NaOH and 0.1N HCl buffer system. After 4 days of incubation at 30˚C, the amount of xylanase was assayed as mentioned in section 2.7.1.

2.8.3. Effect of temperature on the production of xylanase

After inoculation the media were incubated at various temperatures ranging from 20˚C to 35˚C with 5˚C interval to determine the optimum temperature for the production of xylanase by isolate. After 4 days of incubation, the amount of the xylanase was assayed as mentioned in section 2.7.1.

2.8.4. Effect of different carbon source on the xylanase production

The basal medium was supplemented with various carbon sources at concentration of 5%. These carbon sources were: maltose, starch, glucose, sucrose, cellulose and xylan, wheat straw without any supplementation (as control). After 4 days of incubation at 30˚C, the amount of the xylanase was assayed as mentioned in section 2.7.1.

2.8.5. Effect of different nitrogen sources on the xylanase production

Different nitrogen sources, with concentration of 5% were used to see their inductive effect on the enzyme production. These includes ($(NH4)_2NO_3$, peptone, casein, tryptone, malt extract and xylan (as control). After 4 days of incubation at 30˚, the amount of the xylanase was assayed as mentioned in section 2.7.1.

2.8.6. Effect of metal ions on the xylanase production

To determine the effect of metal ions,on xylanase production, the basal medium was supplemented with different metal ions with final concentration of 5%. These include NaCl, KCl, $MnCl_2$, $ZnCl_2$, $CuCl_2$ and $CaCl_2$ and xylan. After 4 days of incubation at 30˚C, the amount of the xylanase was assayed under the standard assay conditions.

2.9. Partial Purification of Xylanase

The crude enzyme was purified from the culture supernatant fluid using ammonium sulphate in a buffer of pH 5.3 (Fialho and Carmona, 2004). For this purpose, various concentrations of ammonium sulphate, i.e. 30, 40, 50, 60, 70 and 80% were used for the precipitation of enzymes. Nine volumes of the respective levels were mixed in one volume of crude enzyme filtrate and kept at least for 10minutes at -20˚C (over-night). After thawing the mixture was centrifuged at 10000 rpm for 10minutes at 4 ˚C. The supernatant was then carefully discharged while the precipitate was dried by inversing the tube on tissue paper. The resulting precipitate was re-suspended in citrate buffer (pH 5.3) and analyzed for xylanase activity. The optimum xylanase activity at a specific concentration of ammonium sulphate reflects the best concentration to attain maximum enzyme recovery. The purification fold was calculated by dividing the specific activity recovered by the starting specific activity.

2.10. Characterization of Xylanase Produced by Pleurotus sp.

2.10.1. Effect of temperature on xylanase activity

Thermal stability was investigated by incubating the 100 µl xylanase of Pleurotus sp. in 900 µl of 0.1% xylan, at temperatures ranging from 20˚C to 90˚C with 10˚C intervals and pH 6.0 for 10 minutes. The residual activity of the enzyme was determined following the standard procedure.

2.10.2. Effect of pH on xylanase activity

The pH of reaction mixtures was adjusted with 100 mM of the following buffer solutions: citrate buffer (pH 2.0-5.0), phosphate buffer (pH 6.0-9.0) and 0.1M of NaOH buffer (pH 10.0-12.0). 900 µl of 0.1% xylan dissolved in respective buffers was incubated with xylanase (100 µl), incubated at 30˚C for 10 min and subsequently assayed.

2.10.3. Effect of substrate concentration on xylanase activity

Rate of xylan hydrolysis was determined by incubating 900 µl substrate (xylan) at various concentrations (2, 4, 6, 8, 10 and 12 µg/ml) with 100 µl of partially purified xylanase. The enzyme incubated without xylan served as control for 10minutes. The kinetic constants k_m and V_{max} were estimated following Lineweaver and Burk method (Bruno *et al*., 1994).

