Addis Ababa University

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Title: Production and characterization of xylanolytic enzyme from white rot fungi Isolated from decayed woods

A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

By: Tariku Abena

Supervisor: Addis Simachew (Ph.D)

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School of Graduate Studies, INSTITUTE OF BIOTECHNOLOGY

Advisor's Approval Sheet

This is to certify that Tariku Abena's MSc. Thesis research entitled "**Production and characterization of xylanolytic enzyme from white rot fungi isolated from decayed woods**" has been conducted under my supervision and guidance for the award of the degree of Master of Science in Biotechnology at Addis Ababa University. The results presented by him are genuine and the candidate himself has written the script of the Thesis. Therefore, I kindly request the Institute of Biotechnology of Addis Ababa University for final approval and acceptance of the Thesis.

Addis Simachew (PhD) Signature _____ Date_____

Candidate's Declaration

I certify that the work contained in the Thesis entitled **"Production and characterization of xylanolytic enzyme from white rot fungi isolated from decayed woods"** is original and has been done by myself under the general supervision of my Advisor **Addis Simachew (PhD),** Institute of Biotechnology. This work has not been submitted in part or in full for any other diploma or degree of this or any other University.

Tariku Abena Signature_____ Date _____

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School of Graduate Studies, Institute of Biotechnology

Examiners' Approval Sheet

This is to certify that the Thesis prepared by Tariku Abena, entitled: **"Production and characterization of xylanolytic enzyme from white rot fungi isolated from decayed woods"** is submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology complies with the regulations of the University and meets the standard with respect to originality and quality signed by the examining committee:

Signed by the Examining Committee:

Dr.Addiss Simachew (Advisor) _____ _____

Signature Date

We, the undersigned, members of the board of examiners of the final open defense by Tariku Abena have read and evaluated his Thesis entitled **"Production and characterization of xylanolytic enzyme from white rot fungi isolated from decayed woods"** and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirement for the degree Maters of Science in Biotechnology.

Examiners

Diriba Muleta (Ph.D) _____ _____

Signature Date

Fistum Tigu (Ph.D) _____ _____

Signature Date

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List of Abbreviations

BSA…….bovine serum albumin

CAZY……carbohydrate–active enzyme

CRD…….completely randomized design

DNS……Dinitrosalicylic Acid

EIAR……Ethiopian Institute of Agricultural Research

HMWLI……high molecular weight with low isoelectric point

Km……...michealis constant

LMWHI……..low molecular weight with high isoelectric point

MSM……Mineral Salt Medium

NABRC……..National Agricultural Biotechnology Research Center

NCBI……..national center for biotechnology information

PI……...Isoelectric point

SmF…….……..……..submerged fermentation

SSF……..……solid state fermentation

U……unit

Vmax……maximum velocity

WRF……...……..……white rot fungi

WS …….……..…Wheat straw

Abstract

Utilization of enzymes like xylanase, in different industrial processes across the globe gained more attention than the chemical catalyst because they are highly selective, easy to control and have a negligible environmental impact since they produce very small amount of byproducts. Therefore, this study was undertaken with the objective of production, purification and characterization of xylanase from white rot fungi. In the present investigation, 68 white rot fungi (WRF) isolates were screened for xylanolytic potential using qualitative (plate assay) and quantitative (ferementation) methods. The optimum growth conditions for xylanase production were determined by growing fungal isolates at different: - pH values, incubation temprature and carbon and nitrogen sources. The feremetation was conducted under SSF and SmF condition using wheat straw as sole carbon source. The enzyme was assayed using 3, 5-dinitrosalysalic acid (DNS) method by measuring the amount of xylose released in µmol/ml/min at 540nm. Partial purification of the enzyme was conducted by ammonium sulfate precipitation method and protein concentration determined. Partial purified xylanas was characteriazed under different physico-chemical condtions to determine the optimum pH, temperature and the effect of metal ions. Among 68 WRF qualitatively screened, 17 isolates with ≥4.55 cm hydrolysis zone on mineral salt medium (MSM) or basal medium were selected and subjected to qualitative screenng. Out of quantitatively screened isolates, five WRF isolates with high xylanase yield (73.63±0.0283–63.6±0.01247 U/ml) were selected and used for further study. It was found that, the optimum xylanse production was at pH 5.0 and 28 ^{o}C temperature. Among carbon and nitrogen sources, xylan and yeast extract respectively, enhanced xylanase production. Solid state fermentation (SSF) favored xylanase production than that of submerged fermentation (SmF). The partial purified xylanase showed higher specific activity than the crude extract. Highest xylanase activity (80.9–61.274 U/ml) was recorded in the pH range of 5.0–6.5 and at 50 ^{o}C. Among the metal ions tested, Mg^{2+}, Ca^{2+} and Mn^{2+} enhanced relative activity of xylanse (127.28–110.06%) while Cu^{2}+, Fe^{2+} and K^{+} showed inhibitive effect with 43.4–17% loss of relative activity. In this study, kinetics parameters (Km and Vmax) were determined from Lineweaver-Burk plot. Xylanase from the isolates showed 0.32–0.545 mg/ml Km and 86.95–113.63 µmol $min^{-1}mg^{-1}$ Vmax. Xylanase from white rote fungal isolates exhibited properties suitable for applications in animal feed and different food industries like bakery, fruit juice and wine industries.

Key words: - Decaying woods, Enzyme assay, Kinetics, Lineweaver-Burk plot

1. Introduction

Nowadays, lignocellulosic wastes are considered as a chief component of renewable biomass on the earth (Saini *et al*., 2015). The lignocellulosic biomass is made up from components such as, cellulose (40%–50%), hemicelluloses (25%–30%) and lignin (15%–20%) (Chaurasia, 2019). Hemicellulose contains branched heterogenous polysaccharides of pentose (xylose and arabinose), hexoses (glucose, mannose, and galactose) and sugar acids (e.g. acetic, galacturonic and glucuronic) (Dimarogona *et al*., 2012). Hemicelluloses are not chemically homogeneous and exist in different composition percentages, (Lee *et al*., 1997; Xiao *et al*., 2001). Hardwood hemicelluloses contain mostly xylose or xylans, while softwood hemicelluloses contain mostly glucomannans (Matsagar *et al*., 2015). Xylans are the most abundant hemicellulose consisting D-xylose as monomeric unit and L-arabinose as trace components (Bastawde, 1992) together with some substituents including acetyl, arabinosyl and glucuronysyl (Whistler and Richard 1970).

The primary factors limiting utilization of crop residues as sources of animal or human feedstuffs or chemical feedstocks are low digestibility, low protein content, high crude fiber and low palatability due to the high fibrous contents like cellulose, hemicelluloses and lignin (Theander and Aman, 1984). Thus, there have been attempts to increase digestibility of crop residues and to break the linkage between cellulose, hemicellulose and lignin by mechanical, chemical or biological treatments **(**Mahmood and Rahman, 2008; Shrestha *et al*., 2004). However, the mechanical and chemical pre-treatments primarily increase process cost, and some of these methods are considered extremely harsh and produce inhibitory compounds that are not environmentally friendly (Howard *et al.*, 2003).

In nature, there are efficient lignocellulosic fungi and bacteria that have capabilities to produce several ligninolytic enzymes for the degradation of lignocellulosic wastes (Sanchez, 2009; Renugadevi *et al*., 2011). Fungi that are active in the biodegradation of wood can be classified into three main groups according to their methods of degrading biomass, specifically white-rot, brown-rot and soft-rot fungi. White-rot and brown-rot fungi belong to Basidiomycetes, while soft-rot fungi belong to *Ascomycetes* (Hatakka, 2001). These fungi such as the brown, white and soft-rot fungi have been used to breakdown lignin and hemicellulose in waste materials such as

agricultural residues and even for cleaning of chemically uploaded textile waters (Seker *et al*., 2006).

The 'white-rot fungi' named from the white, bleached appearance when the lignin has been degraded from woody materials (Pointing 2001) are obligate aerobes and comprise many basidiomycetes and few ascomycetes (Pointing 2001; Asgher *et al*., 2008). White-rot fungi (WRF) are lignocellulolytic microorganisms that can decompose and metabolize all plant cell constituents (cellulose, hemicellulose and lignin) by their enzymes (de Koker *et al*., 2000). This is due to their capability to synthesize the relevant hydrolytic enzymes such as endo–1,4–D–glucanase (EC 3.2.1.4), exo–1,4–D–glucanase (EC 3.2.1.91), and xylanase (EC 3.2.1.8) and oxidative extracellular enzymes (Elisashvili et al 2008). The extracellular nature of WRF enzyme allows it to access non-polar and insoluble compounds (Levin *et al*., 2003). This makes WRF enzymes very attractive for different industrial and biotechnological applications such as the production of biofuel from plant biomass, biopulping, biobleaching and the degradation of recalcitrant environmental pollutants.

Xylanases (EC 3.2.1.x) are glycosidases which catalyze the endohydrolysis of 1,4– ß–Dxylosidic linkages in xylan. They are produced by various organisms including bacteria, algae, fungi, protozoa, gastropods, and anthropods (Bhardwaj *et al*., 2019). Fungi are often selected for the production of xylanase, as their yield is much higher than bacteria and in addition they also produce several auxiliary enzymes required for the degradation of xylan (Polizeli *et al*., 2005). Fungal xylanases have an adevantage over bacterial xylanases, since they are active at high temperature, low temperature, acidic pH, alkaline pH and even at high salt concentration (Bonuplisantos *et al*., 2015; Pandya and Gupte 2012).

Xylanases are group of enzymes composed of *endo*-1,4-β-D-xylanases (EC 3.2.1.8), β-D-xylosidases (E.C. 3.2.1.37), α-glucuronidase (EC 3.2.1.139) acetylxylan esterase (EC 3.1.1.72), α-L-arabinofuranosidases (E.C. 3.2.1.55), *p*-coumaric esterase (3.1.1.B10) and ferulic acid esterase (EC 3.1.1.73) (Bhardwaj *et al*., 2019). Xylanases are extensively used in the textile industries for fabric softening and to improve textile brightness (Polizeli *et al*., 2005), in the paper industry to reduce lignin content and to increase the brightness of the pulp (Kumar and

Shukla 2015), in food industries to improve the organoleptic characteristics of the fruit juices (Kumar *et al*., 2014), in baking process to decrease the firmness and increase the volume of the dough (Jiang *et al*., 2005) and reduce the use of chemical additives such as bromates (Kulkarni *et al*., 1999). Xylanases used as feed additives in animal feed to improve the nutritive value by solubilizing and degrading the insoluble polysaccharides (Goswami and Pathak, 2013).

Several researche activities have been conducted on WRF to isolate new strains with an enormous secretion of ligninolytic potential of industrial applications (Saito *et al*., 2018); waste-degrading fungi with xylanolytic potential, soil and marine fungi that can produce cellulase-free thermostable xylanase and active at alkaline pH (Chipeta *et al*., 2005; King *et al*., 2011Raghukumar *et al*., 2004). Screening for efficient delignifying and hydrolytic WRF fungi from natural environment has been recommended as one of the methods of getting such efficient isolates (Su *et al*., 2018; Kumari *et al*., 2018).

Recently, in Ethiopia, different researche activities have been conducted on lignocellulolytic WRF isolated from different areas of natural forests of the country. For Example; hydrolysis of sawdust samples by wood rot fungi (Megersa and Feleke 2020; Megersa et al., 2019), production and characterization of Mangnase peroxidase and Lignin peroxidase (Megersa *et al*., 2017b); Selection and optimization of lignocellulosic substrate for laccase production from *Pleurotus* species (Teshome and Melaku 2017), qualitative assays and quantitative determinations of laccases of WRF (Megersa *et al*., 2017a). Research finding was reported by Fkiru *et al.*, (2017) indicated that the maximum amount of enzyme produced from Oyster Mushroom (Pleurotus Species) was 2.38 U/ml. This is not enough amounts, therefore searching for WRF with higher amount xylanase production potential. Therefore, the aim of this study was to screen WRF isolated from decaying woods collected from Menagesh-Suba and Chilimo forest for their xylanolytic potential and characterize xylanase enzymes from thos isolates with high xylanolytic potential.

2. OBJECTIVES of the study

2.1. The general Objective objective of the current study was

- To produce and characterize xylanase enzyme from white rot fungi that isolated from decayed woods.

2.2. The specific objectives of this study were:

- To screen white rote fungi isolates based on their xylanolytic enzyme production potential
- To produce xylanase enzyme from selected WRF isolates under SmF and SSF conditions using wheat straw as sole carbon source.
- To optimize the growth condition for production of xylanase enzyme
- To purify xylanase enzyme, from WRF isolates with high xylanolytic potential.
- To characterize and determine the stability of partial purified xylanase under different physico-chemical conditions.

3. Literature review

3.1. White rot fungi

The white-rot fungi (WRF) are under kingdom fungi grouped in the division of Eumycota (true fungi), subdivision *Basidiomycotina*, and class *Hymenomycetes*, subclass *Holobasidiomycetidae* (Hawksworth *et al*., 1995). They are a heterogeneous group of fungi that takes more than 90% role in wood-rotting or decaying than any other basidiomycetes (Eriksson *et al*., 1990; Hatakka and Hammel, 2010). In nature angiosperms are common living environments for these fungi than gymnosperm tree species. Even though some WRF degrade lignin of wood plants selectively, there are other white-rot fungi such as *Trametes versicolor*, *Heterobasidion annosum*, and *Irpex lacteus* that degrade the cell wall components simultaneously (Eriksson *et al*., 1990; Wong, 2009).

Naturally, wood is mainly degraded by fungi that can synthesize enzymes to give them a potential to decay complex-wood plant cell wall components such as; cellulose, hemicellulose, and lignin. Fungal wood decomposition can be classified as white rot, brown rot, and soft rot categories (Schwarze 2007; Schwarze *et al*., 2000). The white-rot fungi, which degrade the plant cell wall components, are mainly associated with hardwoods, includes *Fagus sylvatica* (Schwarze 2007; Schwarze *et al*., 2000). Most of these fungal species cause simultaneous rots, in which all wood components, are degraded at a similar rate. In the less common selective rots, lignin is degraded prior to the other two components (Cellulose and Hemicellulsoe) (Schwarze 2007; Schwarze *et al*., 2000).

In the aspects of economic and environmental concerns, pretreatment of lignocellulosic biomass with microorganisms acting on cell wall components of plants, specifically white rot fungi, has acquired a revived interest as a substitute to thermal/chemical pretreatment. Various research outcomes have been reported on the practicability of fungal pretreatment for improving enzymatic digestibility of various biomass feed stocks, such as corn stover (Xu *et al*., 2010), wheat straw (Dias *et al*., 2010), rice straw (Bak *et al*., 2009), cotton stalks (Shi *et al*., 2009), and woody biomass (Yu *et al*., 2009). White-rot fungi produce xylanases (arabinofurosidases, acetyl–xylan esterases, glucuronidases, β–xylosidases and endo–xylanases) to access and utilize the energy from hemicellulose in lignocellulosic substrates (Chukwuma *et al*., 2020).

3.2. Hemicellulose

Hemicelluloses consist of heterogeneous groups of polysaccharides existing in an irregular form. They are fully integrated into the structure of the cellulose and located between the cellulose fibrils and lignins (Peng *et al.* 2012). The components of hemicellulose include different sugars, like the five carbon sugars or pentose sugars (xylose, rhamnose, and arabinose), six carbon sugars or hexoses (glucose, mannose and galactose), and uronic acids (4-*o*-methyl glucuronic, D-glucuronic, and D-galacturonic acids) (Pandey *et al*., 2014;; Prasad and Sethi 2013; Tarasow *et al*., 2013). The homopolymer or a heteropolymer backbone of hemicellulose is mostly linked by ß–1, 4–glycosidic bonds and occasionally by ß–1, 3–glycosidic bonds (Bajpai 2016). Since hemicellulose are amorphose due to their branched nature, they a hydrolyzed easily into their constituent sugars compared to cellulose. Chemical or enzymatic methods can be employed for the hydrolysis of hemicellulose into its monosaccharide forms (xylose, arabinose, galactose, glucose and/or mannose) (Branco *et al*., 2019). However, utilization of chemical method is not cost effective and not not environmentally friendly compared to enzymatic method (Howard *et al.,* 2003) Hemicelluloses are not chemically homogeneous and exist in different composition percentages (Xiao *et al.* 2001). Hardwood hemicelluloses contain mostly xylose or xylans, while softwood hemicelluloses contain mostly glucomannans (Matsagar *et al.* 2015). Xylans are the most abundant hemicellulose, having a backbone of ß-D-xylopyranose units.

3.3. Xylans

Xylan, the dominant hemicellulose is a hetero-polysaccharide integrated in the part of lignocellulosic structure consisting of ß-1, 4 linked D-xylosyl residues and accounts for about 33% of all renewable organic biomass (Girio *et al*. 2010; Liu *et al*., 2015; Motta *et al*., 2013;). Xylan that are mainly found in secondary cell wallof plant, covalently attach with lignin's phenolic residues which interact with some polysaccharides, such as pectin and glucan (Beg *et al*., 2001; Scheller and Ulvskov, 2010). The homo-polymer of xylan that linked through ß-1, 4-glycosyl bonds is composed of a repeating β-1,4 xylose residue backbone, a reducing end sequence (RES) of xylose, rhamnose and galacturonic acid and is highly modified with acetyl and (methyl)glucuronic acid side groups (Kulkarni *et al*., 1999; Rennie and Scheller, 2014; Scheller and Ulvskov, 2010). The hard woods of angiosperms contains large quantities xylans (15–30% of its cell wall content), while softwoods from gymnosperms and annual plants composed (7–10%) and (<30%) of xylans respectively (Singh *et al*., 2003). In hardwoods xylan

exists in the form of O–acetyl–4–O–methylglucuronoxylan, in softwoods as arabino-4-O-methylglucuronoxylan, while in grasses and annual plants it found as arabinoxylans (Kulkarni *et al*., 1999).

