How to Cite: Shehzadi A, Raza A, Haider Q, Adnan M, Ali SR, Ijaz A. 2017. Xylanase; Production to Characterization and Substrate Interactions. International Journal of Applied Biology and Forensics 1(4): 110-118

REVIEW ARTICLE

Xylanase; Production to Characterization and Substrate Interactions

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Abstract

The enzyme xylanase produce the xylose by breaking beta 1, 4 xylane, which is the most abundant polysacharide after that to cellulose and a major component of plant cell wall. Xylanases (EC 3.2.1.8) have also an important role in the bioconversion of hemicelluloses into their constituent sugars. Xylanases have been produced by many microorganisms like fungi, yeast and many bacterial species by Solid State Fermentation (SSF) or submerged system method. The xylanases obtained from different sources have different molecular masses. The xylan hydrolysis occurs at optimum pH 5 for most of the fungal xylanases. Different techniques are used for purification of this enzyme such as gel filtration chromatography, ion- exchange chromatography, fast performance liquid chromatography, ammonium sulphate precipitation etc. The kinetic study of enzyme extracted from different sources was made by plotting reaction rate vs. substrate concentration to determine that whether the enzyme obeys Michaelis-Menten kinetics, and constants were determined from a Lineweaver-Burk plot. Xylanases are economically very important enzymes as they have number of applications in paper, pulp, feed and beverage industries.

Keywords: Xylanase, Xylan, *Trichoderma harzianum*, Gel-filtration Chromatography, Paper Industry.

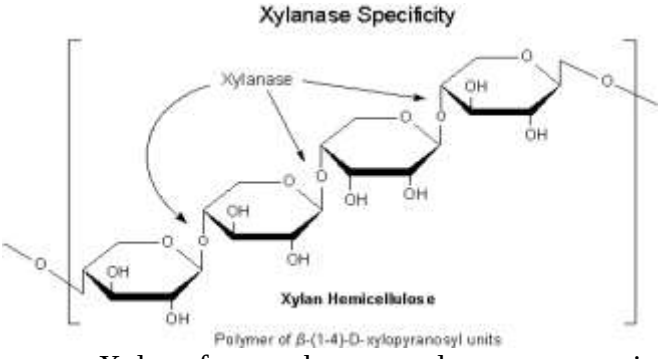
Introduction

Xylanase is the name given to the class of enzyme which hydrolyzes the plant cell wall component beta-1, 4-xylan into xylose (Beg et al, 2001). Xylan is the most abundant polysaccharide second to cellulose in a plant cell wall and a major component of cell wall that consisting on β-1, 4-linked xylopyranosyl residues which form interphase between lignin and other polysaccharides (Kulkarni et al, 1999; Beg et al, 2001). The molecular structure and hydrolytic pattern of xylanases have been extensively reported and the mechanism which is involved in the hydrolysis of xylan to xylose has also been proposed (Subramaniyan, 2002). Microbial xylanases (1, 4-β−D-xylan xylanohydrolase enzyme, EC 3.2.1.8) are the ideal catalysts for xylan hydrolysis because it has high specificity for reaction, mild reaction conditions, insignificant substrate loss, and side product generation (Coral, 2002).

The plant cell wall have 30-50% cellulose, 20-30% hemicelluloses (a group of carbohydrates in

which xylan forms the major class) and 20-30% lignin proportion (Burton et al, 2010). These three are main component of plant cell wall are closely associated with each other. The component of cell wall makes covalent and non-covalent interaction to make cell wall functional. Non covalent interaction is ionic bond between pectic homogalacturonanosyl (Caffall and Mohnen, 2009) and hydrogen bond in covalent interaction between cellulose chains or cellulose and xylan. Xylan is a heteropoly-saccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuron and α-arabinofuranosyl residues associated to the backbone of β-1, 4, -linked xylopyranose units and has properties of binding by covalent and non-covalent interactions with lignin, cellulose and other polymers (Doering et al, 2012; Kulkarni et al, 2012). Xylan, having a linear backbone of b-1, 4-linked xylose is present in all earthly plants and it accounts for 30% of the cell wall material of annual plants, proportion having 15-30% of hard woods and 7-10% of soft woods. A heteropolysaccharide xylan has O-acetyl, arabinosyl and 4-O-methyl-D-glucuronic acid substituent's (Scheller et al, 2010).