3. RESULTS AND DISCUSION

3.1. Screening for Xylanase Producing Pleurotus Ostreatus

The *Pleurotus Ostreatus* culture obtained from Laboratory was screened for Xylanase production based on their growth on potato dextrose agar (PDA) supplemented with 1% wood birch Xylan at 30˚C. The study organism confirmed, based on clear zone of hydrolysis, which confirm its ability to produce xylanase enzyme. Moreover, additional qualitative tests revealed that the culture was also found to display amylolytic and proteolytic activities

3.2. Xylanase Production on Different Lignocellulosic Substrates

When different substrates were used in both solid state fermentation and submerged fermentation medium, the highest enzyme production was obtained on bean straw (Figure 1) (2.38 U/ml, 1.77 U/ml respectively) and the least on eucalyptus tree bark (0.36 U/ml, 0.58 U/ml in SSF and SmF, respectively). This indicated that bean straw is the most suitable substrate for xylanase production by *Pleurotus* spp. when compared with other study substrates. This is probably due to higher protein content of bean straw. Wheat straw was the second highest in SSF, while the third in SmF.

Based on the substrate selection criteria bean straw was not selected for further study in this experiment. Study substrates were analyzed by economical point of view, its availability around study area and management to use it as substrate. Bean straw is more scare, more expensive and need high management (Easily perishable). According to Bakri *et al*., (2003) wheat straw is an inexpensive, agricultural by product, which contains a lot of xylan Therefore; further studies were conducted using wheat straw as .carbon source. Eucalyptus tree bark is natural substrate for the study organism, but the study showed that it results lowest production of xylanase. This is due to nutrient content found in it, which the organism highly need protein nutrient to grow (why it is highest in bean straw).

The production of xylanase by *Pleurotus* sp. was higher in SSF, using different substrates as carbon source, when compared to the results obtained in SmF with the same substrates (Figure 2). A higher efficiency on enzymatic production SSF is described by several authors for various enzymes and microorganisms (Kamra and Satyanarayana, 2004; Da Silva *et al*., 2005). While in submerged fermentation (SmF), the fungus is exposed to hydrodynamic forces; in SSF growth is restricted to the surface of the solid matrix. Another factor is that the use of solid systems (SSF) provides the fungus with an environment closer to its natural habitat (wood and decayed organic matter), which stimulates the fungi to produce more hemicellulolytic enzymes (Da Silva *et al*., 2005). Since this enzyme has an ability to hydrolyze hemicellulosic substrates and function at wide range of temperature and pH, it can be a great attractive to be utilized in industry after pretreatment of hemicellulose from agricultural wastes to fermentable sugars.

3.3. Optimization of Growth Medium for Xylanase Production Under SSF

3.3.1. Time course of xylanase production

The time course of xylanase production by Pleurotus ostreatus was investigated and maximum production was observed on the 4th days (Figure, 2), which was similar to xylanase production from Aspergillus niger (Widjaja et al., 2009); and different from that of Penicillium canescens in SSF which resulted in maximum production after 6 days of incubation (Jayalakshmi et al, 2007). Further incubation after this did not show any increment in the level of enzyme production, probably due to increase in toxic waste substances and depletion of nutrients in the media, which leads to decreased growth and enzyme production.

From the application point of view, faster production of an enzyme could be advantageous which may allow appreciable reduction in the production cost of the enzyme and products can be found in short period of time. Moreover, an organism that produces maximum xylanase activity in short incubation time offers significant advantage in reducing the risk of contamination

3.3.2. Effect of pH on the production of xylanase

The results revealed that optimum of pH 4.0 to be the best for xylanase production (Figure, 3), which was similar with result obtained by Fujimoto *et al*., (1995) by *Aspergillus aculeatus* xylanase. The pH value of the culture medium affected the permeability of cells as well as stability of enzyme (Mase *et al.,* 1996). The effect of hydrogen ion on enzyme may be due to the stability of the enzyme at particular pH and denaturation of the enzyme proteins occur at pH differ, the optimum pH of such enzyme. Acidic pH probably helps to keep away bacterial competition, as most bacteria require high water activity and a pH of around neutrality and above (Kalra and Sandhu, 1986).

3.3.3. Effect of temperature on the production of xylanase

The results revealed that the optimum temperature of production of xylanase was found to be 30°C (Figure, 4). The incubation temperature affects the level of enzyme production under SSF. Maximum production at lower temperatures may be advantageous because it can reduce the rate of evaporation during incubation. The fact that the organism produces maximum enzyme at mesophilic temperature hence facilitates the production of the enzyme without the need of incubation instrument and reduce the cost of enzyme production.