The biosynthesis of xylan including other hemicelluloses (xyloglucan and glucomannan) and pectins takes place in the medial Golgi network (Meents *et al*., 2018; Scheller and Ulvskov 2010). Xylan is synthesized by the membrane bounding proteins that rely on nucleotide sugar, acetyl-CoA as well as S-adenosylmethionine (SAM) precursors facilitates the xylan synthesis through backbone formation, RES and backbone modifications (Manabe *et al*., 2013; Ebert *et al*.,2015). When biosynthesis is completed, the xylan is packaged in to vesicles in the trans-Golgi and transported to the cell wall where it coats and crosslinks the cellulose microfibrils (BusseWicher *et al*., 2014; Simmons *et al*., 2016). Xylan is the major component of hemicellulose in the plant cell wall that provides mechanical strength for plants and has many applications that directly or indirectly determines human life. For example, tarabinoxylans are type of xylan that found in rye and wheat affect the processing, quality, and nutritional values of breads, and the brewing properties of grains (Vinkx and Delcour, 1996). The corn fiber-bran arabinoxylans of starch have served as food additives, thickeners, and stabilizers as well as film formers and emulsifiers (Doner and Hicks, 1999; Yadav *et al*., 2009). Xylan also serves as a major constituent of lignocellulosic biomass for biofuel production (Carroll and Somerville, 2009) and animal feed (Bedford and Partridge, 2010). Lignocellulosic biomass from softwood and hardwood trees is most commonly used for construction, pulp and paper products and for biorefinery applications that entail separating the biomass into its individual components to produce various bioproducts (Pei *et al*., 2016; Xavier *et al*., 2010; Zhu *et al*., 2016).

The existance of xylan in heterogenic and complex form makes its hydrolysis difficult; as a result complete breakdown of xylan into its components requires a large variety of enzymes (Subramaniyan and Prema, 2002). Modification of xylan structure involves the action of cell wall bound xylanases and transglycosylases in response to stress (abiotic, biotic and tension) that occurred during secondary cell wall (SCW) formation (Derba-Maceluch *et al*., 2015; Minic *et al*., 2004). Mostly the complete xylanolytic enzyme systems have been found among fungi, actinomycetes, and other bacteria (Collins *et al*., 2005) and among these xylanolytic systems, the

most relevant enzyme for xylan hydrolysis is endo–1, 4–ß–D–xylanase (E.C. 3.2.1.8), which randomly cleaves ß–1, 4–glycosidic bonds of the xylan backbone releasing xylose and xylooligosaccharides (Kamble and Jadhav 2012; Terrasan *et al*., 2016; Van Zyl *et al.,* 2015).

3.4. Xylanolytic enzymes

As the chemical nature of xylan is very heterogenic and complex, the complete breakdown of this biomass demands action of different hydrolytic enzymes (Subramaniyan and Prema 2002). The hydrolytic enzymes that includes endoxylanase (endo–1,4–ß–xylanase, E.C.3.2.1.8) that randomly cleave the xylan backbone , ß-xylosidase (xylan–1,4–ß–xylosidase, E.C.3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose, α–glucuronidase (α–glucosiduronase, E.C.3.2.1.139), α–arabinofuranosidase (α–L–arabinofuranosidase, E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72) that involved in the removal of side groups of the polymer, are collectively xylanolytic enzyme system that involved on the hydrolysis of xylan (Juturu and Wu 2012).

The endoxylanases and ß–xylosidases (commonly xylanases) are the two major xylanolytic enzymes that hydrolysed of xylan into its consituents. Endoxylanases produces xylo-oligomers by breaking the homopolymeric backbone of 1, 4–linked ß–D–xylopyranose, whereas ß–xylosidases act on xylooligomers and release xylose (Ahmed *et al*., 2009; Knob *et al*., 2010). The endoxylanases are the most important as they are directly involved in cleavage of glycosidic bonds by liberating short xylo-oligosaccharides (Verma and Satayanarayana 2012).

3.4.1. Mode of action of xylanase

Two different mechanisms such as retention and inversion involved in the hydrolysis process of xylan polymer by xylanase enzymes (Lombard *et al*., 2014; Subramaniyan and Prema 2002).

The retention process is a double displacement mechanism in which α–glycosyl enzyme intermediate and oxo-carbonium intermediate formed and glutamate residues involved as a catalyst for its hydrolysis. The involvement of this double displacement mechanism for the anomeric retention of product has been firstly proposed by Koshland (1953) and also later reported by Clarke *et al*., (1993). Primarily, the two carboxylic acid residues present in the active site result in α–glycosyl enzyme intermediate formation by the protonation of the substrate with the action of carboxylic acid residue (an acid catalyst) and another carboxylic acid that causes

the nucleophilic attack. This collectively ended up with β to α inversion as a result of the α–glycosyl enzyme intermediate formation (Bhardwaj *et al*., 2019). In the second displacement, the anomeric carbon gives rise to product with the β configuration (α to β inversion) by a transition state of oxo-carbonium ions (Collins *et al*., 2005; Lombard *et al*., 2014). Generally the double displacement mechanism involves: an acid catalyst which protonates the substrate, carboxyl group of the enzyme, a covalent glycosyl enzyme intermediate with carboxylate in which the anomeric configuration of the sugar appears in opposite that of the substrate, oxo-carbonium ions are involved for the formation of covalent intermediates from both direction and various non-covalent interactions (Clarke *et al*., 1993).

While inversion is a single displacement mechanism, in which only one carboxylate ion offers for overall acid catalyzed group departure. The enzymes that use this single displacement mechanism (inversion) are like enzymes families 8 and 43. These enzymes invert the anomeric center with glutamate and aspartate as the major catalytic residue and also act as the base for activating a nucleophilic water molecule to attack the anomeric carbon to cleave the glycosidic linkages and actualize inversion of anomeric carbon configuration (Collins *et al*., 2005; Motta *et al*., 2013; Lombard *et al*., 2014). Most of the polysaccharide hydrolyzing enzymes like cellulases, cellulohydrolases and xylanases are known to hydrolyse their substrates with the retention of the C1 anomeric configuration (Gebler *et al*., 1992).

3.4.2. Sources of xylanase

Xylanase is produced by different living organisms like: archaea (Wainø and Ingvorsen 2003), bacteria (Chakdar *et al*., 2016), fungi (Chadha *et al*., 2019; Singh *et al*., 2019), actinomycetes (Hunt *et al*., 2016), insects (Brennan *et al*., 2004), algae (Jensen *et al*., 2018), plants and immature seeds (Sizova *et al*., 2011), protozoa (Béra-Maillet *et al*., 2005), molluscs (Yamaura *et al*., 1997) and nematodes (Mitreva-Dautova *et al*., 2006). The xaylanase producing microorganisms cover diverse ecological niches like marine and terrestrial habitats with decayed plant biomass, thermophilic and mesophilic environments, as well as rumen of ruminants (Chadha *et al*., 2019; Singh *et al*., 2019).

There are many advantages that make microorganisms, the choice of interest than that of plants and animals. These includs: rapid multiplication in short period of time, small space requirement,

high potential in waste biomass biodegradation, their production under controlled and closed systems, ease to handle and cheap production cost, since the cheap substrates are used as carbon sources (Mitreva-Dautova *et al*., 2006). The microbial xylanase also have advantages over conventional chemical catalysts such as; high catalytic activity, high specificity to its substrate, high yield of product, easily biodegradable, environmental friendly, and are economically viable (Gote, 2004). Bacteria and fungi are widely used for industrial production of xylanase; therefore here the focus of this study is on the fungal xylanase.

3.4.2.1. Fungal xylanases

Most of filamentous fungi are producers of xylanases and other auxiliary enzymes that involve in the degradation of xylan into its components (Polizeli *et al*., 2005). The fungal genera that commonly produce xylanase includes; *Fusarium* (Saha 2002), *Trichoderma* (Ayadi *et al*., 2015), *Aspergillus* (Pal and Khanum 2010), and *Penicillium (*Dwivedi *et al*., 2009). The basidiomycete, white-rot fungi (WRF) are also the producers of extracellular xylanases that act on various hemicellulosic biomasses that are used as food sources (Buswell and Chang 1994) and produce metabolites for pharmaceutical, cosmetic and food industries (Qinnghe *et al*., 2004). These enzymes producing white-rot basidiomycetes includes, *Phanerochaete chrysosporium* (Castanares *et al*., 1995), *Coriolus versicolor* that produces a complex xylanolytic enzymes (Abd El-Nasser *et al*., 1997) and *Cuninghamella subvermispora* (de Souza-Cruz *et al*., 2004). Fungi are chosen than bacteria for xylanase production, due to their potential to yield higher amount of enzymes, produce several xylan substitute degrading auxiliary enzymes (Polizeli *et al*., 2005) and also fungal xylanases are active at high and low temperature, acidic and alkaline pH and high salt content (Bonughisantos *et al*., 2015; Pandya and Gupte 2012).

3.4.2.2. Bacterial xylanases

Bacteria are abundant producers of xylanases among other xylanase producing microbes. Bacterial genera, such as *Cellulomonas* (Laurie *et al*., 1997), *Bacillus* (Gupta *et al*., 2015), *Microbacterium* (Kim *et al*., 2005) and *Paenibacillus* (Liu *et al*., 2018) have been reported as prolific producers of xylanase. From these bacterial genera, *Bacillus* species are widely studied and reported for their xylanolytic enzyme producing potential. For example, *Bacillus subtilis* (Banka *et al*., 2014), *Bacillus halodurans* (Gupta *et al*., 2015), *Bacillus pumilus* (domas *et al*., 2014) are some of the *Bacillus* species reported as xylanase enxyme producers. Xylanase enzyme producing bacteria have been isolated from different environments including; soil sample

(Horikoshi and Astukawa 1973), from marine sample (Menon *et al*., 2010), human gut microflora (Mirande *et al*., 2010), from water sample of hot-springs (Baysal *et al*., 2003) and poultry and mushroom compost (Walia *et al*., 2014; Taibi *et al*., 2012).

3.4.3. Classification of xylanase

The xylanase can be categorized into three types based on their; molecular mass and isoelectric point (PI), crystal structure and kinetic property (Motta *et al*., 2013; Liu and Kokare 2017). As the classification based on molecular mass and isoelectric point, the xylanase enzymes grouped into two, i.e., (a) high-molecular weight with low isoelectric (acidic) point (HMWLI) and (b) low-molecular weight with high isoelectric (basic) point (LMWHI). But this classification has a drawback since it cannot describe all xylanases, because not all xylanases belong under the category of (HMWLI) and (LMWHI) (Collins *et al*., 2002). Therefore, as the solution of this drawback a more suitable system like; primary structure (crystal) and comparison of the catalytic domains was developed (Collins *et al*., 2005; Henrissat and Coutinho 2001). For more curated information on the characteristics and classification of enzymes, the Carbohydrate-Active Enzyme (CAZy) database that involved in the breakdown, modification, and assembly of glycosidic bonds in carbohydrates and glycoconjugates is an appropriate. This database consists of genomic, sequence annotation, family classifications, structural (3D crystal) and functional (biochemical) information from publicly available resources like; National Center for Biotechnology Information (NCBI) (Lombard *et al*., 2014). The CAZy database (http://www.cazy.org) classification categorizes the xylanases into glycoside hydrolase (GH) families that includes: 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62. Among these families the 16, 51 and 62 GH families have two catalytic domains with bifuctional properties, unlike the 5, 7, 8, 10, 11, and 43 GH families that have a single distinct catalytic domain (Collins *et al*., 2005).

3.5. Fermentation process for xylanase production

The production of xylanase depends on the selected microorganism, composition of growth medium, physicochemical growth parameters and the enzyme producing fermentation method. The growth medium contributed 30–40% to the total cost of enzyme production (Mitreva-Dautova *et al*., 2006). The xylanase production can be affected by the type and concentration of medium components such as carbon source, nitrogen source, micronutrient, and macronutrients.

The physiochemical growth parameters such as inoculum size, temperature, pH, agitation/aeration, the water content of substrate, incubation period , trace elements and vitamins significantly influence the xylanase production (Abhishek *et al.*, 2017; Thomas *et al.*, 2017). The production of xylanases involves two types of fermentation processes such: submerged fermentation and solid-state fermentation (Agnihotri *et al.*, 2010; Motta *et al.*, 2013).

3.5.1. Xylanase production under submerged fermentation (SmF)

Submerged fermentation (submerged culture) uses liquid fermentation media in which the substrate is dissolved in water. Even though space, energy and water requirements are limitations connected with submerged fermentation process, it is widely used in industrial processes, due to higher yield of production, lower risk and cost of contamination and easy to monitor the fermentation process (Jain *et al.*, 2013). Additionally submerged fermentation is widely used beuase of lack of equipment for enzyme production under solid state fermentation (Subramaniyam and Vimala, 2012). About 90% of the total xylanase production worldwide uses the submerged fermentation (SmF) process (Polizeli *et al.*, 2005). The submerged fermentation of aerobic microorganisms is a well-known and widely used method for the production of cellulase and xylanase and it has been found that SmF is normally preferred when the preparations require more purified enzymes (Garcia-Kirchner *et al.*, 2002).

3.5.2. Xylanase production under solid–state fermentation (SSF)

Solid state fermentation is a fermentation process that involves a solid matrix without free flowing water that used as physical support and source of nutrition for microbial growth (Pandey et al. 2000; Singhania *et al.*, 2009). The SSF system provides many advantages over SmF, such as mimicking the natural habitat in which the microorganism grows, reducing water activity which can minimize microbial contaminations, greater enzyme stability, and less energy requirements than submerged cultures, production of enzymes with higher specific activities, easier downstream processing (Beg *et al.*, 2001; Jain *et al.*, 2013; Singhania *et al.*, 2009). Also the solid state fermentation provides higher enzyme yield than that of submerged fermentation (Agnihotri *et al.*, 2010).

The SSF process is especially suitable for the growth of fungi, as these organisms are able to grow at relatively low water activities, unlike that of bacteria and yeast that are not able to grow at low water activity (Corral and Villasenor-Ortega 2006). The production of enzymes like

xylanase from fungal orgin by the SSF processe have been received special attention due to its economic and engineering advantages (Pandey *et al*., 1999). There are several research reports that indicate suitability of solid-state fermentation than liquid state conditions for high yield of xylanase enzyme (Roy *et al*., 2013; Singh *et al*., 2013; Nair *et al*., 2008). Solid-state fermentation processes involves utilization of agricultural, forestry and food processing residues and wastes such as sugar cane bagasse, wheat bran, wheat straw, corn cobs, rice bran, rice husks, saw dust and other similar substrates for xylanase production (Corral and Villasenor-Ortega 2006; Pandey *et al*., 1999).

Even though SSF have tremendous advantages, there are limitations and challenges that negatively affect this process. These limitations and challeges includes, different gradients (moisture, temperature, substrate concentration, and others), bioreactor (static bed bioreactor), heat dissipation, mass transfer, and control of fermentative parameters (Khanahmadia *et al*., 2006; Singhania *et al*., 2009). In this respect, more research on design, modeling, operation, and scaling up are necessary to allow the employment of SSF processes involving bioreactors.

3.6. Purification of xylanase

Enzymes are one of the most important biomolecules that have a wide range of applications in industrial as well as biomedical fields. Therefore; purification of these biomolecules is an important prerequisite for the effectiveness of enzymes in their industrial applications. The purification of xylanases from different microorganisms started since 1982 (Walia *et al*., 2014). Conventional purification methods such as: ultrafiltration, ammonium sulphate precipitation, gel permeation chromatography and ion exchange chromatography are commonly used for xylnase enzyme purification (Irfan and Sayed, 2012; Kamble and Jadhav, 2012; Walia *et al*., 2014).

Several researchers rely on these methods for xylanase enzyme purification from different sources. For example; Li et al. (2010) have used DEAE 52 column and CM Sepharose Fast Flow chromatography for the purification of xylanase produced by *Streptomyces rameus* L2001, Taibi and coworkers (2012) were purified endo-xylanase enzyme with a molecular mass of 70 kDa from *Penicillium* using ammonium sulfate fractionation, gel filtration on BioGel P10, DEAE cellulose and CM Sephadex chromatographies (Taibi *et al*., 2012). Lopez and Estrada (2014) purified xylanase from *Trichoderma reesei* using ammonium phosphate precipitation, followed

by DEAE-Sepharose CL-6B and Ultrogel AcA 44 (LKB) chromatographies. There is also another research report on the purification of two xylanases Xyl I and Xyl II from *Trichoderma inhamatum* strain using ion exchange chromatography (diethylaminoethanol DEAE-Sepharose and subsequent gel filtration chromatography (Sephadex G-75) (Silva *et al*., 2015). Recently de Oliveira Simões and coworkres (2019) were extracted crude enzyme from *Myceliophthora heterothallica* by precipitating with 96% ethanol and performed xylanase purification with two steps of chromatography: gel filtration (Sephadex® G-75) and anion-exchange (Resource™ Q) (de Oliveira Simões *et al*., 2019).

However, the above mentioned conventional xylanase enzyme purification methods have drawbacks like; highly time consummation, not cost effective and low yield of protein (Iqbal *et al*., 2016; Ramakrishnan *et al*., 2016). To overcome these problems, a single step liquid–liquid fractionation technique (aqueous two-phase system) has been developed (Glyk *et al*., 2015). The advantages of aqueous two-phase system (ATPS) purification technique over conventional purification techniques includes: low energy consumption with high yield, use of cheaper materials, higher resolution and not affecing the nature of protein (Glyk *et al*., 2015; Iqbal *et al*., 2016; Ramakrishnan *et al*., 2016). Due to these advantages some researchers: Garai and Kumar (2013) have used this technique for purification of alkaline xylanase from *Aspergillus candidus,* Recently Ng *et al.* (2018) have purified xylanase from *Bacillus subtilis* using alcohol/salt ATPS, Gómez-garcía *et al*. (2018) and Bhardwaj *et al*. (2019) have used ATPS with PEG/salt system for purification of xylanase from *Trichoderma harzianum* and *Aspergillus oryzae* LC1 respectively.