Approximately 60-70% of the xylose units perform esterification reaction with acetic acid at the hydroxyl group of carbon 2 and/or 3 and on an average every tenth xylose unit carries an a-1, 2-linked uronic acid. Many microorganisms, particularly bacteria and fungi are reported to produce xylanases typically endo-xylanases (Subramaniyan, 2002). Xylanases are genetically single chain glycoproteins, ranging from 6–80 kDa, are active on pH between 4.5–6.5 and temperature between 40 and 60 °C (Butt, 2008).



Xylan forms the second most occurring renewable polysaccharide in the biosphere besides hemicelluloses. It has been predicted that approximately 500 million tons of such material could be annually available from the residues of major crops (Rifaat, 2005). Microbial Xylanase was isolated by Biely firstly in 1985 (Krishnaveni, 2011).

Xylan is major structural polysaccharide in plants as they constitute up to 30% of the dry weight of the cell wall of monocotyledons and a minor component of the cell wall of dicotyledons. The xylanases are also having important function in the bioconversion of hemicelluloses into their component sugars (Seyis and Aksoz, 2005).

Xylanase Production

Enzymes are potential biocatalysts for a large number of reactions which enhances the rate of the reaction without being used in the particular reaction. All living things, especially animal, plant and microorganisms are sources of enzymes. However, microorganisms are the most important source of various enzymes for commercial applications of industrial enzymes (Yang et al, 2010). Microbial xylanases represent one of the largest groups of industrial enzymes and they have attracted a great deal of attention during the past few decades (Azeri, 2010).

Even though many microorganisms consisting bacteria (Yang et al, 2010) such as actinomycetes and filamentous fungi have been reported for xylanase production and few of them known as alkaliphilic or thermophilic. Many microorganisms such as bacteria and fungi easily hydrolyze xylase by synthesizing endo xylanases. Xylanases have been identified in Bacillus, Streptomyces and other bacterial species (Coral, 2002). Bacillus specie produces high levels of Xylanase activity at alkaline pH and high temperature. For example Bacillus SSP-34 produced a Xylanase activity of 506 IU/ml in the optimized medium. Another Bacillus sp. Bacillus circulans AB 16 formed 19.28 U/ml of Xylanase when developed on rice straw medium (Subramaniyan, 2002)

The optimum pH for xylan hydrolysis is around 5 for most of the fungal xylanases although they are normally stable at pH 3 – 8. Most of the fungi produce xylanases, which can bear temperature lower than 500ºC. In common, with fungi identified to be secrets xylanases have an initial

Table 1: Characteristics of Xylanases Produced by Different Microorganisms

Microorganisms	Molecular mass kDa	Optimum pH	Optimum Temperature /°C
Acrophialophora nainiana	22	7	55
Aspergillus awamori	39	5.5-6	40-55
Aspergillus nidulans	34	6	56
Aspergillus nidulans KK-99	not determined	8	55
Aspergillus oryzae	35	5	60
Aspergillus sojae	32.7	5-5.5	50-60
Aspergillus terreus	not determined	7	50
Aspergillus terreus	not determined	4.5	45
Myceliophthora sp.	53	6	75
Penicillium capsulatum	22	3.48	48
Streptomyces sp	24.5,37.5	6.0–8.0	55-60
Thermomyces lanuginosus	24.7	6-6.5	70
Trichoderma harzianum	20	5	50
Trichoderma longibrachiatum	37.7	5.0–6.0	45
Trichoderma viride	22	5	53

Butt et al, 2008

cultivation pH lower than 7. The optimum pH of bacterial xylanases is some extent higher than the optimum pH of fungal xylanases. There has been increased usage of Xylanase preparations having an optimum pH smaller than 5.5 produced invariably from fungi (Subramaniyan et al, 2000).