3.3.4. Effect of varying carbon sources on xylanase production

Efficient production of xylanase is dependent upon the choice of an appropriate inducer substrate and the medium composition. In this study, the production medium was supplemented with different carbon sources, maintaining other parameters constant, to find out their effect on the amount of xylanase. The results showed varying levels of xylanase depending on the kind of substrate (carbon source) used. Xylanase production was significantly increased when the production medium containing wheat straw was supplemented with xylan.

The report shows that production of xylanase was good on wheat straw without supplementation. It is

might due to presence of high amount xylan in wheat straw. According to Kulkarni *et al*. (1999) xylanase production is inducible by xylan rich substrates, which is similar to the present finding. Glucose, maltose and starch were found to reduce xylanase production while on the other hand sucrose completely inhibited its production (Figure 5), reported that the reason was believed to be in the presence of easily metabolisable substances, production of xylanolytic enzymes were decreased (repressed).

3.3.5. Effect of different nitrogen sources on the xylanase production

Enzyme production by Pleurotus sp. was seriously affected by the type of nitrogen source used in the growth medium (Figure, 6). This study showed that peptone was the best of the six nitrogen sources tested in resulting relatively higher level of xylanase production, it increase xylanase production by 14% which has an important role in enzyme synthesis, probably because this complex nitrogen source contains elements that are necessary for the metabolism of fungus when compared with production on control (xylan). In contrast, low xylanase production was observed in the medium supplemented with malt extract, which is similar with the result reported by Khandeparkar et al., (2006) and Kuhad et al., (2006), most of microorganism need nitrogen for growth and production of enzyme.

3.3.6. Effect of metal ions on xylanase production

Figure 7 shows that, the influence of metal ions on the production of xylanase by Pleurotus sp. It is generally known that xylanases may be inhibited or activated by metal ions or other reagents. A lot of impurities like metal ions, which can inhibit the production and activity of xylanase, exist in industrial wastes. Pleurotus spp. xylanase production was stimulated by addition of K^+, which increase 13.6% when compared with control; whereas addition of Zn^{+2} and Cu^{+2} inhibited the xylanase production. Similarly, xylanase production by *Penicillium glabrum, Penicillium sclerotiorum* and *Aspergillus ficuum* was inhibited by these ions (Knob *et al.*, 2013). Ponnusami *et al*. (2013) also reported that xylanase production by *Bacillus subtilis* was inhibited and stimulated by Cu^{+2} and Zn^{+2}, respectively. This may be due to its interaction with sulphydril groups, suggesting that there is an important cystein residue in or close to the active site of the enzyme.

3.4. Partial Purification of Xylanase

In the present study, xylanase was partially purified with 30-80% ammonium sulphate precipitation. The results showed that 40% ammonium sulphate saturation resulted in the highest xylanase activity with 1.5 purification fold. Similar studies also reported a xylanase with 1.25 fold purity from a fungus *Paecilomyces themophila* upon partial purification using 20-50% ammonium sulphate saturation (Lite *et al*., 2006). Enzymes could be used without purification for commercial applications (Marta *et al*., 2000) because of the fact that crude extracts have synergistic activities and the presence of some other factors in the crude extract that stabilizes the xylanase enzyme.

Higher activity of crude enzyme is very important especially when the enzyme is to be applied in its crude form not in pure condition (Kulkarni *et al*., 1999). However, purification enhances the efficacy of the xylanase and hence purity is needed.

3.5. Characterization of Partially Purified Xylanase

3.5.1. Effect of temperature on xylanase activity

Figure 8 shows that, the influence of temperature on the activity of Pleurotus sp. xylanase. Pleurotus isolates'xylanase in this study gave promising results, with maximum activity when incubated at 50˚C (11.47 Unit/ml) which is similar with xylanase from streptomyces sp. (Georis et al., 2000; Bajaj and Singh 2010), Aspergillus terreus (Ghanen et al., 2000), Aspergillus aculeatus (Fujimoto et al., 1995). When the temperature was increased or decreased from 50˚C, the activity was gradually reduced probably due to enzyme denaturation and conformation change.