3.7. Application of xylanase

3.7.1. Animal feed industry

The xylanase also involved in the transformation of nutritional values of agricultural residues and animal feeds. Xylanase enzymes are applied as animal feed additives to improve feed nutritional value by solubilizing and degrading the insoluble feed constituents (Goswami and Pathak, 2013). There are several research findings that demonstrating the application of xylanases in animal feed to improve its nutritional value and increase the weight gain of animal. The research by Zhang et al. (2014) on boilers fed wheat-based diet supplemented with xylanase, indicated that the supplementation of xylanase to wheat-based diets degraded the arabinoxylan

backbone into its monomers (arabinose and xylose) in the ileum, jejunum and duodenum, and enhances digestibilites of nutrients by decreasing digesta viscosity. Paloheimo and coworkers reported utilization of xylanase as feed additives showed the weight gain improvement and enhanced feed conversion ratio (Paloheimo *et al*., 2010). The research finding by Passos et al. (2015) also demonstrated addition of xylanase in corn and soybean meal based diet of growing pigs improved the nutrients digestibility, digesta viscosity.

3.7.2. Paper and pulp Industry

There are two major processes involved in paper and pulp industry such as; pulping and bleaching that essentially generate organic and inorganic pollutants including: lignins, tannins, resins and chlorine compounds that are environmental pollutants (Chandra and Abhishek, 2011; Chandra *et al.*, 2011). In the present Chlorine dioxide that commonly used as a bleaching agent in paper and pulp industries, forms organo-chlorin compound when combined with organic matter which harms all lives by causing genetic and reproductive damages (Bajpai, 2012; Sharma *et al*., 2014).

3.7.2.1. Bioleaching

Bleaching is the process of removing lignin from pulp using chemicals, gases, steams, enzymes etc to produce bright and white finished paper (Beg *et al*., 2001). Utilization of ezymes in paper and pulp industries not only avoids use of hazardous chemicals like chlorine in bleaching process (Garg *et al*., 2011), but also it combats the limitations of mechanical pulping processes (Sharma *et al*., 2016). The conventional pulping strategies have faced different challenges such as: precipitation or re-precipitation of xylan on the fiber, entrapment of lignin and variation in brightness (Mathur *et al*., 2005).

To overcome the above mentioned problems related to traditional pulping and bleaching strategies an eco-friendly treatments specifically enzymatic treatment have been used. Among these enzymatic treatment, xylanase is the one gained great attention in pulp and paper industries (Ho and Jamila 2014; Golugiri *et al*., 2012). Xylanase enhances the bleaching process by hydrolyzing the bond covalently associate xylan with cellulose and lignin and cause the separation of cellulose and lignin (Woolridge, 2014; Yang *et al*., 2019; Roncero *et al*., 2005). The application of xylanases improve pulp brightness, purity of dissolved pulp, remove metal

cations, reduce the overall paper cost, decrease time consumption of pulping process, improves the permeability of fiber surface, restore bonding and avoid chlorine requirement (Nagar *et al*., 2013; Yang *et al*., 2019).

There are several reports indicating the potential application of xylanase enzyme in paper and pulp industries. Sridevi et al. (2017) demonstrated that pretreatment of paper pulp with xylanase produced by *Trichoderma asperellum* showed reduced kappa number, higher brightness and removed chromophores and hydrophobic compounds. Nair et al. (2010) reported application of xylanase produced by *Aspergillus sydowii* SBS 45 on Kraft pulp, increased brightness of pulp from 29.42 to 70.42% and reduced kappa number from 15.93 to 1.61. Gautam and coworkers demonstrated that xylanase from white rot fungi, *Schizophyllum commune* ARC-11 resulted maximum decrease in kappa number (14.51%) with a maximum improvement, 2.9% in pulp brightness when applied on ethanol-soda pulp from Eulaliopsis (Gautam et al. 2018). Pretreatment of hardwood kraft pulp with xylanase produced by *Paenibacillus campinasensis* BL11 increased brightness (as much as 4.4 and 3.9%) and viscosity (as much as 0.5 and 0.3 cP) of pulp after full chlorine dioxide bleaching for untreated and oxygen delignified hardwood kraft pulp (Ko *et al*., 2010). Raj et al. (2018) suggested that xylanase produced on wheat bran from alkaliphilic *Bacillus liceniformis* used as pre-treatment of kraft pulp showed 19% reduction in kappa number, compared to control pulp, after 2 h treatment and the scanned electron Microscopy (SEM) and infrared spectroscopy (FTIR) analysis of xylanase-treated pulp revealed significant morphological and structural changes on pulp fibers.

3.7.3. Textile industry

The textile industries comprise three main processes such as desizing (to remove the size or adhesive substance), scouring (to remove the inhibitory material from desized fibers) and bleaching (to increase the whiteness) (Rouette 2001). The conventional method used for these processes is chemical-intensive, non-specific and involves the application of high temperature under alkaline condition. Therefore, it is not enviromental friendly (Bhardwaj *et al.*, 2019). In order to combat these problems related to utilization of traditional processing methods, several research recommendations to use more environmental friendly enzyme-based treatment have emerged (Agrawal *et al*., 2004; Lenting *et al*., 2002, Lenting and Warmoesken 2004). Among the enzymes used in textile industries, xylanase is the one invoved in desizing,

bioscuring and bio-stoning of denim garments fabric softening, releasing the extra dye and bio-bleaching (Polizeli *et al*., 2005; Sing 1999). Taking this in mind, several researcher groups conducted different researches to find cellulase free xylanase enzyme with high thermal and alkaline pH stability that used for desizing and scouring processes in texitile industries (Losonczi *et al*., 2005; Bajpai 2014). There are also reports indicated the utilization of xylanase extracted from *Bacillus pumilus and Bacillus stearothermophilus* SDX for de-sizing of cotton and micropoly fabrics and bioscouring (Battan *et al*., 2012; Saurabh *et al*., 2008).

3.7.4. Application of xylanase in food Industry

3.7.4.1. Baking industry

Baking industries use wheat flour as a raw material for making bread. The wheat grain contains the hemicellulose (arabinoxylan) exsisting as water–solubile and water–insoluble forms (Courtin and Delcour, 2002). The water insoluble fibers in wheat grain hinder the quality of bread (Cavella *et al*., 2008). In the backing process, xylanase amended to the dough in order to convert water-insoluble hemicellulose into water–soluble form, which helps to improves the rheological properties of dough such as: increase the volume, creating more uniform and finer crumbs and decreasing dough firmness (Butt *et al*., 2008, Camacho and Aguilar 2003). Omar Al-Widyan *et al*. (2008) reported that xylanases produced from different types of microorganisms significantly improved loaf volume, loaf colour and crumb texture and firmness of the bread. Similarily, Driss *et al*. (2013) demonstrated that the addition of xylanse decreased springiness and gumminess of bread. Xylanase also enhances the bread quality and extend the shelf life by reducing the staling rate (Harris and Ramahlingam 2010).

3.7.4.2. Fruit juice clarification

The presence of polysaccharides such as celluloses, pectins, hemicelluloses, and surface- bound lignin in the fruit juice negatively affects its quality i.e. haz color and viscosity (Danalache *et al*., 2018; Lee *et al*., 2006). Since the acceptability of cloudy and turbid juices are very low, fruit juice industries use different plant cell wall degrading enzymes such as pectinase, cellulase, and xylanases to remove these undesired properties (Pinelo *et al*., 2010; Kumar *et al*., 2014). Nowadays, utilization of xylanase enzyme for the clarification and extraction of fruit juices has

gained more attention and many research outcomes have been published that showing the potential of xylanase enzyme for extraction and clarification fruit juices produced from different sources. The potential of xylanase from *Streptomyces* species was investigated and the clarification of juices from different fruits such as: orange (20.9%), mousambi (23.6%) and pineapple (27.9%) clarity was reported by Rosmine et al. (2017). Kumar with his research groups demonstrated the potential application of immobilized xylanase from *Bacillus pumilus* VLK-1 for clarification of orange and grape juice with 29%, 26% clarity respectively (Kumar *et al*., 2014). The fruit juices from orange, grape, kiwi, apple, pomegranate, peach and apricot were treated with endo-β-1,4-xylanase extracted from *Pediococcus acidilactici* GC25 and the turbidity of the juices decreased (Adiguzel *et al*., 2019). All these research outcomes indicated the potential and applicability of xylanase enzyme in the fruit juice industries.

3.7.5. Biofuel production

Nowadays second-generation biofuels from renewable resources as an alternative to fossil fuel gained more attention, because it does not compete with food production and can provide environmental and economic benefits (Sharma and Sharma, 2016; Viikari *et al*., 2012). Xylanase, together with other hydrolytic enzymes, used for the depolymerization of the carbohydrate polymers (lignocellulosic biomass) to produce biofuels (Beg *et al*., 2001; Lee, 2009). There are research reports that indicats the role of xylanase in the saccharification of lignocellulosc biomass for biofuel production (Ramanjaneyulu *et al*., 2017; Basit *et al*., 2018). For production of biofuels from lignocellulosic biomass different integrated stratagies that improves ethanol production have developed. These processes include: simultaneous saccharification fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CB) (Sun and Cheng, 2002; Malhotra and Chapadgaonkar, 2018). Cellulase and xylanase enzyme used for bioethanol production by saccharification and co-fermentation (SSCF) of anhydrous ammonia-pretreated Napier grass (Yasuda *et al*., 2014). Song and coworkers demonstrated using xylanase together with cellulase improved the conversion efficiency of the reducing sugars, derived from corn stover (48.5%) and rice straw (31.1%) (Song *et al*., 2016).

4. Materials and methods

4.1. The WR Fungal culture

The fungal isolates used in this study were obtained from Microbial Biotechnology Research laboratory, National Agricultural Biotechnology Research Center (NABRC), Ethiopian Institute of Agricultural Research (EIAR). All white rote fungal isolates were previously isolated from decaying woods obtained from Menagesh-Suba and Chilimo forests based on their morphological characteristics and were preserved on potato dextrose agar (PDA) slant at -20^{o}C. (Adaba Tilahun and Mulatu Worike, unpublished)

4.2. Inoculum preparation and refreshment of fungal isolates

For the development of inoculum, potato dextrose agar (CONDA, Madrid, Spain) (pH 5) was prepared, sterilized at 121^{o}C for 15 min and poured into the Petri plates. After solidification of the medium, the plates were inoculated with 6 mm diameter plug of white rot fungal isolates and incubated at 28^{o}C for 7 days. Thereafter, 68 isolates were screened qualitatively and quantitatively for their xylanolytic properties.

4.3. Qualitative screening of fungi for production of xylanase

All the 68 white rot fungal isolates that obtained from NABRC were screened for their xylanolytic potential qualitatively as described by Manju and Narsi (2016) on Mandel and Reese agar media with slight modification. The mineral salt medium consisting g/l: (1.73 K_2HPO_4, 1.73; KH_2PO_4, 0.68; $(NH_4)_2SO_4$, 4; yeast extract1.4; $MgSO_4.7H_2O$, 0.1; NaCl, 4; CaCl, 0.02; $MnSO_4$, 0.01 and $FeSO_4$ 0.03 agar, 20 and xylan, 10 and the pH was adjusted to 5 using 2N NaOH or HCl. The prepared medium was inoculated at center of the Petri plate by taking culture disk of 5 mm size plug from 6-day old culture plate and incubated at 28^{o}C for 7 days. Thereafter, plates were stained by flooding with 10–15ml 1% (w/v) congo red solution for 15 min. The plates were distained by flooding with 1 M NaCl for 15 min and the clear zone diameter was measured in cm using digital ruler.

4.4. Quantitative screening of fungal isolates

In secondary screening, the cultures with high zone of hydrolysis were selected and further screened in submerged state fermentation (SmF) for the production of xylanase. Erlenmeyer flasks (250 ml) containing 100 ml of liquid mineral salt medium (MSM) were autoclaved at 121 ^{o}C for 15 min, inoculated with 5 agar plugs of fungal mycelium and incubated at 28^{o}C in an

orbital shaker (120 rpm) for 7 days. The extracellular enzyme present in culture filtrate was obtained by filtration through Whatman No.1 filter paper and the culture filtrate was centrifuged at 10,000×*g* for 10 min and the clear supernatant was a source of crude enzyme for further analysis.

4.5. Production of xylanase under SmF and SSF condition using wheat straw as carbon source

4.5.1. Preparation of Agricultural waste residue–wheat straw (WS)

Wheat straw (WS) was obtained from Holeta Agricultural Research Center (HARC) farm. The agricultural residue was air dried and then oven dried in a hot-dry oven at 60^{o}C for 12 h in order to remove any moisture from the substrate and to facilitate the physical treatment (grinding). After drying the wheat straw was ground with blender. The powder that used for SmF was sieved through 1mm sized mesh but for SSF it was used without sieving.

4.5.2. Production of xylanase under SmF and SSF

Submerged fermntation was performed in duplicate in 250 ml Erlenmeyer flasks containing 100 ml of MSM medium amended with 2% WS as a carbon source (pH 5). After autoclaving at 121^{o}C for 20 min, flasks were inoculated using 5 plugs of 7 days old pre-inoculum. The inoculated flasks were incubated at 28^{o}C with shaking on an orbital shaker at 120 rpm for 7 days. Supernatants were decanted and re-centrifuged for 12 min at 13, 000 rpm. The extra cellular enzyme was obtained as described in section 4.4.

While, for solid state fermentation 10 g non-sieved WS powder and 30 ml of MSM solution was mixed in 250 ml Erlenmeyer flask to achieve 70% moisture content.

Then the flasks containing the fermentation medium were autoclaved at 121^{o}C for 20 min and inoculated with five disks of 7 days old actively growing fungal isolates of 6 mm diameter and incubated at 28 ^{o}C for 10 days. After the end of incubation period, the enzyme was extracted by a simple contact method (Krishna and Chandrasekaran 1996). The fermented samples were shaken (150 rpm) with 0.05 M sodium citrate buffer (pH 5.0) by applying substrate to buffer ration (1:20) concentration for 1 h at room temperature and filtered through Whatman No. 1 filter paper. The filtrates were centrifuged at 10,000×*g* for 10 min at 4^{o}C to separate the enzyme

from other components of the medium. The clear cell free supernatant was used as crude enzyme for determination of xylanase activity.

4.6. Xylnase assay

Xylanase activity was assayed as described by Bailey *et al*. (1992) and Geweely (2011) using 1% birch wood xylan as substrate in 0.05M sodium citrate buffer (pH 5). Xylanase activity was measured by incubating 2.25 ml total reaction mixture containing 0.75 ml of diluted (ratio of 1ml crude enzyme to 100 ml buffer) crude enzyme solution and 1.5 ml of the substrate with buffer reaction solution at 50°C for 30 min. The reaction mixture containg enzyme with buffere was used as enzyme blank and the reaction mixture of substrate with buffer was used as reagent blank. The reaction was stopped by adding 2ml 3, 5–dinitrosalicylic acid (DNS) reagent followed by heating for 5 min at 100°C and then rapidly cooled by rensing with cold water. After cooling, the absorbance was measured by UV-spectrophotometry (JENWAY 6300, Altay scientific S.P.A Juscolana 242, 0004 Grottaferatal, Rome (Italy)) at 540 nm. The reagent blank was used to make the specterophotometeric reading zero and xylanase activity was determined from xylose standard curve.

4.6.1. Preparation of xylose standard curve

Series of xylose standards was prepared in order to express the xylanase activities into enzyme unit. The xylose standard solution was prepared by the method of Bailey *et al*. (1991). For this 150 mg of xylose was dissolved in 100 ml 0.05 M citrate buffer and the series of dilutions were made. The assays of each standard dilution were in duplicate. For this 3.0 ml DNS and 0.5ml standard dilutions were mixed and the reaction mixture boiled at 100^{o}C for 5 min. Then tubes were rinsed with cold water and the absorbance was measured at 540 nm using Uv–spectrophotometer (JENWAY 6300, Altay scientific S.P.A Juscolana 242, 0004 Grottaferatal, Rome, Italy). The graph was plotted using xylose concentration on x–axis and optical density value (OD) at 540 nm on y–axis. From the above standard solution the graph was plotted and the linear regression equation was derived as $y = 0.231x + 0.1797$. The regression coefficient was $R^2 = 0.9933$ indicating that the xylose concentrations and OD at 540 nm readings were powerfully positively correlated (Appedix 28). The amount of xylose liberated quantified using this regression equation and one unit of xylanase activity (U) was defined as the amount of enzyme that liberates 1 µmol of reducing sugar (xylose) per min under the standard assay conditions.

4.7. Optimization of culture condition for xylanase production by WRFs

4.7.1. Effect of incubation temprature on xylanase production

The effect of incubation temperature on xylanase production was determined by incubating the MSM–wheat straw medium inoculated with 7 days –old mycelia at 22, 25, 28, 31, 34, 37, 40, 43 and 46^{o}C for 7 days. All activities conducted in triplicate and at the end of the incubation period the enzyme extracted as described in section 4.4 and enzyme activity was measured under optimum condtions following the standard enzyme assay method.

4.7.2. Effect of pH on the production of xylanase

The effect of pH on xylanase production was carried out by incubating the culture flasks containing 150 ml of MSM supplemented with 2% WS and was inoculated with five mycelial discs (6 mm) at different pH (3.0, 4.0, 5.0, 6.0, 8.0 and 9.0) and incubated at 28^{o}C for 7-days. Then the enzyme was extracted as described in section 4.4 and enzyme was assayed following the standard enzyme assay method.

4.7.3. Effect of carbon sources on production of xylanase by WRF

The effects of different carbon sources (cellulose, lactose, Carboxyl methyl cellulose (CMC) and xylan) on the production of xylanase from the identified fungal isolates were studied. The carbon sources were supplemented to the MSM at 1% level and inoculated with 7 days old mycelial discs (6 mm diameter) and incubated at 28℃ for a period of 7 days at 120 rpm in orbital shaker. After 7 days incubation crude enzyme was extracted and assayed for xylanse index as described in section 4.6.

4.7.4. Effect of nitrogen sources on xylanse production

To determine the suitable nitrogen source for the maximum production of xylanase by the identified fungal species urea, yeast extract, ammonium sulfate and peptone was used as nitrogen source at 0.2% concentration level. The five mycelial discs (6 mm diameter) from 6-days-old culture was inoculated in 150 ml of MSM and incubated at 28℃ for a period of 7 days. Following the completion of incubation extracellular enzyme was extracted and its activity was determined as described in section 4.6

4.7.5. Production of xylanse under optimized production condtion

After finilazing optimization of production parameters the ferementation process was conducted by combining all optimum (aproprate) parameters together. Briefly, MSM was supplemented

with 2% WS as sole carbon source, 0.2% Yeast extract as nitrogen source , 1% brich wood xylan as xylanse inducer and pH adjusted to 5. Then the medium inoculated with five mycelial discs (6 mm) fungal mycelium and incubated at 28^{o}C for 7–days. Finally, enzyme harvested as as described in section 4.6 and subjected for purification.

