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A Review of xylanase production by Aspergillus niger AD-81 using saccharum munja and municipal solid waste as substrate under submersed state fermentation

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Abstract

Hemicelluloses are a group of heteropolysaccharides which accounts for 33% by dry weight of the total lignocellulosic biomass. Xylan is one of the most important and abundantly found hemicellulose which has diverse structures according to the source from which it is extracted. Due to its structural diversity, xylan is hydrolysed by a class of xylanase enzymes into its monomeric xylose subunits. These xylose residues when treated with yeasts get converted into ethanol which can be used as a supplement along with natural fuels. xylanase production by biological means not only reduces the cost of production but also decreases the formation of inhibitors which are formed during chemical pretreatment of xylan that hampers the process of fermentation. Besides bioethanol production xylanases have also got many other productive uses in various industries such as paper pulp bleaching, oil extraction, food additives, bakeries, detergents, fodder industries, etc. Twelve strains were isolated from environmental samples like cow dung, leaf litter, waste water, soil, termites, etc.

Keywords : *Hemicellulose ,xylanase , lignocellulosic*

Introduction

Enzymes are proteins that act as organic catalysts i.e., they speed up a biological reaction without getting used up in the reaction. Based upon the reactions they are involved in, the enzymes are broadly classified as oxidoreductases (involved in the oxidation-reduction reactions), transferase (transfer a functional group from one moiety to another), hydrolase (involved in the hydrolysis), lyase (involved in the lysis), isomerase (catalyze isomer formation), ligase (involved in the ligation reaction), and translocase (catalyze the transport across cell membrane).

Xylanase

Xylanases (E.C.3.2.1.8) are hydrolases that catalysis the hydrolysis of plant xylan (Sharma and Sharma, 2016). The complete breakdown of xylan into xylooligosaccharides and xylose involves the action of several xylanolytic enzymes including endo-1,4-xylanases (1,4- β –xylan xylanohydrolase; EC 3.2.1.8) and β -D- xylosidase (1,4-b-xylan xylohydrolase; EC 3.2.1.37) (Gomez et al., 2008; Juturu and Wu, 2014). Xylan being an important constituent of the plant cell walls, the action of xylanase, thus, helps to dissolve plant cell walls during several physiological processes such as seed germination.

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Classification of xylanases

The xylanases were classified into two groups based upon the molecular mass and the Isoelectric pH (pI) of the enzymes with a cut off of pI 7.0 and 30kDa (Wong et al., 1988). However, many xylanases esp., fungal xylanases did not conform to this style of classification and the need for a new classification system arose. To overcome the shortcoming, the xylanases were classified based upon the structural differences in the primary conformation of the enzymes (Henrissat et al., 1993). The classification system also considered the substrate specificity and was used to classify all glycosidases in general. Endoxylanases were grouped into two different families that differ in their structure as well as the catalytic properties (Biely et al., 1997).

Substrate Xylan

Xylan is a branched heteropolymer of β-1, 4-linked D-xylopyranosyl residues (Figure 1). The xylans are the chief component of the secondary xylem. The primary wood of monocots also carries xylans as a major component, while the primary wood of dicots has a low abundance. Xylan in combination with polymers such as lignin and cellulose contributes to the cell wall integrity (Kulkarni et al., 1999; Beg et al., 2001).