Usually, xylanases from filamentous fungi show optimum temperature between 40˚C and 55˚C (Knob and Carmona, 2010; Dobrev and Zhekova, 2012), that was in agreement with present study. Nevertheless, other fungal xylanases show optimum temperature at 60°C or above (Fawzi, 2010). Incubation temperature for enzyme substrate reaction plays a critical role in enzyme activity; the stability of the enzyme in a wide range of temperature shows its potential application in different industries (Seyis and Aksoz, 2003).

3.5.2. Effect of pH on xylanase activity

The *Pleurotus* sp. xylanase showed optimal activity at pH 6.0 (Figure 9) in agreement with xylanase obtanined from *Bacillus* sp. K1 (Ratanakhanokchai *et at*., 1999) and *Bacillus* sp. C-125 (Honda *et al*., 1985). Similarly, most of xylanases from different fungi show optimal activity in pH between 5.0 and 7.0 (Madlala *et al*., 2001). A pH stability is an interesting enzyme property due to the great industrial importance. Xylanses which are active in the acidic pH range (pH 4.8-6) are considered to be suitable for application as animal feed supplement and relatively high temperature will be of great advantage for the hydrolysis of wastes, especially in pulp and paper industry waste as it contains more dissolved xylan. (Tony *et al*., 2005).

3.5.3. Effect of substrate concentration on xylanase activity

The present xylanase apparent kinetic parameters were calculated using Lineweaver Burk plot. The regression equation for the Lineweaver Burk slopes of *Pleurotus* sp. xylanase was y =16.098x+0.0863 (R^2 = 0.895) an apparent V_{max} of (11.58µmole/min) and apparent K_m value of 186.56µg/ml, According to Sa-Pereira *et al*., (2002), generally K_m value for xylanases obtained from some microbial sources was relatively low (25-1700µg/ml) which is agreed with present study. The lower the $K_{m,}$ the higher affinity of the enzyme towards its substrate (Hamilton *et al*., 1998) and the larger the velocity, the higher will be the amount of substrate binding which is a desirable quality for an enzyme, Monisha *et al*., (2009) reported that V_{max} of 0.000068µmole/min for partially purified xylanase from *Bacillus pumilus* which was comparatively lesser than the present study.

4. SUMMARY, CONCLUSION AND RECOMMENDATIONS

4.1. Summary

This study was conducted to screen xylanase producing oyster mushroom species collected from Eucalyptus tree bark around Holetta, to evaluate the in vitro production of xylanase by Pleurotus spp. using different lignocellulosic substrates, and to partially purify and characterize the xylanase produced by Pleurotus spp. with respect to changes in pH, temperature, and concentration of substrates.

A total of 100 mushroom specimens were randomly collected from eucalyptus tree bark in the premise of Holetta Agricultural Research Center campus. 100% of the collected mushroom specimens were identified morphologically and biochemically as Pleurotus sp. and also screened for xylanase production depend on the clear zone formation on malt extract agar plate containing xylan as sole carbon source.

Screened mushroom specimen were cultivated using both solid state fermentation and submerged fermentation systems supplemented with different substrates (wheat straw, teff straw, bean straw and Eucalyptus tree bark) to identify the most suitable medium for the production of xylanase.

The highest enzyme production was obtained on bean straw (2.38U/ml and 1.77 U/ml in solid state fermentation and submerged fermentation, respectively) and the lowest was obtained in media containing Eucalyptus tree bark (0.36 U/ml and 0.58 U/ml in solid state fermentation and submerged fermentation, respectively).

Optimal production of xylanase was obtained when Pleurotus ostreatus was grown in solid state fermentation using wheat straw supplemented with 5% birch wood xylan, peptone and KCl salt at pH 4.0 and a temperature of 30°C under stationary conditions for four days.

The xylanase from *Pleurotus* sp. was partially purified to homogeneity using different concentrations (30 to 80% (wt/vol) of ammonium sulphate. Xylanase having highest specific activity (11.47U/ml) and total protein content of 0.631mg/ml was recovered from the culture supernatant when precipitated with 40% (wt/vol) ammonium sulphate.

The optimum activity was observed at 50°C and pH 6.0. The enzyme was very stable at a wide range of temperature and pH. Its apparent K_m and V_{max} were 186.67 µg/ml and 11.58µmole/min, respectively, showing its high affinity towards its substrate.