4.8. Partial purification of enzymes

Ammonium sulphate precipitation method was performed based on Kamble and Jadhav (2012) with some modifications. Primarily ammonium sulfate crystals were dried overnight at 120℃ and ground finely using mortar and pestle. Then the saturated ammonium sulfate solution was prepared as the method described by Wingfield (2001) and added 766.8 g of powdered (NH4)2SO_4 per 1L of dH_2O. The saturated ammonium sulfate was added to the cell-free culture supernatant to attain 30-80% saturation. The precipitation was done by diluting 100 mL crude enzyme extract partially into ammonium sulphate with concentration 0-30%, 30–40%, 40–50%, 50–60%, 60–70%, and 70–80% (v/v) gradually. The following equation is used to calculate volume of saturated $(NH_4)_2SO_4$ solution to be added to 100 ml of solution to increase saturation from initial saturation (S1) to the saturation of interest (S2)

$$\text{Volume (ml)} = 100(S2–S1)/1–S2$$

Where S1 = initial saturation S2 = saturation of interest (final saturation).

The saturated ammonium sulfate solution was added slowly to the crude enzyme. The suspension was stirred for 1 h and kept overnight at 4℃. The sample was centrifuged at 3000 × *g* for 10 min, supernatant was discarded and the pellets was collected and dissolved in a desired buffer using a volume of buffer equal to the volume of extract. Desalting was carried out against phosphate buffer saline (PBS) pH 8 using dialysis membrane with molecular weight cut-off (10 kDa). The dialysis was done at room temperature by changing the buffer in every two hours three times and then the dialysis took place at 4^{o}C for overnight.

4.8.1. Protein concentration of the partial purified xylanase

Protein content was measured according to the method of Lowry *et al*. (1951). Primarily, the stock solution of standard protein was prepared by dissolving 4 mg/ml bovine serum albumin (BSA) in distilled water and the standards was prepared by diluting the stock solution as shown in Table–1.

Table 1: Standard concentration of BSA for determination of protein concentration

H_2O (ml)	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3
BSA (ml)	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Concentration of BSA (mg/ml)	0	0.4	0.8	1.2	1.6	2	2.4	2.8

To determine the protein concentration, 0.1 ml of 2N NaOH and 0.1 ml of sample or standard solution mixture was boiled in water bath at 100°C for 10 min in order to hydrolyze protein. After cooling the hydrolyzate at room temperature 0.1 ml of freshly mixed complex-forming reagent (2% Na_2CO_3, 1% $CuSO_4$ and Sodium potassium tartrate) was added and stand at room temperature for 10 min. Then 0.1 ml Folin-reagent (Phosphotungstic acid and Phosphomolybdic acid) was added, mixed by vortexing and incubated for 60 min at room temperature. Finally the absorbance was measured at 750 nm and standard curve that used for determination of protein concentration of the sample was plotted (Lowry *et al*., 1951). Afer the protein concentration was determined using the regression equation of the standard curve, specific activity of the enzyme (U/ml), yield (%) and purty of the enzyme was determined using the following equations (Burgess and Deutscher, 2009).

$$Specific\ activity(U^{-1}mg) = \frac{\text{Enzyme activiy } (U^{-1}ml)}{Protein\ concentation\ (mg^{-1}ml)}$$

$$Yield\ (\%) = \frac{\text{activity of partial purified enzyme } (\frac{U}{ml})}{activity\ of\ crude\ enzyme\ (\frac{U}{ml})} x100$$

$$Purity = \frac{specific\ activity\ of\ purifed\ \ enzyme(\frac{U}{mg})}{\text{specific activity of crude enzyme } (U/mg)}$$

4.9. Characterization of partial purified xylanase

4.9.1. Effect of temperature on the xylanase activity and stability

The optimum catalytic temperature of partially purified xylanase was determined by assaying the enzyme activity in the range of 30–90 °C. The temperature stability was determined by measuring the residual xylanolytic activity after being treated with temperatures from 50 to 90 ^{o}C for 60 min without the substrate, then after enzyme assay was conducted under standard assay condition.

4.9.2. Effect of pH on the xylanase activity and stability

To determine the optimum pH of partial purified enzyme, the assay was conducted using different buffers with pH values ranging from 3.5 to 9.5 and incubated at 50°C for 30 min. The enzyme was mixed with buffers with varying pH values at 1: 10 (v/v) ratio. The buffers used were as follows: 0.1M citrate buffer (pH 3.5–5.0), 0.2 M phosphate buffer (pH 6.0–7.5), and 0.2 M Tris–HCl buffer (pH 8–9.5) (Guo-Qiang Guan *et al*., 2016). All the buffers were prepared according to Gomori (1955) method. For pH stability, the enzyme was pre-incubated without the substrate in different buffers with pH values range from 3.5 to 9.5 for 24 h at 4°C and thereafter all the activity were performed under optimum conditions of pH and temperature (de Oliveira Simões *et al*., 2019).

4.9.3. Effect of metal ions on partially purified xylanase activity

Effect of metal ions on xylanase activity was evaluated by incubating reaction mixtures containing 1.0% xylan with K^{+}, Mn^{2+}, Mg^{2+}, Fe^{2+} , Ca^{2+} and Cu^{2+} at the concentrations of 1.0 mM. The study on effects of different metal ions was carried out using KCI, $MgSO_4$, $MnSO_4$, $CaCl_2$, $FeSO_4$, $ZnCl_2$ and $CuSO_4$. Inhibition/activation degree of xylanase activity was expressed as percentage of a control sample incubated in absence of any additive.

4.9.4. Determination of xylanase activity under optimized physico-chemical condtion

After characterization of partial purified xylanase activity under different physico-chemical conditions, its activity also determined using all optimum parameters at a time. Briefly, the assay was conducted by mixing 1.5 ml 1% birch wood xylan as substrate in 0.05M sodium citrate buffer (pH 5) with 0.75 ml diluted (1:100) enzyme. Then the reaction mixture incubated at 50^{o}C for 30 min. After the end of incubation period, 2ml DNS added, boiled for 5 mim. Finally, after

cooling, the OD measured using UV-spectrophotometer and enzyme activity determined using the regression equation from xylose standard curve.

4.9.5. Determination of kinetics parameters

The study of kinetics parameters (Km and Vmax) was conducted by measuring the enzyme activity at different xylan concentration (0.1–1.2 mM). The effect of various concentrations of birchwood xylan (P-ACXYL, Megazyme) on xylanase activity was determined. The reaction mixture containing 0.75 ml of the enzyme solution and 1.5 ml of 0.1–1.2 mM birchwood xylan in 50 mM citrate buffer (pH5.0) was incubated for 30 min in a water bath at 50 ^{o}C. After incubation the enzyme activity was measured and the kinetic constants, Km and Vmax, were estimated using the linear regression method of Lineweaver and Burk (1934).

5. Data analysis

The study was carried out with completely randomized design (CRD) experimental design. Each experiment was conducted in triplicate and evaluated with R program (Rx64 3.6.3) and graphs were ploted using ggplot2 package in R. The results were expressed as means ± standard deviations. Statistical comparisons were made by one-way analysis of variance (ANOVA) and means were separated by LSD test. Differences were considered significant when the *P* values are <0.05.

6. Results

6.1. Qualitative screening of WRF isolates

Among qualitatively screened 68 fungal isolates, all the isolates showed xylanolytic potential as examined based on Congo red staining (Fig. 1). However, isolates were different in their diameter of the hydrolyzed zones that ranged from 2.8 cm to 6.4 cm (Table–2). Seventeen isolates produced distinct high clearing zones greater than 4.55 cm around their colonies, were selected for further quantitative screening.

Table 2: Zone of hydrolysis produced by white rot fungal isolates on Mandel and Reese agar medium supplemented with 1% xylan as a carbon source

S/N	Isolates	Zone of hydrolysis (cm)
1	WR-68	6.40^{a}
2	WR-36	6.15^{a}
3	WR-39	6.10^{ab}
4	WR-5	5.75^{bc}
5	WR-35	5.60^{c}
6	WR-51	5.10^{d}
7	WR-10	4.85^{de}
8	WR-42	4.85^{de}
9	WR-8	4.85^{de}
10	WR-45	4.80^{de}
11	WR-55	4.75^{de}
12	WR-33	4.65^{e}
13	WR-11	4.60^{e}
14	WR-41	4.60^{e}
15	WR-48	4.60^{e}
16	WR-7	4.60^{e}
17	WR-2	4.55^{e}

- *The superscript letters at to right side within columns indicate that, the values are not significantly different at ($P<0.05$)*
- *Least Significant Difference (LSD)= 0.3829253*

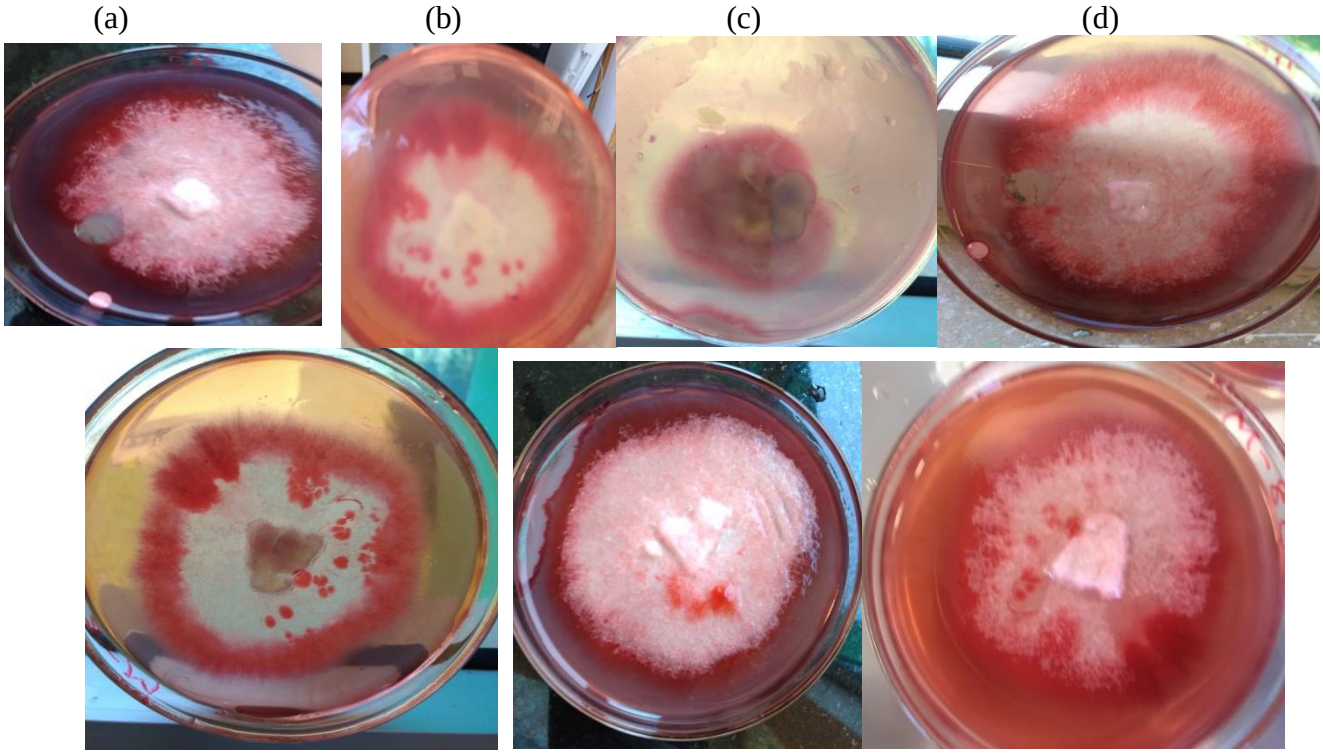


Figure 1: The zone of hydrolysis produced by white rote fungal isolates on Mandel and Reese agar medium containing xylan (a) WR–51; (b) WR–35; (c) WR–2; (d) WR–36; (e) WR–5; (f) WR–68; (g) WR–39

6.2. Quantitative screening of WRF isolates

The white rot fungal isolates that shown clear zones diameter greater than 4.55±0.05 cm in the qualitative screening were further subjected to quantitative screening to confirm the plate assay agar screening method under submerged conditions for xylanase production. The xylanase activity of all isolates determined from xylose standard curve. The isolates showed activity from 12.84 U/ml to 72.53 U/ml (Table 2). Five isolates showed higher than 65.459 U/ml xylanase activity.The highest xylanase activity observed was 72.53 U/ml. Finally five isolates that recoded maximum xylanase production were selected for SmF and SSF using wheat straw as sole carbon source for all experiments of this study.

Table 3: xylanse activity of seventeen white rot fungal isolates that quantitatively screened under SmF on MSM–xylan broth medium.

S/N	Isolates	Enzyme activity (U/ml)
1	WR-35	72.53006[a]
2	WR-36	70.70226[ab]
3	WR-68	69.93266[ab]
4	WR-5	68.68206[b]
5	WR-39	65.45936[c]
6	WR-55	29.57672[d]
7	WR-51	26.88312[d]
8	WR-10	21.01491[e]
9	WR-2	19.2352[ef]
10	WR-42	18.75421[efg]
11	WR-7	18.17701[efgh]
12	WR-48	17.69601[fgh]
13	WR-11	17.64791[fgh]
14	WR-8	17.16691[fgh]
15	WR-33	16.20491[gh]
16	WR-41	15.24291[hi]
17	WR-45	12.83790[i]

- *The superscript letters at to right side within columns indicate that, the values are not significantly different at (P<0.05)*
- *Least Significant Difference (LSD)=2.947635*

6.3. Production of xylanase under SmF and SSF condition

In this work all isolates showed promising xylanase production potential both in SmF and SSF. The isolates showed xylanse activity from 63.23 U/ml to 75.3 U/ml Under SmF and from 68.7 U/ml to 87.99 U/ml under SSF. Howevere, xylanase production in SSF was much higher the submerged fermentation (SmF). All isolates except WR–36 improved their xylanase index under SSF than the level of enzyme achieved in SmF as shown on Figure (3). Under SSF WR-39 scored highest xylnase activity (87.99±0.2452 U/ml) followed by WR–5 and WR–35 (86.62± 0.2356 and 84.42±0.4137 U/ml), respectively. However, WR–68 showed slight improvement.

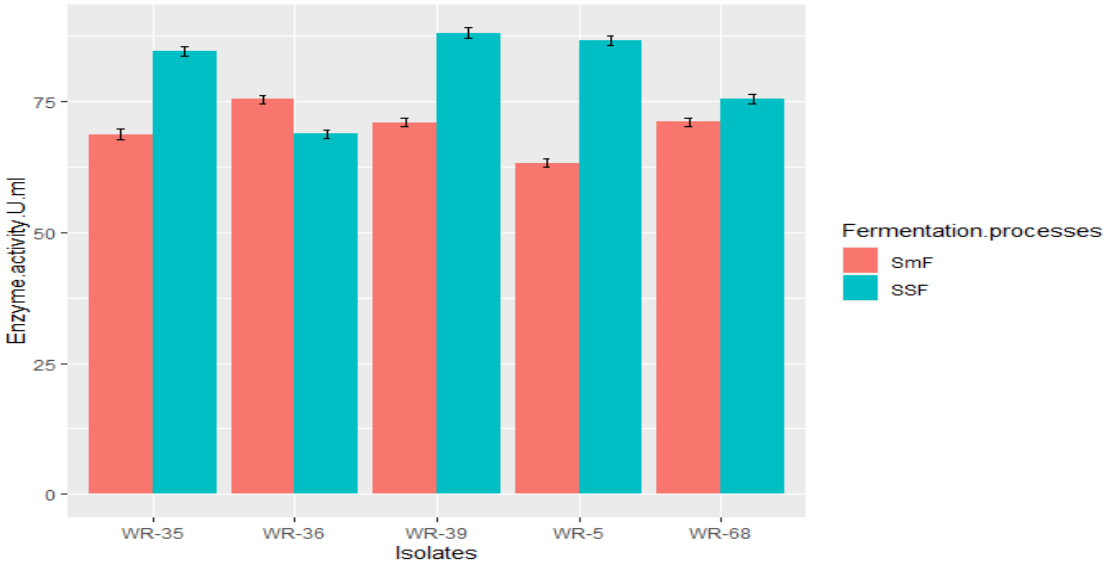


Figure 2: comparison of xylanase enzyme production under SmF and SSF by selected white rot fungal isolates using wheat straw as carbon source

6.4. Optimization of culture condition for xylanase production

6.4.1. Effect of incubation temperature

The result of this study indicated that the incubation temperature 25, 28 and 31^{o}C were good for all WRF isolates (Fig. 4). The optimal temperature for all isolates to produce xylanase was 28^{o}C. The isolates showed greather than 77 U/ml xylanase activity at 28^{o}C. Hence the subsequent studies were peformed at 28 ^{o}C.

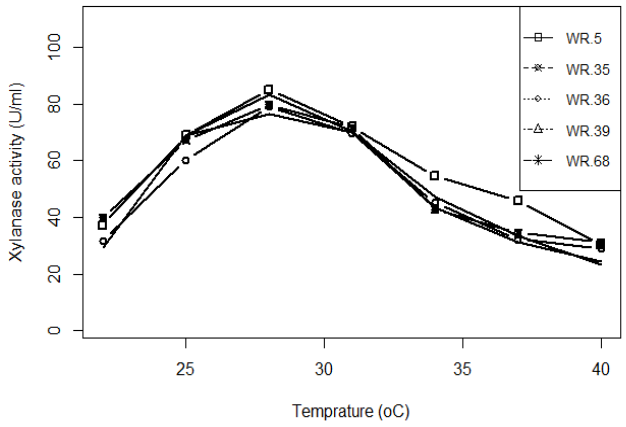


Figure 3: Effect of temprature on xylanase production

6.4.2. Effect of pH on xylanase production

The fungal isolates exhibited xylanase production at pH range from 5 to 6.5 with relatively highest xylanase production at pH 5 (Fig. 5). The activity decreasing rapidly below pH 5 and above pH 6.5 values (Fig. 5).