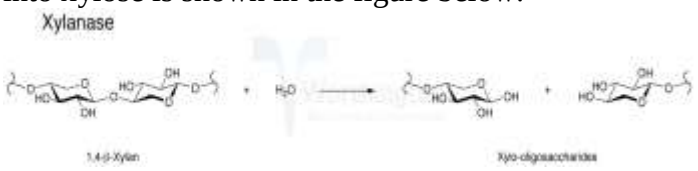
The most important enzyme for xylan conversion is endo-1, 4-xylanase (EC 3.2.1.8), which initiates the conversion of xylan into xylooligosaccharides (Muller et al, 2008). Xylanases from different sources differ in their requirements for temperature, pH, *etc*. for optimum functioning (Butt, 2007). A thermostyable xylanase was also isolated from an *Aspergillus niger* wild type strain with a molecular mass of about 36 kDa. The optimum pH for activity was found to be 7.5 at 60 °C (Coral, 2002). Most commercial xylanases are produced by *Trichoderma*, *Bacillus, Aureobasidium, Aspergillus*, *Penicillium*, and *Talaromyces* sp. (Knob, 2008).

Xylanase can also be produced by a submerged system method or Solid State Fermentation (SSF) using bacteria, filamentous fungi and yeast. The manufacture of Xylanase by the SSF method offers several advantages over the submerged system simply because of its low operational and production cost. The reduction in the production cost is due to the simple and low cost production facilities. At the same time, SSF uses inexpensive or cheap agro waste materials as substrate (Archana and Satyanarayan, 1997). The use of agro wastes not only helps to overcome the problem of solid waste management but also allows the development of biotechnological processes from cheap natural resources (Pang et al, 2006).

Xylanase production by solid state fermentation (SSF) using varying lignocellulosic substances has been reported using different fungal and bacterial strains. Solid state fermentation is widely used for fungal cultivations and few reports are available for bacterial cultivation. The medium generally used in SSF for enzyme production is wheat bran (Archana and Satyanarayan, 1997).

Mode of action of Xylanase

The mode of action of xylanase or how xylanase breakdown beta-1, 4-xylan into xylose is shown in the figure below:-



Xylanase Characterization

The characterization of xylanase from bacterial and fungal sources generally occurs as a lower molecular weight protein although higher molecular weights have also been reported. The bacterium *B. pumilus* from which xylanase was isolated is present in agricultural land soil. Extracellular xylanase was obtained from the cell-free culture supernatant of *B. pumilus* grown on Berg's mineral salt medium containing pure xylan as substrate (Monisha, 2009). Media containing wheat bran, wheat straw, rice husk, saw dust, bran of maize and gram, and groundnut as xylan source have been reported.

Xylanase obtained from *B. pumilus* ASH by fermentation, without purification was used for commercial application. However, purification would enhance the extent of its efficacy as a biobleaching agent and hence purity is warranted. The enzyme obtained had a molecular weight of 19 kDa with 3.79 fold purity by using 75% ammonium sulphate saturation. The tempo of the purity of an enzyme depends upon the molecular weight of the enzyme (Monisha, 2009).

The optimum activity of xylanase was found to be maximal at 35°C and at pH 7.0. While xylanase from a thermoalkaliphilic bacterium showed optimum activity at 50°C. Optimal action of xylanase obtained from B. *circulans* and *B. amyloliquefaciens* was at 50°C (Seyis and Aksoz, 2005). The industrial importance of an enzyme will be more if the temperature input for its optimal activity is less (Monisha, 2009).

Xylanase was also produced by *Penicillium sclerotiorum*. The strain of *Penicillium sclerotiorum* produces high levels of xylanase with low cellolytic activity. In order to check the influence of some pure carbohydrates on xylanase production different substrates are added and among them only xylan induced xylanase production. For others species of genus *Penicillium*, xylan also showed to be the best inducer. The optimum temperature for xylanase activity was 50°C. Similarly, in other studies with *Penicillium* spp., it was concluded that the optimum temperature varied between 40 and 50°C and it was active at acidic pH with an optimum at 4.5 and was stable in acid and neutral pH range. These are desirable properties for application in the pulp and paper, as well as in food industry (Knob, 2008).