4.2. Conclusions

This study was concluded that a reasonably higher amount of xylanase can be produced by *Pleurotus* sp. cultivated in SSF system than in SmF system.

The cultivation system can easily be modified to enhance productivity and facilitate the scale up process for mass production of xylanase. Using this system, maximum production of xylanase can be achieved in 4 days at pH 4 and a temperature of 30°C when a basal medium containing wheat straw is supplemented with xylan, peptone and KCl.

Furthermore, the xylanase produced by the test organism can be extracted and partially purified with 40% ammonium sulphate precipitation to get optimum activity at 50°Cand pH 6.0.

The enzyme seems to be very stable at a wide range of temperature and pH in addition to having high affinity toward its substrate. These characteristics hopefully would make this enzyme potentially very attractive for animal feed processing and other industrial applications

4.3. Recommendations

- This study is recommended that further research be conducted on molecular level characterization of *Pleurotus* sp.
- Optimization of *Pleurotus* sp. xylanase production would be done, to be used in industrial level.
- Based on the *pleurotus* sp. xylanase characteristics; the enzyme was potentially very attractive for animal feed processing and other industrial applications.
- This study is also strongly recommended that researches have to be done on large scale production,

further purification and characterization of xylanase from *Pleurotus* sp.

5. ABBREVIATIONS

BSA	Bovine Serum Albumin
DNS	Dinitrosalicylic Acid
EIAR	Ethiopian Institute of Agricultural research
EC	Enzyme Commission
HARC	Holetta Agricultural Research Center.
K_m	Michael Menten Constant
MEA	Malt Extract Agar
NABL	National Agricultural Biotechnology Laboratory
PDA	Potato Dextrose Agar
SmF	Submerged Fermentation
SSF	Solid State Fermentation
V_{max}	Maximum Velocity
U	Enzyme Unit

6. ETHICAL CONSIDERATION

All activities conducted were achieved to uphold the honour, dignity and ethical standards of the study.

In addition, in order to avoid unfavorable criticism we permit the proper officials and only after the associate has been informed of the nature of the criticism that every experiment was done.

No group of researchers or any part may make representation to Government, its members or officials or any other authority on matters affecting the interests of any living organisms employed in experiment or advocating a change in ethical standard and policy without the knowledge and consent of the Director acting on behalf of the Board.

7. CONSENT OF PUBLICATION

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8. AVAILABILITY OF SUPPORTING DATA

The data set(s) supporting the result of this article is/ are included within the article and also the data set(s) be cited where appropriate in the manuscript, and included in the reference list

9. COMPETING INTERESTS

"Not applicable" I have no competing interests to declare

10. FUNDING

"Not applicable"

11. AUTHORS' CONTRIBUTIONS

FG: conducting all parts of research laboratory works, analysis and write up; AK: Major Advisor, supervises each and every activity of the work and MA: Co- advisor, supervises all laboratory works

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13. AUTHORS' INFORMATION
1. **Fikiru Getachew:** MSc.in Biotechnology; Lecturer and Researcher at Dilla University
2. **Melaku Alemu:** PhD in microbial biotechnology ;Researcher at Ethiopian National Agricultural Biotechnology Laboratory, Holeta.
3. **AmehaKebede**: PhD in microbiology (Prof.) researcher and lecturer at Haramaya University

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15. LIST OF FIGURES

Figure 1: Xylanase production by different lignocellulosic substrates under SSF and SmF.
Figure 2: Effect of incubation time on the production of xylanase under SSF.
Figure 3. Effect of pH on the xylanase production under SSF.
Figure 4: Effect of temperature on the xylanase production under SSF.
Figure 5: Effect of different carbon sources supplementation on the xylanase production under SSF
Figure 6: Effect of different nitrogen sources supplementation on the xylanase production under SSF.
Figure 7: Effect of different metal Ion supplementation on the xylanase production under SSF
Figure 8: Effect of Temperature (°C) on the activity of xylanase, under SSF
Figure 9: Effect of pH on the xylanase activity at different pH ranging from 2 to 12.under SSF
Figure 10: Rate of substrate degradation at pH 6.0 and 50°C