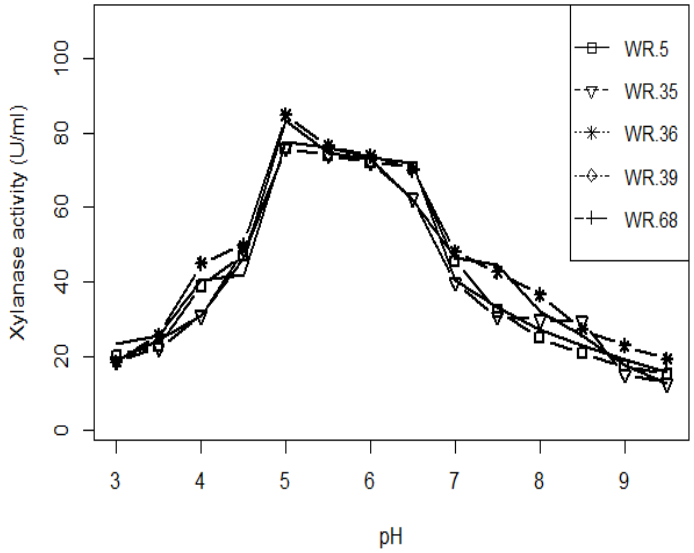


Figure 4: Effect of pH on xylanase production from WRF isolates

6.4.3. Effect of different carbon source

All the fungal isolates showed maximum xylanase production when xylan was used as carbon source, followed by CMC and xylose that were not significantly different at ($P>0.05$) in their xylanase production profile (Table 4). While lactose was achieved relatively moderate xylanase production. However, the lowest xylanase production was recorded from cellulose.

Table 4: Effect of carbon source on xylanase production by WRF isolates (values are mean ± SD of 3 replicates)

	Xylanase activity (U/ml)				
Carbon sources	WR–5	WR–35	WR–36	WR–39	WR–68
Xylan	80.466±0.827^{a}	75.031±0.068^{a}	76.52±1.2^{a}	82.7±0.757^{a}	80.707±2.6^{a}
CMC	74.36±0.6^{b}	69.307±0.245^{b}	71.42±0.65^{b}	71.905±1.246^{b}	73.97±0.802^{b}
Xylose	73.4 ±0.18^{b}	69.21±0.235^{b}	70.606±0.12^{b}	71.04±0.235^{b}	73.63±0.204^{b}
Lactose	58.4±1.886^{c}	61.13±1.80^{c}	68.345±0.68^{c}	67.768±0.136^{c}	67.816±0.068^{c}
Cellulose	41.7±0.36^{c}	38.76±0.18^{d}	30.68±1.88^{d}	46.075±0.89^{d}	44.824±0.18^{d}

- ***The same superscript letters at top right side within columns indicates that, the values are not significantly different, whereas, different subscript letters stand for values significantly different P < 0.05).***
- ***Pairwise group comparisons of different carbon sources was conducted by Fisher-LSD test.***

6.4.4. Effect of nitrogen sources

Among the nitrogen sources used yeast extract was found to be the best for xylanase production followed by ammonium sulfate (Table 5).. The highest amount of xylanase produced in the presence of yeast extract was 75.6565±1.027 U/ml. When fermentation medium was supplemented with urea, relatively moderate amount of xylanase was produced in comparison to yeast extract. The smallest xylanase production was achieved by peptone (40.0144±0.9425 U/ml). All the nitrogen sources were showed significantly different effect on xylanase production at alpha = 0.05. Exceptionally non-significant effect recorded between ammonium sulfate and urea in the case of isolate WR–39.

Table 5: Effect of nitrogen source on xylanase production by WRF isolates (values are mean ± SD of 3 replicates)

	Xylanase activity (U/ml)				
Nitrogen sources	WR–5	WR–35	WR–36	WR–39	WR–68
Yeast extract	74.887±0.446[a]	75.464±0.544[a]	75.6565±1.027[a]	74.454±2.651[a]	72.578±0.93[a]
Ammonium sulfate	59.062±1.896[b]	62.140±1.533[b]	69.02±0.513[b]	60.024±0.836[b]	59.4949±2.78[b]
Urea	52.809 ±1.651[c]	54.30±0.471[c]	59.93±1.64[c]	56.176±0.12[b]	54.4±2.204[c]
Pepton	52.809±1.433[d]	42.85±2.452[d]	40.014±0.942[d]	43.622±1.87[c]	46.315±1.883[d]

- ***The same superscript letters at top right side within columns indicate that, the values are not significantly different, whereas, different subscript letters stand for values significantly different at (P < 0.05).***
- ***Pairwise group comparisons of different nitrogen sources was conducted by Fisher-LSD test***

6.5. Comparative study of crude and partial purified xylnase enzyme activity

Xylanase from WRF isolates showed maximum activity at 70% ammonium sulfate fraction. Partial purified xylanases were achieved higher activity than unpurified. Isolates WR-36 and WR-39 showed the highest xylanse activity after purification followed by WR-5 which achieved a moderate improvement. However, WR–35 and WR–68 showed slight improvement of xylanse activity (Fig. 7).

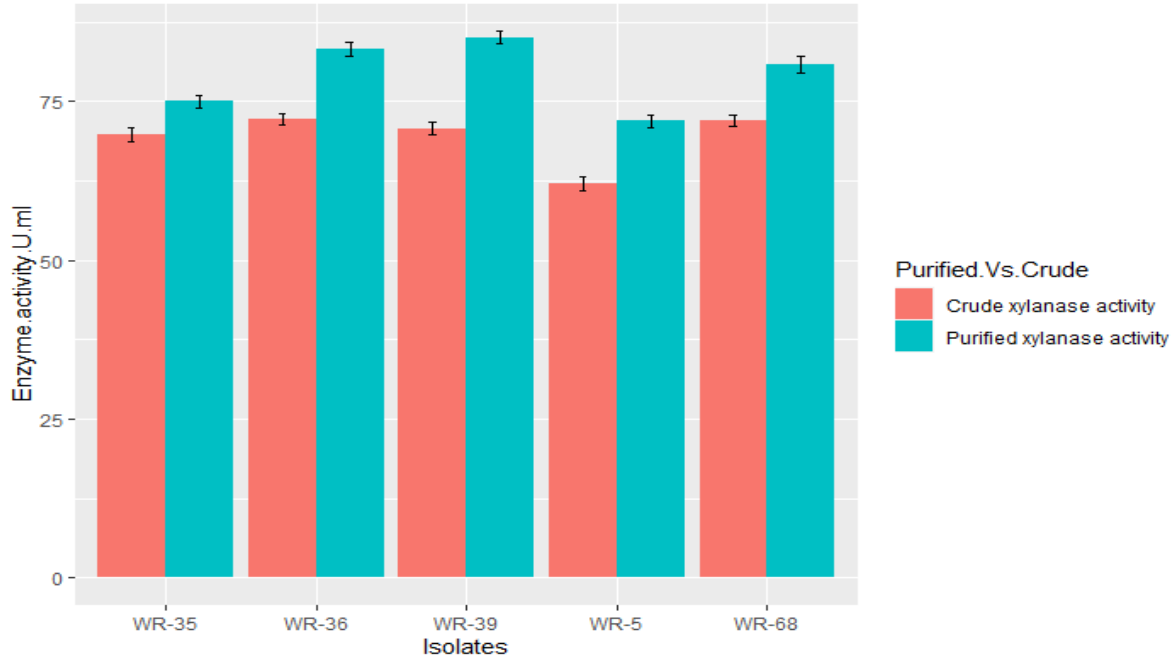


Figure 5: Comparative study of crude and partial purified xylanse enzyme

6.6. Determination of protein concentration and specific activity

The protein content of the enzyme was estimated by using Lowery method from BSA standard curve (Appendix 29). Protein concentration of partial purified xylanase was determined from the regression equation (Y= 0.5356x + 0.0123) of the standard curve. Xylanase enzyme from all isolates was improved its activity and specific activity after 70 % ammonium sulfate precipitation as shown in Table 6. Partially purified xylanase enzyme from isolates WR-39 and WR-68 yielded highest specific activity (86.423 and 86.678) and 120.197 and 112.14 % yield with 2.003 and 1.841 fold of purity respectively.

Table 6: Summary table for crude and partial purified xylanase enzyme

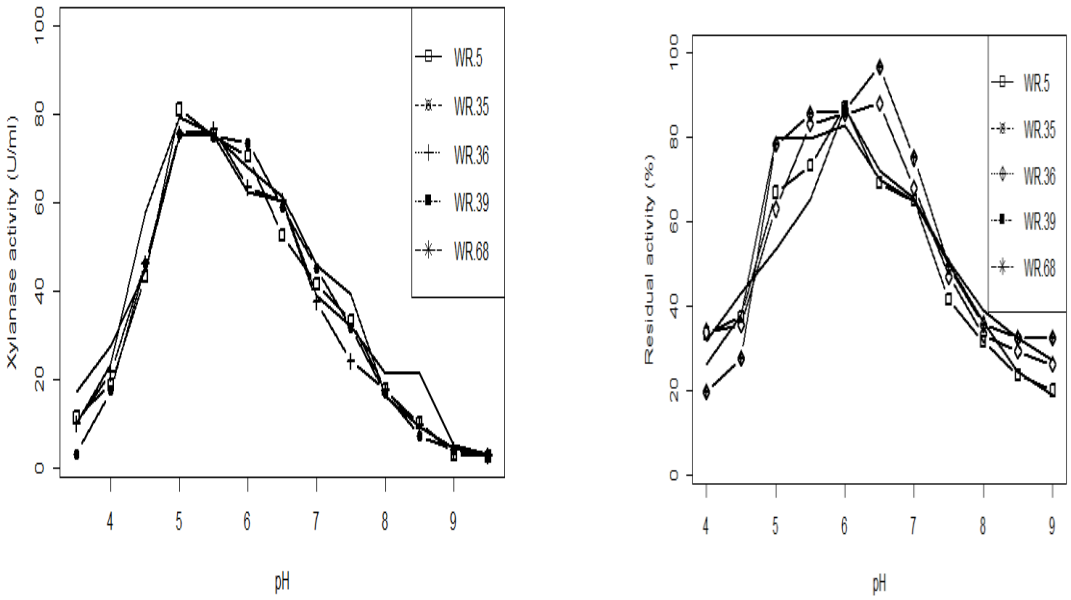
	Activity (U/ml)		Total protein (mg/ml)		Specific activity (U/mg)		Yield (%)	Purity
Isolates	Crude	Purified	Crude	Purified	Crude	Purified		
WR–5	62.092	71.953	1.45	0.946	42.822	76.0117	115.881	1.775
WR–35	69.738	74.983	2.334	1.304	29.879	57.496	107.521	1.9242
WR–36	72.145	83.256	2.463	1.837	29.291	45.317	115.401	1.547
WR–39	70.747	85.036	1.64	0.983	43.138	86.423	120.197	2.003
WR–68	72.013	80.755	1.53	0.932	47.067	86.678	112.1397	1.841

6.7. Characterization of partial purified xylanase

6.7.1. Effect of pH on partial purified xylanase activity and stability

The result of this test indicated that, the xylanase enzyme from all WRF isolates recorded highest activity in the pH range of 5-6.5 (Fig 10a). The maximum activity was observed at pH 5 for all, except xylanase obtained from WR–36 which showed the maximum activity at pH 5.5. Xylnase enzyme activity from all the WRF isolates were declined on either side of optimum range from acidic to neutral pH and alkaline pH as shown in Figure 10-a.

According to the results of this test, xylanase enzymes from all the WRF isolates retained more than 60% residual activity in pH range 5 to 7. However, xylanase from each fungal isolate exhibited maximum residual activity at different pH values. For example, xylanases from WR–5, WR–39 and WR–68 exhibited maximum residual activity (87.065 %, 82.5% and 86.816 %) at pH 6 respectively. Whereas, xylaneses from WR–35 and WR–36, respectively retained 87.726% and 96.535% residual activity at pH 6.5 (Figure 10-b)



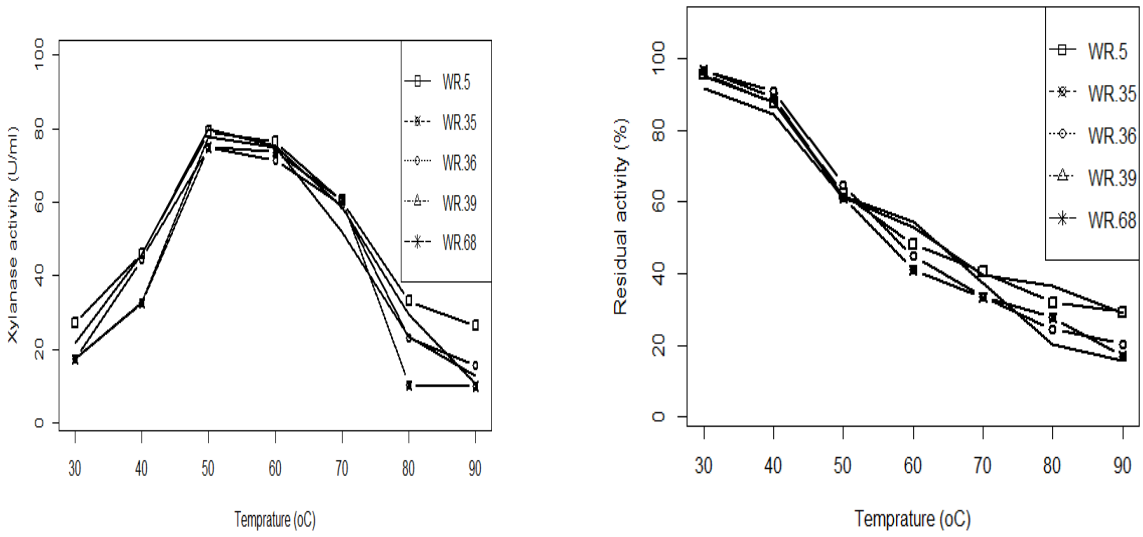
(a) **(b)**

Figure 6*:* Effect of pH on partial purified xylnase activity (a) and stability (b)

6.7.2. Effect of temperature on partial purified xylanase activity and stability

The result indicated that, xylanases from all isolates showed good performance in the temperature range of 50–60 ^{o}C, but maximum xylanase activities (79.17, 74.9, 74.84, 77.82 and 80.034 U/ml) for WR–5, WR-35, WR–36 and WR–68, respectively were recorded at 50 ^{o}C. Below 50 ^{o}C and above 60 ^{o}C the enzyme activities were declined as shown on figure (11-a).

The result from thermostability test showed that, xylanases from all sources retained more than 90% and 80% of their residual activities at 30 and 40^{o}C respectiviley. The residual activities for all xylanases were about 60% at 50 ^{o}C. Except WR-39 and WR–68 that were retained 50% of their activity at 60^{o}C , xylanase from all isolates lost residual activity above 50^{o}C (Fig. 11-b).



(a) **(b)**

Figure 7: Effect of temperature on partial purified xylanase activity (a) and stability (b)

6.7.5. Effect of metal ions on partial purified xylanase

Among the metal ions tested in this study, Mg^{2+}, Ca^{2+} and Mn^{2+} were positively modulated relative activity of xylanase (Fig. 10). The incorporation of these metals enhanced relative activity of partial purified xylanse by 10–27%. Inhibition of enzyme activity was observed in the presence of Cu^{2+} and K^{+} which caused ~ 40 % loss of xylanase activity from all isolates. Fe^{2+} relatively showed slight inhibition, causing ~ 20% loss of enzyme activity (Fig 10).

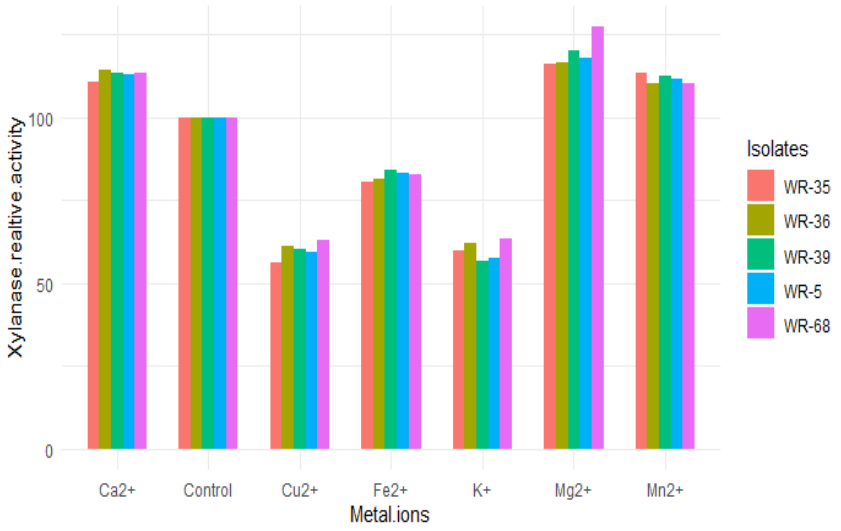


Figure 8: Effect of metal ions on partial purified xylanase activity

6.8. Determination of kinetics parameters

The result showed that, xylanase activity was increased with the increase in substrate concentration. However, further increment in substrate concentration didn't affect xylanase activity and the rate of increase in velocity was leveled off after certain substrate concentration (Fig.11).

The Vmax and Km were calculated from the regression equation of Lineweaver–Burk plots (Appendices. 17–21). The regression coefficients (R^2) were (0.9912, 0.992, 0.9894, 0.9955 and 0.9964) for WR–5, WR–35, WR–36, WR–39 and WR–68 respectively. This indicates that the reciprocal of xylan concentration and enzyme (xylanase) activity were positively correlated. The Vmax and Km for xylanases from (WR–5, WR–35, WR–36, WR–39 and WR–68 (106.38, 113.63, 86.95, 109.89 and 99 µmol $min^{-1}mg^{-1}$) and (0.415, 0.545, 0.339, 0.527 and 0.32 mg/ml) respectively.