Trichoderma species are also reported to produce enzymes involved in the degradation of cellulose, xylan and pectin to fermentable sugars. Among them the soft rot fungus Trichoderma has been shown to be efficient producers of xylanase. The xylanase produced from *Trichoderma harzianum* has maximal activity at optimum temperature 60°C and optimum pH 5 **(**Rifaat et al, 2005**).**

Penicillium expansum is a filamentous fungus that produces extracellular xylanase. The xylanase was stable at temperatures between 20 and 40°C. The activity dropped to less than 48% between 50 and 70°C. Thermal stability however, to higher temperatures could be increased after the addition of 1 mg/mL of bovine serum albumin (BSA) preventing the enzymatic inactivation significantly and the xylanase was stable at pH 5.5-6.5 (Querido et al, 2006). Xylanase activity was also correlated with the microorganism producing that xylanase. For example, activity of the xylanase enzyme isolated from *Enterobacter* spp. was directly correlated with the growth of that enzyme. It showed highest activity at pH 8 and at temperature 80°C (Sharma, 2009).

Two xylanases, namely xylanase I and xylanase II were isolated from *Streptomyces* sp. The apparent molecular weights of xylanase I and xylanase II were estimated by gel filtration on Bio-Gel P-60 to be 5,000 and 30,000 Da, respectively. They had similar temperature optima of 60ºC. Xylanase I had optimal pH of 5.5 while xylanase II had a slightly broader optimal pH range of 5.5-6.0. However, at the higher pH of 8.0 approximately 40% of xylanase I activity and 80% of xylanase II activity were still observed (Wateewuthajarn, 2000).

Effects of different substrates on xylanase activity

Different substrates have different effect on xylanase activity. For example the effect of different metal ions and carbohydrates on xylanase activity extracted from *Trichoderma harzianum* was observed. Among those metal ions, Mn^{2+}, Ca^{2+} and Zn^{2+} enhanced the xylanase activity whereas K+ reduced the xylanase activity. Mg^{2+} and Cu^{2+} had no significant effect on the activity. In order to investigate the effect of sugars on the activity, six

different sugars (glucose, galactose, fructose, xylose, lactose and sucrose) were studied. Among these, xylose was found to be increasing the enzyme activity slightly, whereas others decrease the activity. These results were also summarized in the table given below in table 3 (Seyis and Aksoz, 2005).

Table 2: Summary of Properties of Xylanase 1 and Xylanase 2

Feature	Xylanase 1	Xylanase 2
Molecular weight(Da)	5,000	30,000
Optimum pH	5.5	5.5-6.0
Optimum Temperature(°C)	60	60
Oat spelt xylan	5.67	1.65
Birchwood xylan	2.76	0.63

Wateewuthajarn et al, 2000

Table 3: Percentage Activity of Xylanase on Different Substrate

Metal Ions	Xylanase Activity (%)	Carbon Source	Xylanase Activity (%)
None	100	none	100
Mn	107.6	glucose	76.4
Zn	103.9	galactose	75.8
Cu	99.9	xylose	103.2
Ca	108.6	fructose	81
K	96.6	lactose	96.7
Mg	99.8	sucrose	83.1

Seyis I and Aksoz N, 2005

Xylanase Purification

Different techniques are used for purification of enzyme such as gel filtration chromatography, ion-exchange chromatography, fast performance liquid chromatography, ammo-nium sulphate precipitation etc. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can also be used for the purification of enzyme. There are also reports of purification with hydrophobic interaction column chromatography.

It also depends on the source from which this xylanase was isolated and purified. For example, gel filtration chromatography and ion-exchange chromatography are used in the purification of enzyme isolated from *Aspergillus oryzae* (Do, 2010). The purification of xylanase from *Streptomyces halstedii* takes place by fast-performance liquid chromatography (FPLC) system equipped with an anionic exchange column.