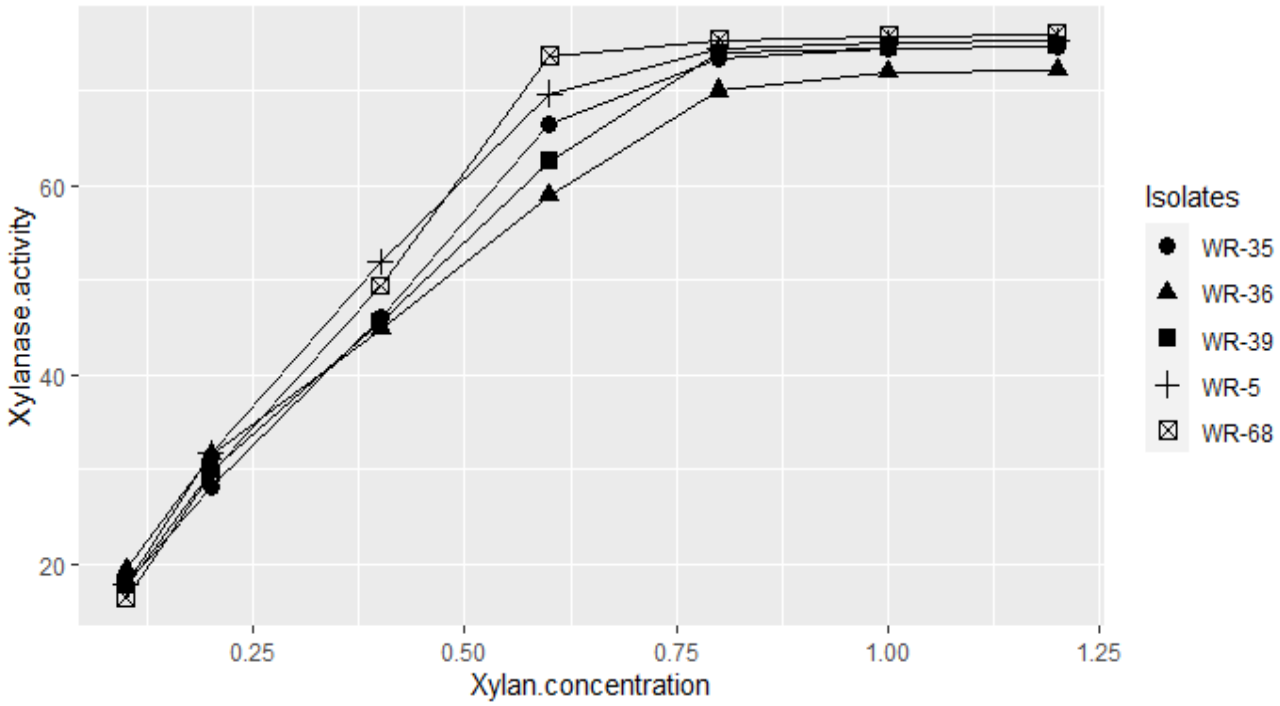


Figure 9: Micheal–Menten plot of xylanase enzymes from selected WRF isolates

7. Discussion

Xylanolytic enzymes have gained a great attention, as they have a potential role in biorefinery processes as well as industrial applicationsin various fields like wine making, textile, paper and pulp industries, animal feed industries, bread making, fruit juice extraction (Chadha *et al*., 2019; Sunkar *et al*., 2020).

Considering these applications, in this study the white rot fungi that isolated from decayed wood were qualitatively screened by plate agar screening method using MSM–xylan medium. All the WRF isolates those were isolated showed xylanolytic potential. The appearance of the clear zone (hydrolysis zone) around the colony was strong evidence for the production of xylanase enzymes. This implies that, the isolates have potential to convert xylan to xylose due to the hydrolytic action of xylanases. Similarly, Ramanjaneyulu et al., (2015) have utilized this technique for screening xylanolytic fungal isolates. However, isolates that produced relatively higher clear zone or colony diameter were selected for further studies.

Qualitative screening based on the clear zone around the colonies of isolates on solid agar medium is not only due to the release of desired enzyme but may sometimes be due to the presence of membrane bound hydrolyses which cause formation of the clear zone when the substrates are being hydrolysed (Bairagi 2016). Hence, the isolates that were selected based on their zone of hydrolysis, were subjected to quantitative screening. Similarly, different researchers have utilized two step screening methods i.e qualitative followed by quantitative screening to identify xylanolytic enzymes. For example Bairagi **(**2016) has conducted primary (agar plate) screening, followed by qualitative (submerged fermentation) screening to identify xylanolytic fungal isolates. Similarly Varghese and coworkers (2017) have utilized the same technique to screening xylano-cellulolytic positive microbes from termite gut and termitarium and also Manju and Narsi Ram Bishnoi (2016) have applied the two step screening technique to determine the xylanase production potential of fungi from degraded wood, fruits and animal dung. Among 17 isolates subjected to qualitative screening, five isolates WR–5, WR–35, WR–36, WR–39 and WR–68 showed maximum xylanase activity and were selected for subsequent studies.

Different environmental factors such as: pH of the medium, incubation temperature, carbon and nitrogen sources and substrate concentration significantly influence the production of fungal enzymes during fermentation (Mardawati *et al*., 2020). Taking this into account, in the present study the appropriate pH of the growth medium, incubation tempratue, carbon sources and nitrogen sources for xylanase production was determined.

The pH level of fermentation medium plays the most important role in the growth of an organism and consequently determines the amount of enzyme produced by influencing nutrients transport. The results of present study indicate that the WRF isolates showed good growth and xylnase production in the pH range of 5 to 6.5. However, all the isolates were recorded the highest xylanse production at pH 5 (Fig.4). This agrees with the findings of Qinnghe et al. (2004) who reorted that the maximum xylanase production from *Pleurotus Oustreatus* was at initial pH 5. Similarly Saleem et al (2014) have also reported white rot fungal isolates maximum xylanase production at same initial pH. Ramanjaneyulu et al (2017) have reported that xylanase production increased with increasing in the initial pH of the medium from 4.0 to 5.5 with maximum yield at 5.0. This may be due to the acid pH levels (5.0–6.5) that were the optimum pH for the growth of WRF isolates and consequently favored xylanase production.

The incubation temperature is another crucial parameter which influence nutrients transport and determines the growth of the microbes and simultaneously affects their enzyme production. In this study, the increment in xylanse production was observed at (25, 28 and 31^{o}C) with maximum yield of xylanas at 28^{o}C for all isolates. The amount of xylanase decreased below 25 ^{o}C and above 31 ^{o}C. Similarly, Guan et al (2016) reported that temprature from 28°C to 30°C favoured production of xylanase production from *Cladosporium oxysporum*. Higher xylanse yield at lower temperature range (28^{o}C–31^{o}C) is adevatageouse since it reduces energy requirement and facilitates the production of xylanse without the need of incubation instrument.

Nitrogen (N) is an important component of media utilized by microorganisms for the synthesis of amino acids, nucleic acids and proteins (enzymes) (Bechem 2012). Hence, incorporation of

nitrogen sources in the fermentation medium influences the growth of the fungi and consequently enzyme production. This investigation has demonstrated that supplementation of the medium with nitrogen source significantly influenced xylanase production with 95% level of confidence. The maximum amount of xylanase was obtained when yeast extract supplemented as nitrogen source. This observation is in line with Ramanjaneyulu et al (2017) who revealed that yeast extract yielded the highest amount of xylanse enzyme. Similarly Guan et al (2016) have reported organic nitrogen tryptone and yeast extract gave the high xylanase production. Similar observation also reported by Muthezhilan et al (2007) who revealed that highest the xylanase produced by *Penicillium oxalicum* when yeast extract applied in growth medium. The present finding also agrees with earlier investigation in which an organic nitrogen (yeast extract) and inorganic nitrogen (ammonium sulfate) were yielded the highest amount of xylanase from white rot fungal isolate (Maan Poonam *et al*., 2016).

The WRF isolates produced maximum amount of xylanase when xylan used as carbon source. The xylanase induction effect of xylan is due to its low molecular mass fragments such as xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers that play a key role in the regulation of xylanase biosynthesis (Motta et al. 2013). Similarly Rajeeva Gaur et al (2015) have reported that, birchwood xylan was found to be the best for xylanase production out of different carbon sources tested for xylanase production from *Bacillus vallismortis* RSPP-15. Another investigation also revealed that maximum xylanase production by *Trichoderma harzianum* 1073 D3 was occurred with xylan, followed by avicel, CMC, maltose and lactose (Isil Seyis and Nilufer Aksoz 2005). Similar result was also obtained by Rosmine et al., (2018) who indicated that xylan was the optimum carbon source inducing the highest level of xylanase production from *streptomyces* species.

Among five isolates tested under SmF and SSF all isolates except WR–36 yielded the highest amount of xylanse under SSF condition compared to that of SmF. This is in agreement with Agnihotri et al. (2010) revealed that fungal strain, *Coprinellus disseminatus* SW-1 NTCC 1165 yielded the highest amount of xylanase (88.79%) under SSF compared to that of SmF. Similarly Liu et al (2020) have demonstrated that *Phanerochaete chrysosporium* produced higher cellulase and hemicellulase during SSF than SmF. Another investigation in line with this finding reported

by Fikiru Getachew et al. (2017) who revealed that production of xylanase by *Pleurotus* species was higher in SSF, using different substrates as carbon source, when compared to the results obtained in SmF with the same substrates. Similarly, SSF of *Trichoderma koenini* using corn cob supplemented with Pineapple peel powder showed enhanced production of xyanase (Bandikari *et al*., 2014). The increased yield of xylanase produced by the isolate observed in SSF may be due to close contact between mycelium and substrate which is not possible during SmF. It may be also due to environmental condition similarity between the SSF condition and the white rot fungal natural habitat (wood and organic material) and this condition would be expected to stimulate fungal isolates to produce higher xylanase enzymes. The morphology of filamentous fungi grown on SSF allows it to colonize the surface of the substrate and penetrate into the substrate matrix in search of nutrients and the fungal biomass inside and on the surface of the substrate secretes metabolites and enzymes.

In the present work, xylanase produced by WRF isolates was partially purified by ammonium sulphate precipitation and xylanase from all the isolates recorded maximum xylanase activity at 70% ammonium sulphate fraction. Since salts lower the effective water concentration and enzymes need water as "grease" for conformational changes during their cycle. Partial purified xylanases from all the isolates were achieved higher enzyme activity and more specific activity than unpurified. There are several earlier findings that are in agreement with this work. Deshmukh et al. (2016) have studied the purification of xylanase from *Aspergillus fumigatus* R1 and observed maximum xylanase activity with 30–55% fraction of ammonium salt precipitation with 24.6% yield and 9.04 purification fold. Guan et al (2016) also reported that ammonium sulfate precipitation led to xylanase specific activity of 10.12 U/mg protein and increased purification fold to 3.61. Similarly Gautam et al (2018) have suggested that, Xylanase from *Schizophyllum commune* ARC-11 was recorded maximum xylanase activity at 50–70% ammonium sulphate fraction with 41.86% yield, specific activity of 632.18 IU/mg and 2.75 fold of purification.

The optimal pH for xylanases activities from all isolates were in the pH range of 5.00-6.5 with maximum xylanase activity at pH 5 for all, but pH 5.5 for xylanase obtained from isolate WR–36. Most of the xylanase activity declined below and above the optimum pH. This is due to

change of the pH outside the optimum range that can cause enzymes to denature, since they are proteins. Enzymes are sensitive to changes in the hydrogen ion concentration that causes denaturation by altering the degree of ionization of an enzyme's acidic and basic side groups located in the active site. It could be concluded from the results xylanase from all the WRF isolates needed an acidic environment to be active, fungal xylanase have a maximum activity under acidic pH conditions (Chen *et al*., 2016). The pH of the medium affects enzyme activity by altering the ionization state of amino acids i.e it affects the state of acidic ionization (carboxyl functional group in their sidechains) or basic amino acids (amine functional groups in their side-chains). The change in ionization state of amino acids causes the change in ionic bonds that help to determine the 3-D shape of the protein and lead to the alteration of protein recognition that inactivated the enzyme (Kurrataa et al. 2015**)**.

In this work, pH stability of the xylanse was conducted and xylanases from all isolates were retained above 60% of their original activity at pH range of 5 to 7 after 24h pre-incubation in buffers with different pH values without substrate. However, the xylanase from different isolates retained its maximum residual activity at different pH. Xylanase from WR–36 and WR–35 retained maximum residual activity (87.726% and 96.535%) at pH 6.5 respectively and xylanse from WR–5, WR–39 and WR–68 were retained 87.065 %, 82.5% and 86.816 % of their original activity at pH 6 respectively. Whereas, similarly Siliva et al (2015) research finding indicated that xylanase obtained from *Trichoderma inhamatum* were showed more than 50% of the maximum activity in the pH range from 4.5 to 6.5, and the activity decreased sharply from this range.

The result indicates that, the optimumal activity at pH range 5–6.5 i.e acidic condiction and wide-ranging pH stability i.e 5–7 makes the xylanase enzyme suitable for application in food industry (fruite juice and bakery), animal feed ndustries and wine industries. Adigüzel and Tunçer (2016) have reported that, paritial purified xylanase with optimal pH 6 and pH stability 4–9 with > 54% activity retaintion enhanced orange and grape juice clarity and improved dough volume. Similarly da Silva et al (2019) have reported that, the endo-xylanase with optimal activity at a range of pH from 5.0 to 6.0 enhanced the clarity of mango, banana and tangerine juices. The acidic pH and temperature profile of the white rot fungal xylanse makes it suitable

for application in animal feed industry. Because both poultry and livestock gastrointestinal tracts have an acidic pH (<6.9).

There are several commercially available fungal xylanases that exhibite similar optimal pH and temperature as the xylanase enzyme produced in this study and have been used in different industiries. For example bioxylanase, produced by *Trichoderma reesei* used for brewing and animal feed industries with optimum pH 5.5–6.0 and temperature (55^{o}C), Allzyme PT, produced by *Aspergillus niger* recommended for the animal feed industry exhibited optimum conditions pH 5.3 and 65^{o}C, 'Gammazym X400OL', produced by *Trichoderma longibrachiatum* and recommended for production of wheat starch, baking and brewing industry exihibted optimum conditions (pH range 4.5–7.0 and 50—60^{o}C), 'Solvay Pentosanase' from *Trichoderma reesei* has optimum conditions (pH 5.3–5.5 and 55^{0}C) and recommended for starch and baking industries (Haltrich *et al*., 1996). Therafore, the enzyme produced in this study from WRF isolates showed desirable characteristics suitable for applications in animal feed upgrading, starch producing, baking, brewing and fruit juice producing industries.

The present investigation indicated maximum xylanase activities (79.17, 74.9, 74.84, 77.82 and 80.034 U/ml) for WR–5, WR–35, WR–36 and WR–68 respectively at 50^{o}C. Below 50^{o}C and above 60^{o}C the enzyme activities were declined. Temperature has a generalized effect on reaction rates, interfering with reagent solubility, enzyme stability and kinetic constants. Two opposite mechanisms activation (constant rate increases with the temperature increases) and denaturation (loss of quaternary and tertiary structures of the enzyme) occur simultaneously as the reaction temperature increases. However, extreme high temperatures can cause an enzyme to denaturation of the protein structure and disruption of the active site (Vitolo 2020).

In this study thermo stability of partial purified xylanases were also deterimined. The results this test indicated that the xylanase enzymes produced from WRF in this study could be used in food processing like fruit juice clarification and bread making and animal feed industries, since most commericial available xylanase used in these industries have the optimum temprature ranging 40–60^{o}C. Adigüzel and Tunçer (2016) have reported that, treatment of grape juice at 50^{o}C and orange juice at 60^{o}C with partially purified xylanase from *Streptomyces species* AOA40

increased the juice clarity by 17.19% and 18.36% respectively and also they reported that treatment of dough with this enzyme resulted in increased oven spring and 17.06% increase of dough volume. da Silva et al (2019) have also repoted that application of partial purified endo-xylanase with 55^{o}C optimum temperature improved the juice quality of mango, banana and tangerine juices with 51.11%, 9.99% and 8.54% increase in the clarity respectively.

Out of the metal ions tested in this study, Mg^{2+}, Ca^{2+} and Mn^{2+} enhanced relative activity of xylanase. The incorporation of these metals enhanced relative activity of partial purified xylanse. The finding of Ping et al (2018) partial in agreement with that revealed Ca^{2+}, Mg^{2+}, Ba^{2+}, K^{+} and Ag^{+} enhanced xylanase activity, however K^{+} slightly inhibited the xylanase activity in present study. The previous study on effect of various metal ions on xylanase activity indicated that enzyme activity was stimulated in the presence of Mg^{2+}, Mn^{2+} and Ca^{2+} whereas and other metal ions, strongly inhibited xylanase activity. This finding is in agreement with present work in which Mg^{2+}, Mn^{2+}, and Ca^{2+} enhanced xylanase activity and Cu^{2+} highly affected xylanase activity (Raja *et al*., 2018). The finding of Zhang et al(2012) also revealed that the presence of Mn^{2+}, Mg^{2+}, Ca^{2+} enhanced xylanse activity while, Cu^{2+} inhibited the xylanase activity.

The inhibition or stimulation effect of the metal ions on enzyme activity may be due to their interaction with sulfhydryl or carboxyl groups of the enzyme that destabilize its molecular structure and led to conformational change and subsequent cause inactivation or activation of the enzyme. According to this result, for future application studies of these enzymes, it is important to add metal ions like Mg^{2+}, Ca^{2+} and Mn^{2+} in the reaction mixture and to avoid the presence of metal ions like Cu^{2+}, K^{+} and Fe^{2+}.

Determination of enzyme kinetics of an enzyme catalyzed reaction is an important parameter to know the effectiveness of the enzyme. The smallest Km value (0.32 mg/ml) achieved by xylanse produced from isolate WR–35 whereas highest Km was achieved by WR–35 which is 0.545 mg/ml and the highest Vmax (113.63 µmol $min^{-1}mg^{-1}$) and lowest Vmax (86.95 µmol $min^{-1}mg^{-1}$) were recorded from WR–68 and WR–36, respectively. The lower Km value of xylanase from WR–68 indicates that it has relatively the highest affinity with substrate compared xylanse from rest isolates, while the highest Km value by xylanase from WR–35 shows lowest affinity to the substrate. Kumar et al (2017) have reported that Km and Vmax for xylanase produced by

Bacillus amyloliquefaciens were 5.6 mg/ml, 433 µmol $min^{-1}mg^{-1}$ proteins respectively. Similarly Huang et al (2019) have reported that the GH11 Xylanase produced by hytridiomycetous fungus, Rhizophlyctis rosea achived the Km for RrXyn11A S and RrXyn11A L was 21.63 mg/ml, 18.83 mg/ml and the Vmax for RrXyn11A S and RrXyn11A L 219.5 µmol $min^{-1}mg^{-1}$, 210.7 µmol $min^{-1}mg^{-1}$ respectively. de Oliveira Simões et al (2019) have indicated that Xylanase produced by the Fungus *Myceliophthora heterothallica* recorded 2.7 ± 0.28 mg ml^{-1} Km and 19± 1 µmol $min^{-1}mg^{-1}$ Vmax.

8. Conclusion

White rote fungal isolates from decaying wood can be a good xylanase source. Based on the results obtained from production optimization it can be concluded that using a cheap agricultural residue (wheat straw) yielded high amount of xylanase under SSF condition. Therefore, wheat straw can be used for large-scale production of xylanase unded SSF condition. The pH and temperature profiles and stabilities indicate that xylanase enzyme can be suitable for application in food industry (fruit juice and bakery), animal feed industry and wine industry. The lower Km value of the xylnase enzyme indicates the enzyme has highest affinity for the substrate (xylan). This implies that small quantity of xylanase will hydrolysis considerably high amount xylan into its monomer.

9. Recommendation

Based on the observations and conclusions of this study, the following recommendations are forwarded.

- Further molecular identification and characterization of the selected WRF isolates is mandatory to identify the isolates at species level
- The enzyme shall be further purified and immobilized to increase the stability of enzyme activity
- Xylanase should be evaluated for their potential applications in different industries such as textile industry, paper and pulp industry, food and animal feed industries and biofuel industries.

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11. APPENDICES

Appendix 1. Components of MSM media

Chemicals **chemical formula** **Amount in g/L**

Dipotasium hydrogen phosphate ……K_2HPO_4…….. …1.73

Potassium dihydrogen phosphate……..KH_2PO_4……..0.68

Ammonium sulfate…….. $(NH_4)_2$ SO_4……1.4

Sodium chloride……..NaCl: ……..4

Magnesium sulfate hepta hydrate……$MgSO_4.7H_2O$……..0.1

Calcium chloride……$CaCl_2$: ……..0.02

Manganese sulfate……$MnSO_4$…….. .0.01

Ferrous sulfate……$FeSO_4$. ……..0.03

Appendix 2. 0.1 M citrate buffer preparation for pH range 3.5 to 5.0

Primarily: 0.1 M solution of citric acid and 0.1 M solution of sodium acetate were prepared by dissolving 2.101 g and 2.941 g in 100 ml distil water respectively. Then the buffer was prepared by mixing the solution of citric acid and sodium citrate in the proportion as indicated on the table below

Appendix 3. Table.1**:** The proportion for Citrate buffer preparation

Citric acid (ml)	40.0	33.0	28.0	20.5
Sodium acetate (ml)	**10.0**	**17.0**	**22.0**	**29.5**
pH	**3.5**	**4**	**4.5**	**5.0**

Appendix 4. 0.2 M phosphate buffer preparation for pH range 6.0 to 7.5

For preparation of 0.2M phosphate buffer 0.2 M solution of monobasic sodium phosphate and 0.2 M solution of dibasic sodium phosphate were prepared by dissolving 2.7 g and 5.365 g in 100 ml dH2O respectively. Then phosphate buffer was prepared by mixing the solution of monobasic sodium phosphate and dibasic sodium phosphate in the proportion as indicated on the table below.

Appendix 5. Table.2: **The proportion for phosphate buffer preparation**

0.2 M monobasic sodium phosphate solution (ml)	**87.7**	**68.5**	**39.0**	**16.0**
0.2 M dibasic sodium phosphate solution (ml)	12.3	31.5	61.0	84.0
pH	6.0	6.5	7.0	7.5

Appendix 6. **0.2 M Tris–HCl buffer preparation for pH range 8.0 to 9.5**

For preparation of 0.2M Tris–HCl buffer, 0.2 M solution of tris (hydroxylmethyl) aminomethane and 0.2 M solution of HCl were prepared by dissolving 2.7 g and 5.365 g in 100 ml dH_2O respectively. Then phosphate buffer was prepared by mixing 50 ml tris (hydroxylmethyl) aminomethane solution with the solution of HCl as indicated on the table below.

Appendix 7. Table. 3: The proportion for Tris–HCl buffer preparation

0.2 M tris (hydroxylmethyl) aminomethane solution (ml)	50.0	50.0	50.0	50.0
0.2 M solution of HCl solution (ml)	**26.8**	**12**	**5.0**	**4.6**
pH	**8.0**	**8.5**	**9**	**9.5**

Appendix 8. **Table.4 : ANOVA results of carbon sources for enzyme production by WR–5 (mean ± SD of 3 replicates)**

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	4	2945.4	736.3	516.5	1.55e-11 ***
Residuals	10	14.3	1.4		

Alpha=0.05 CV = 1.818429 LSD = 2.172216

Appendix 9. Table.5: ANOVA results of carbon sources for enzyme production by WR–35 (mean ± SD of 3 replicates)

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	4	2440.5	610.1	599.6	7.38e-12 ***
Residuals	10	10.2	1.0		

Alpha = 0.05 CV = 1.609101 LSD = 1.835142

Appendix 10. Table.6: ANOVA results of carbon sources for enzyme production by WR–36 (mean ± SD of 3 replicates)

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	4	4150	1037.5	594.6	7.7e-12 ***
Residuals	10	17	1.7		

Alpha = 0.05, LSD = 2.403175

Appendix 11. Table.7: ANOVA results of carbon sources for enzyme production by WR–39 (mean ± SD of 3 replicates)

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	4	2162	540.5	601.8	7.25e-12 ***
Residuals	10	9	0.9		

Alpha =0.05, LSD = 1.72413

Appendix 12. Table.8: ANOVA results of carbon sources for enzyme production by WR–68 (mean ± SD of 3 replicates)

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	4	2297.6	574.4	215.5	1.18e-09 ***
Residuals	10	26.7	2.7		

Alpha = 0.05 LSD = 2.734082

Appendix 13. Table.9: ANOVA results of nitrogen sources for xylanase production by WR–5 (mean ± SD of 3 replicates)

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	3	1510.3	503.4	156.6	1.92e-07 ***
Residuals	8	25.7	3.2		

Alpha = 0.05 LSD = 3.376201

Appendix 14 . Table.10: ANOVA results of nitrogen sources for xylanase production by WR–35 (mean ± SD of 3 replicates)

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	3	1690.1	503.4	169.1	1.42e-07 ***
Residuals	8	26.7	3.3		

Alpha= 0.05 LSD = 3.436697

Appendix 15. Table.11: ANOVA results of nitrogen sources for xylanase production by WR–36 (mean ± SD of 3 replicates)

Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	3	1690.1	503.4	169.1	1.42e-07 ***
Residuals	8	26.7	3.3		

Alpha = 0.05 CV = 2.213504 LSD = 2.548723

Appendix 16. Table. 12: ANOVA results of nitrogen sources for xylanase production by WR–39 (mean ± SD of 3 replicates)

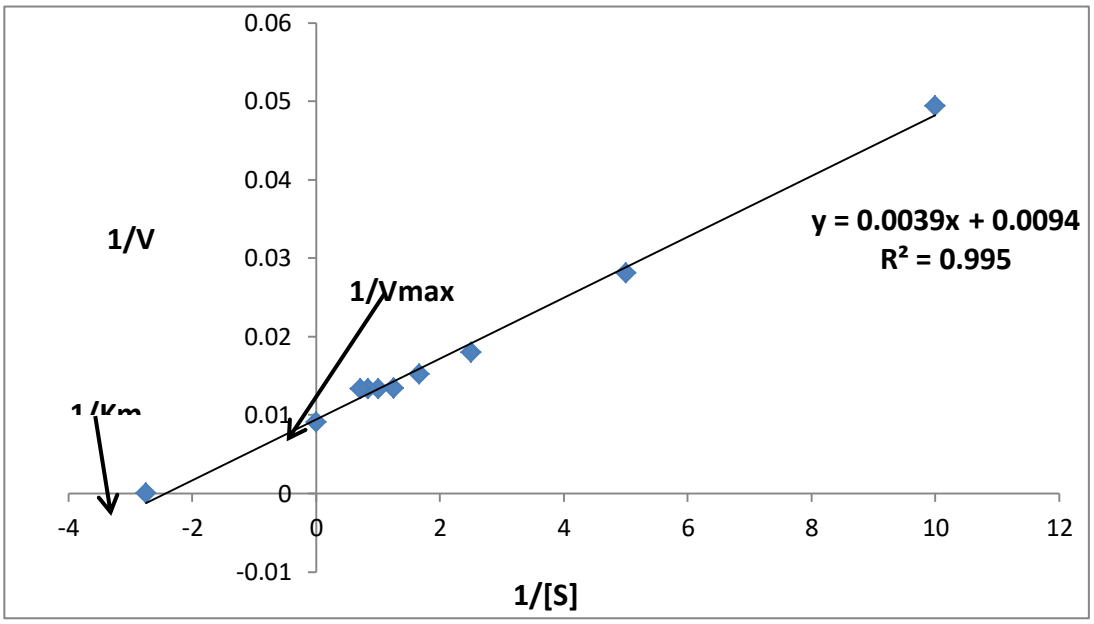
Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	3	1450.8	483.6	114.7	6.49e-07 ***
Residuals	8	33.7	4.2		

Alpha = 0.05 LSD = 3.865488

Appendix 17. Table.13: ANOVA results of nitrogen sources for xylanase production by WR–68 (mean ± SD of 3 replicates)

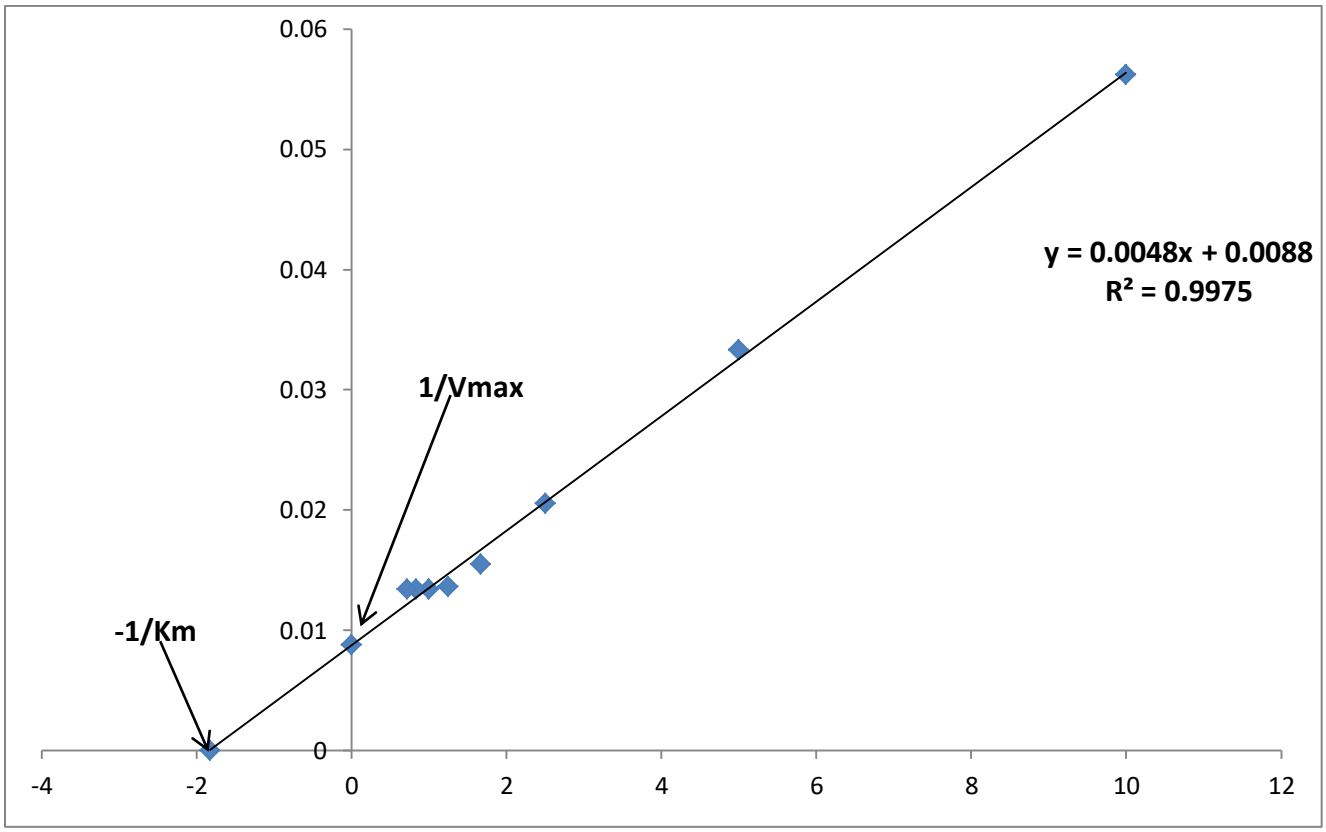
Source of variations	Df	Sum sq	Mean Sq	F value	Pr(>F)
Treatments	3	1450.8	483.6	114.7	6.49e-07 ***
Residuals	8	33.7	4.2		

Alpha = 0.05 LSD = 3.882954



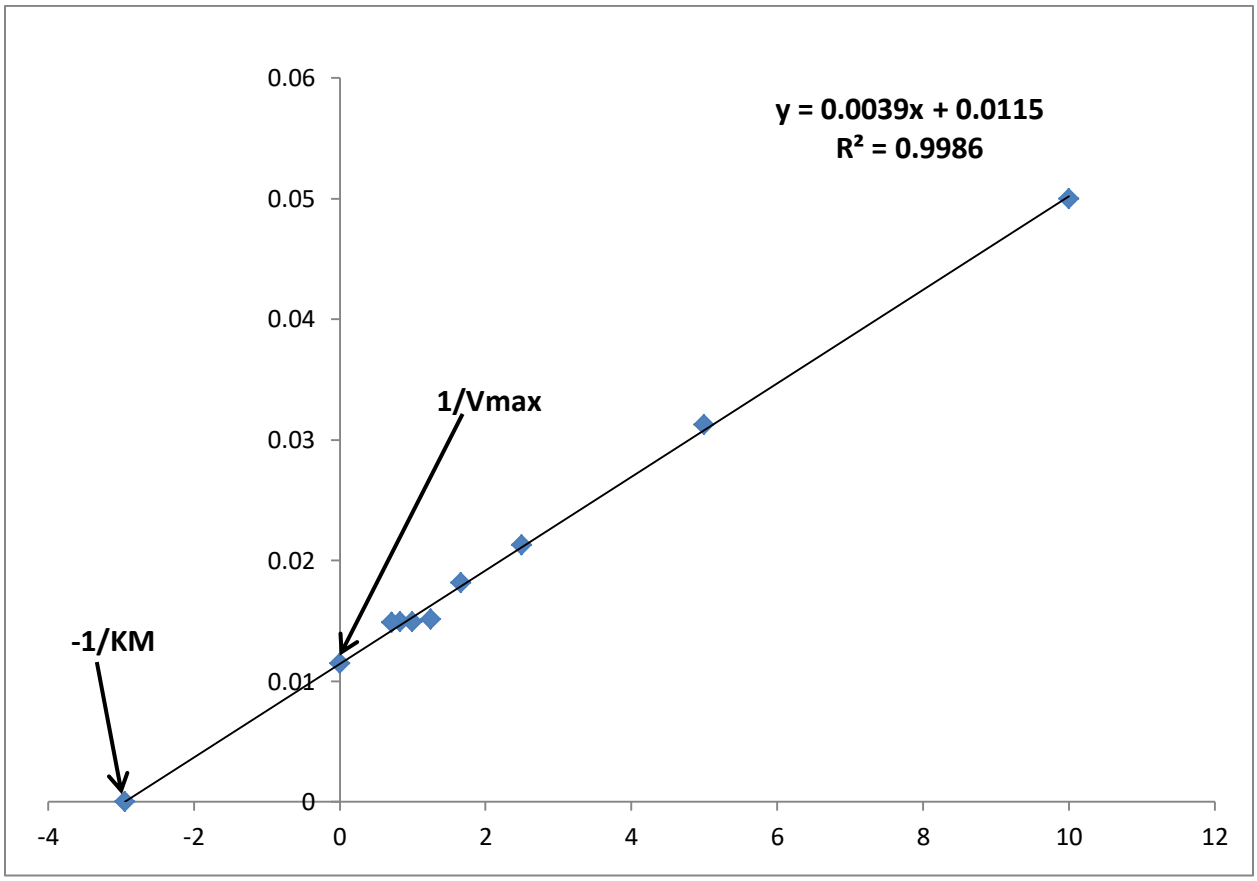
Appendix 18. Figure 1: Lineweanver–Burk plot for WR–5 isolate