Similarly, an extracellular xylanase from *Aspergillus niger* grown on a medium containing D-xylose was purified by disc acrylamide gel electrophoresis with an apparent molecular mass of about 25 kDa using DEAE-Sephadex A-50 and Sephadex G-100 column chromatography. The xylanase was purified 14.79 fold with 29.88% recovery. Optimal temperature and pH for activity was observed at 55°C and 5.5, respectively (Yang, 2010).

Two xylanases, namely xylanase I and xylanase II from *Streptomyces* sp. were purified from culture filtrate to 13.6- and 17.3- fold of purity, respectively by ammonium sulfate preci-pitation, ion-exchange and gel filtration chromate-graphy. The apparent molecular weights of xyla-nases I and II as estimated by gel filtration were 5,000 and 30,000 Da, respectively. Both enzymes had temperature optima of 60°C. The pH optima for xylanases I and II were 5.5 and 5.5-6.0, respectively (Wateewuthajarn, 2010).

Another example of the bacterial specie which produced xylanase was thermoalkalophilic *Enterobacter* sp. The xylanase enzyme was isolated by ammonium sulfate (80 %) fractionation, and purified to homogeneity using size exclusion and ion exchange chromatography. The molecular mass of the xylanase was ~ 43 kDa. The optimal pH of the xylanase activity was 9 and the optimal temperature for the enzyme activity was 100 ºC at this pH (Khandeparkar, 2006).

Xylanase Kinetics

To do the kinetic study of enzyme extracted from different sources reaction rate vs. substrate concentration curve was plotted to determine

whether the enzyme obeys Michaelis-Menten kinetics, and constants were determined from a Lineweaver-Burk plot. For example, Km value and Vmax value of the xylanase produced by *Arthrobacter* sp.was determined. The Km value was obtained from Lineweaver-Burke plot of xylanase activity at 100 °C using various concentrations of xylan as substrate. The Km of xylanase for wheat bran was 0.9 mg/ml, and the Vmax was 3571 µmol min-1 mg-1 (Khandeparkar 2006).The kinetic study of the xylanase enzyme enzyme extracted from *Escherichia coli* was done.The simplified batch fermentation kinetic models based on Logistic and Luedeking-Piret equations which have been described elsewhere were used to describe growth of *E. coli* and xylanase production, respectively.

Cell Growth:

$$\frac{dx}{dt} = [\mu\max(1 - \mathrm{X}/\mathrm{Xmax})]\mathrm{X}$$

Substrate Consumption:

$$\frac{-dS}{dt} = \frac{\frac{1}{YX}}{\mathrm{S}\left[\frac{\mathrm{dX}}{\mathrm{dt}}\right]} + \mathrm{ms.\,X}$$

Xylanase Production:

$$\frac{\mathrm{dP}}{\mathrm{dt}} = \alpha\left[\frac{\mathrm{dX}}{\mathrm{dt}}\right] + \beta\mathrm{X}$$

Where:

X = The cell concentration (g /L)
Xmax = The maximum cell concentration (g/ L)
µmax = Maximum specific growth rate (1/h)
S = The substrate concentration (g/ L)
Yx/s = Yield coefficient of cells on carbon substrate (g /g)
ms = Maintenance coefficient (g /gh)
α = Growth associated xylanase production coefficient (U mg /cell)
β = Non-growth associated xylanase production coefficient (U mg /cell h).

Equations were fitted to the experimental data by non-linear regression through Marquant algorithm using MATLAB software (matlab release 14.0, mathworks, USA). The model parameter values were evaluated by solving these equations with the computer program applied as a search method to minimize the sum of squares of the differences between predicted and calculated values. The predicted values were then used to simulate the profiles of cell, substrate and product formation during the fermentation process (Rusli and Mohamed, 2009).

Xylanase Applications

In recent years, important applications for xylanases in different industrial processes have been found. Xylanases are fast becoming a major group of industrial enzymes finding significant application in paper and pulp industry. Xylanases are of great importance to pulp and paper industries as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent (Subramaniyan, 2002). The applicability of xylanases increases day by day as Rayon, cellophane and several chemicals like cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethyl cellulose, methyl and ethylcellulose) are all produced from the dissolving pulp i.e. the pure form of cotton fiber freed from all other carbohydrates (Subramaniyan, 2002).

Commercial applications of xylanases demand the identification of highly stable enzymes that remain active under routine handling conditions. Many advantages, such as reduced contamination risk and faster reaction rates, have been proposed for the use of thermophiles in biotechnology processes. In general, parameters such as temperature, pH, and enzymatic stability are important for the industrial applicability of any enzyme (Kulkarni et al, 1999).

The importance of xylanases is not bound to the paper and pulp industry and there are other industries with equal importance where xylanase has several applications. Potential applications of xylanases also include bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in consistency of beer and the digestibility of animal feed stock (Tsenga et al, 2002). Application of xylanase in the scarification of xylan in agro wastes and agro foods intensifies the need of exploiting the potential role of them in biotechnology. In all these cases xylan hydrolysis forms a chief factor. Most of the studies on xylanases were focused on single aspect of xylanase technology.We should also consider other aspects because it is necessary for the analysis of future

exploitation of xylanase technology (Subramaniyan, 2002).

Potential application of xylanases in biotechnology include biobleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuels. Only during 1980's the great impact of xylanases has been tested in the area of biobleaching. Future work on the application of these enzymes in paper and pulp, food industry, in environmental science i.e. bio-fuelling, effluent treatment and agro-waste treatment, etc. require a complete understanding of the functional and genetic significance of the xylanases (Subramaniyan, 2002).

Xylanases are extensively used in pre-treatment of forage crops and other lignocellulosic biomass, added to swine and poultry cereal based diets to improve nutrient utilization, flour modification for bakery products and saccharification or agricultural industrial and municipal wastes. Xylanases have been widely used for clarifying fruit juices, wine and also in food processing in combination with celluloses2 and in improving the nutritional properties of agricultural silage and grain feed (Krishnaveni, 2011).

Hemicellulase and endoxylanase enzymes have been extensively studied, since they hydrolyse polysaccharides in the pulp of woods. An attractive application of this hydrolysis process is the removal of xylan from wood pulp for manufacturing of dissolved pulp. The most important enzyme needed for enhancing the bleaching of pulp is endo-β-xylanase. Xylanases enhance the cleaving of reprecipatated xylan formed on the outer surfaces of the cellulose fibers after pulping. This causes increased permeability of the pulp fibers to the bleaching chemicals and allows the passage of larger fragments of residual lignin out of the pulp. (Rifaat, 2005).

Xylanases are hydrolytic enzymes, which can completely hydrolyse xylan. Due to this property xylanases have wide application in the bioconversions of lignocellulose to sugar ethanol clarifying juices and wines, extraction of plant oils coffee and starch and improving nutritional value and green feed and in bread making. Cellulose free xylanases have applied as bleaching agents in pulp (Olempska, 2004)

For commercial applications, xylanases should ideally be produced quickly and in large quantities from simple and inexpensive substrates. Natural xylan sources such as agricultural and forestry wastes, paper industry wastes and various fruit wastes are potential raw materials for xylanase production (Ratanachomsri et al, 2006). Among these, food industry wastes contain high amount of xylan, as it is one of the main polymers in the plant cell wall. These wastes are potential raw material for xylanase production and as xylanases have a wide range of application, economical production of these enzymes is of great importance (Seyis and Aksoz, 2005).

Conclusion

Xylanases are genetically single chain glycoproteins, ranging from 6–80 kDa, are active between pH 4.5–6.5 and at temperature between 40 and 60 °C. Although xylanases have been produced by many different microorganisms but microbial xylanases (1, 4-β−D-xylan xylanohydrolase, EC 3.2.1.8) are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss, and side product generation. Most commercial xylanases are produced by *Trichoderma*, *Bacillus*, *Aspergillus*, *Penicillium*, *Aureobasidium* and *Talaromyces* species.

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