Vmax=106.38 µmol $min^{-1}mg^{-1}$ Km = 0.415 mg/ml ½ Vmax = 53.19



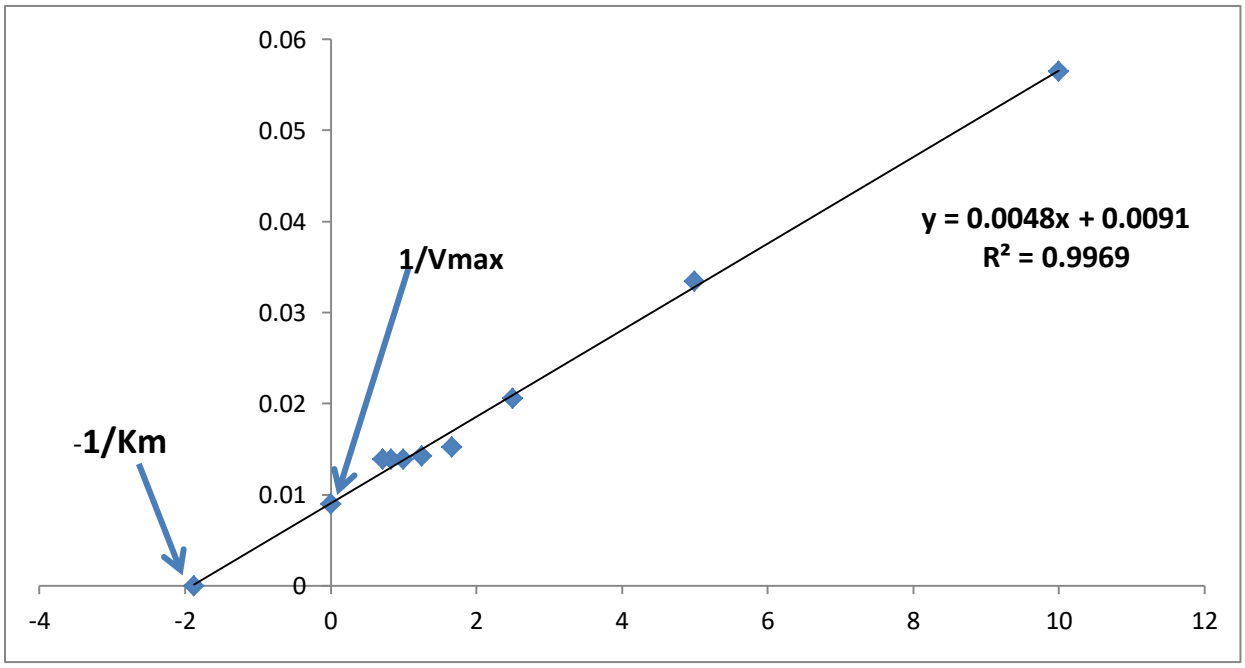
Appendix 19. Figure 2: Lineweaver Burk plot for WR–35

Vmax = 113.63 µmol $min^{-1}mg^{-1}$ Km = 0.545mg/ml ½ Vmax = 56.815



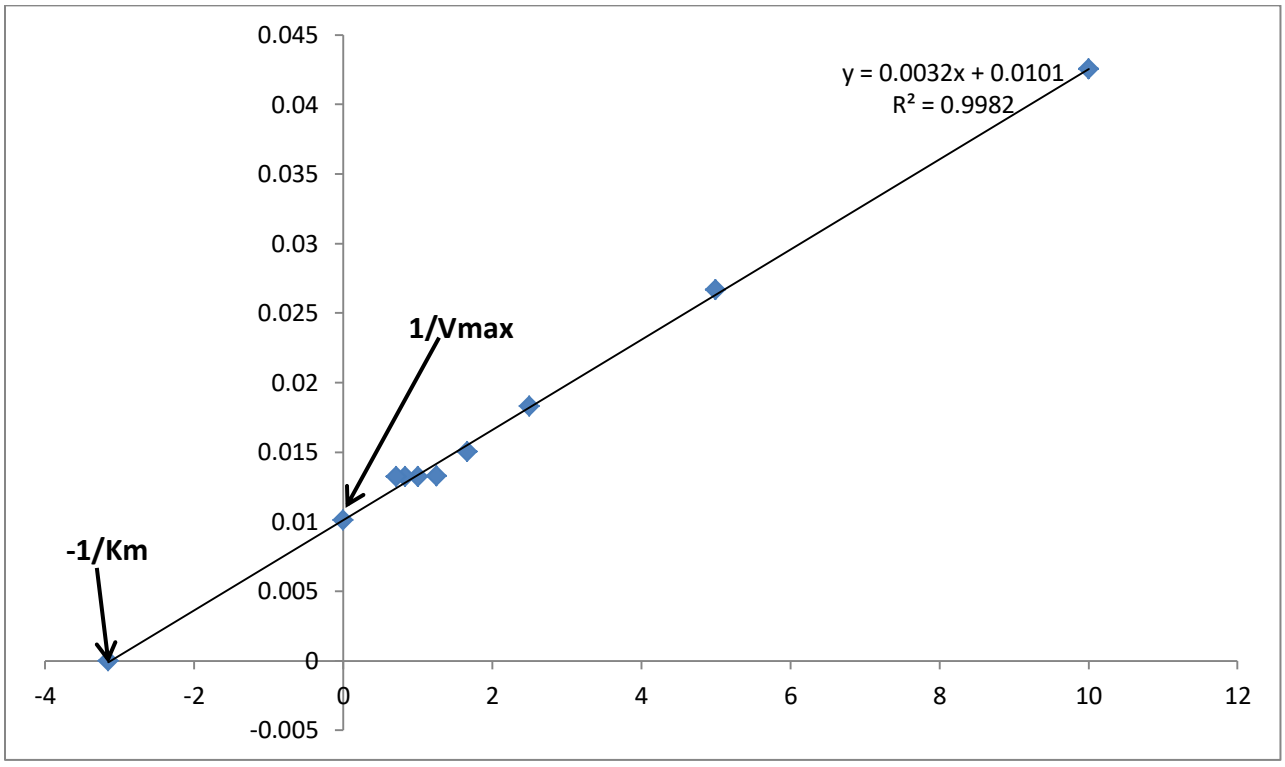
Appendix 20. Figure 3: Lineweaver–Burk plot for WR–36

Vmax=86.95 µmol $min^{-1}mg^{-1}$ Km=0.339 mg/ml ½ Vmax=43.475



Appendix 21. Figure 4: Lineweaver Burk plot for WR–39

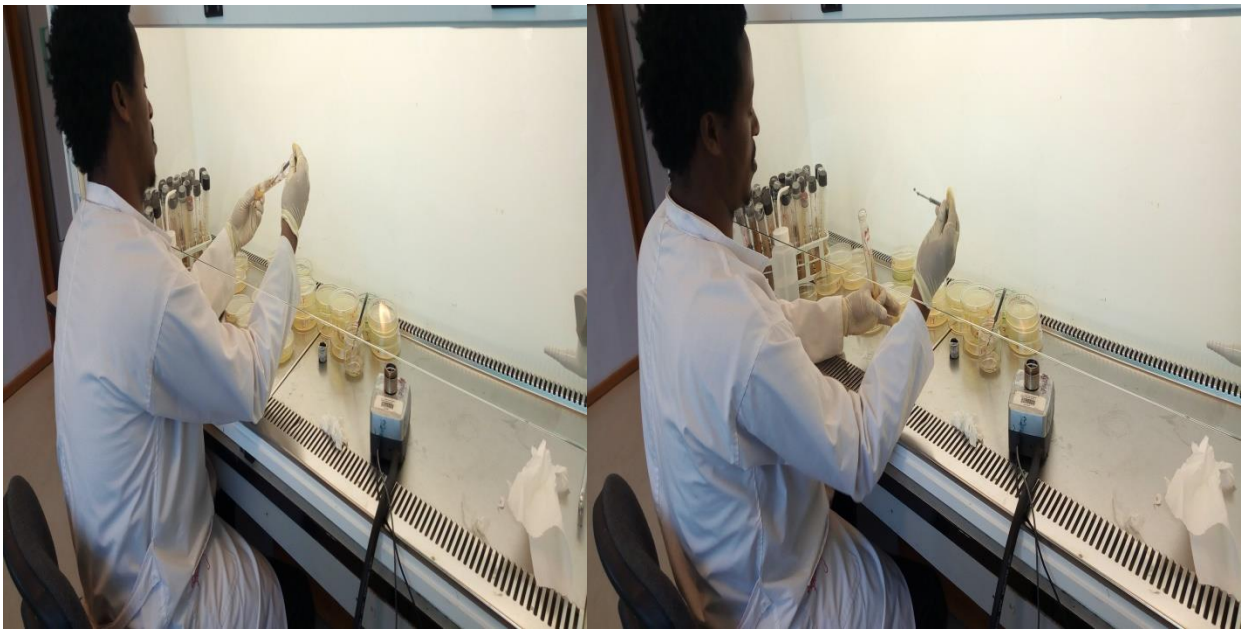
Vmax = 109.89 µmol $min^{-1}mg^{-1}$ Km = 0.527 mg/ml ½ Vmax = 54.945



Appendix 22. Fig. 5: Lineweaver–Burk plot for WR–68

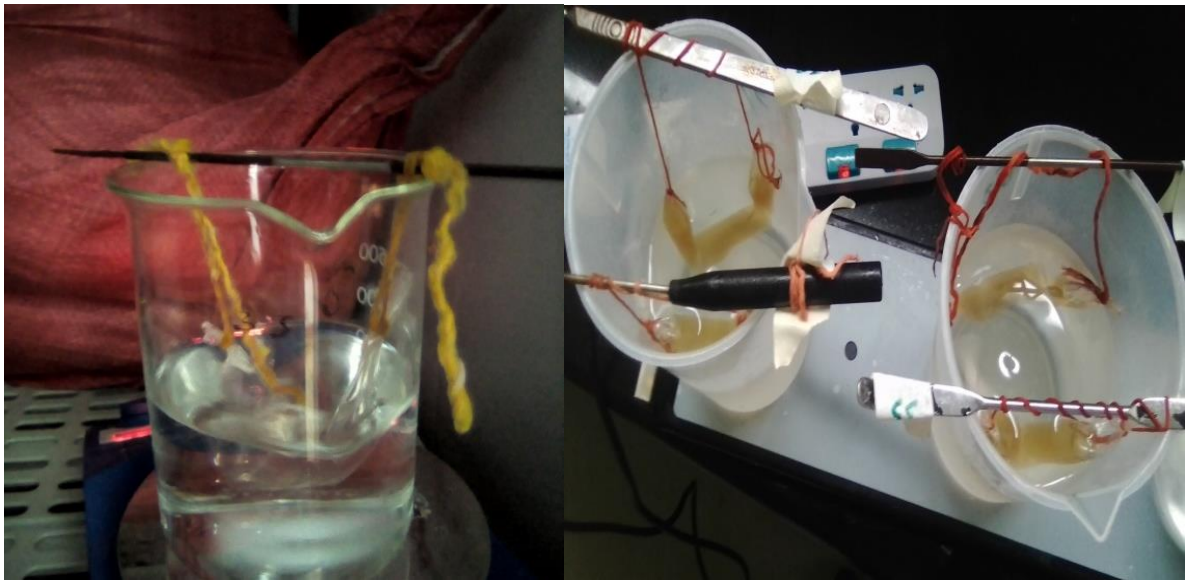
Vmax = 99 µmol $min^{-1}mg^{-1}$ Km = 0.32 mg/ml ½ Vmax = 49.5

Appendix 23. Solid state fermentation of WRF isolates

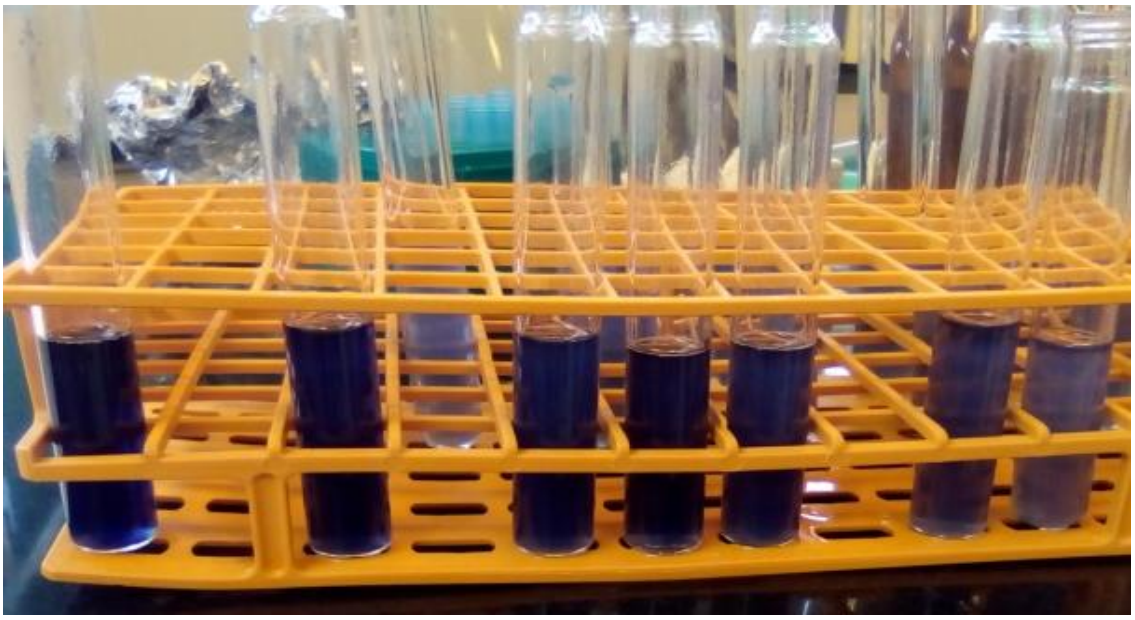


Appendix 24. Refreshing of preserved WRF isolate on PDA

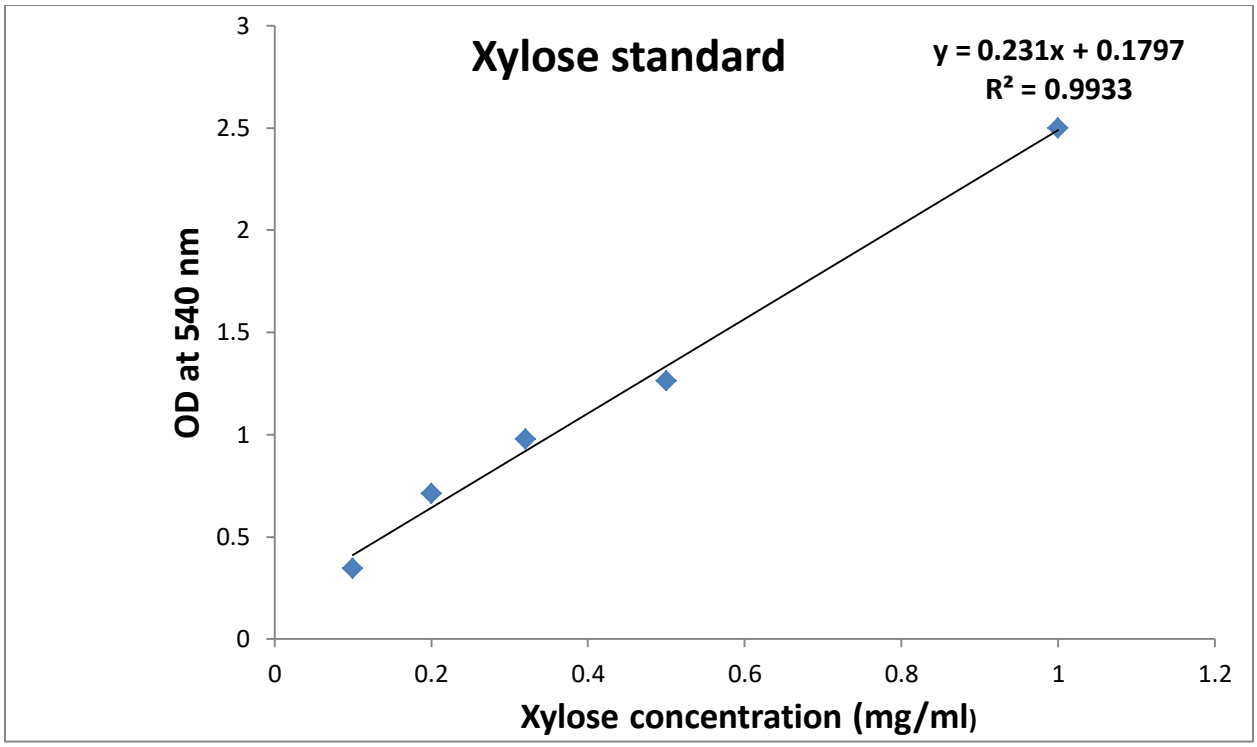
Appendix 25. Media preparation and SmF of WRF isolates on shaker Incubator



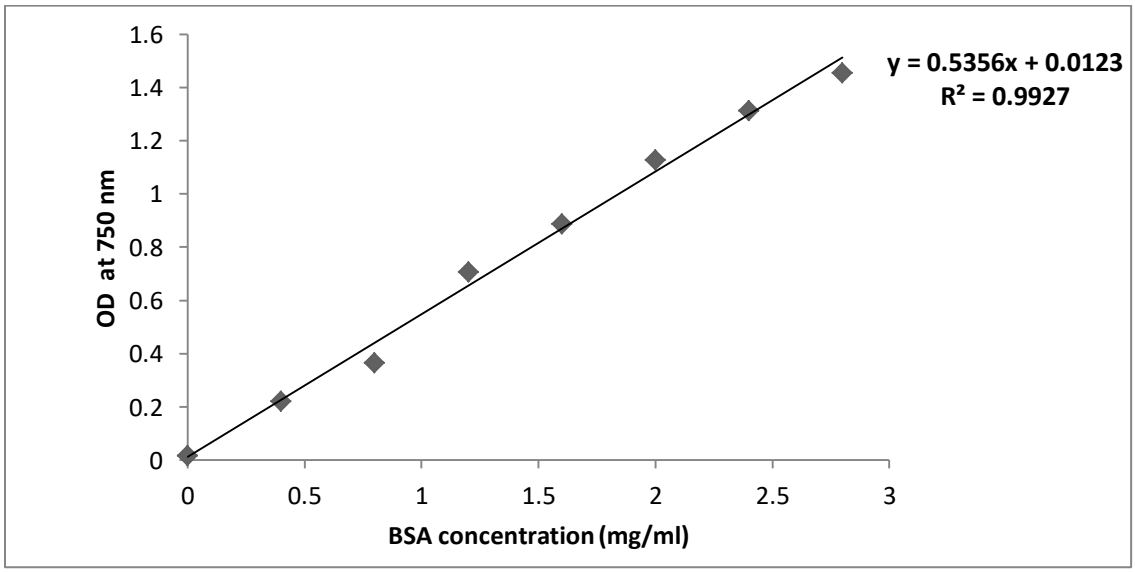
Appendix 26. Dialysis of xylanase enzyme



Appendix 27. Protein concentration determination



Appendix 28: **xylose standard curve**



Appendix 29: BSA standard curve