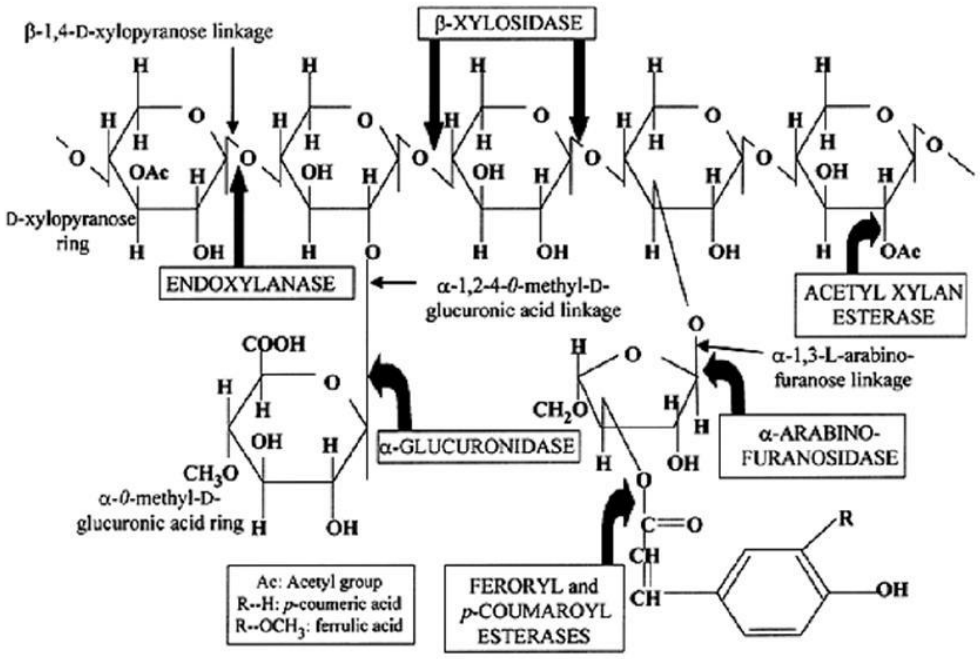

Figure 2.1. Structure of plant xylan showing different substituent groups with sites of attack by microbial xylanases

The 3D structure of its molecule varies with its source (Aspinall, 1980). The side chain of xylan determines the physical and chemical properties of the molecule as well as the mode and extent of enzymatic cleavage (Shallon and Shoham, 2003). Acetyl, arabinosyl, and glucuronosyl are

common residues on its backbone (Bastawde, 1992). Among these, the acetyl group confers some degree of polarity to the otherwise water-insoluble molecule. Apart from the common side-chain residues, arabinosyl, galactosyl, glucuronosyl, and xylosyl residues are also seen in various xylanases (Aspinall, 1980; McNeil et al., 1984). The rhamnosyl, galacturonosyl, and xylosyl residues are found in both the soft and hardwoods (Andersson et al., 1983) whereas, β-(1,3)-linked-arabinofuranose units are found only in the softwoods (Puls and Schuseil, 1993). The Xylans of grasses are generally arabinoxylans (Wilkie, 1979; Aspinall, 1980), while homopolymer of xylosyl residues was found only in the esparto grass (Chanda et al., 1950) and tobacco (Eda et al., 1976).

Industrial production of Xylanases

Condition optima

Optimum pH and temperature conditions

The pH of the culture medium and its incubation temperature are crucial abiotic factors that have a direct bearing on the efficiency of the xylanase production. The effect of the pH on the enzymatic activity of an enzyme, when plotted, gives a bell shape curve. The peak of the bell curve is the pH optima for the particular enzyme. The bacterial xylanases have slightly higher pH optima as compared with the fungal counterparts. The xylanase enzyme is active in the range of pH 2.0 to pH 9.0. However, maximum xylanase activity is generally obtained when the pH of the nutrient medium is adjusted to 5.0. The xylanases obtained from alkalophilic bacteria are selected to show high enzymatic activity at a higher pH range. The fermentation process itself changes the pH of the medium with the progression of time as a result of chemical changes in the medium i.e., uptake of the substrate and secretion of the secondary metabolites in the medium. It is, therefore, very important to constantly monitor the pH of the bioreactor during the progression of the fermentation.

Enzymes are also very sensitive to change in the incubation temperature of the fermentation. An increase in the temperature increases the activity of an enzyme that reaches its peak and decline thereafter. The peak at which an enzyme shows its maximum activity is called temperature optima of that particular enzyme. The xylanases are reported to be active over a broad range of temperature and the optimum temperature for an enzyme depends upon its origin. Most xylanases have an optimum temperature below 50^{o}C (Subramaniyan and Prema, 2002). The temperature optima for the fermentation process is 30^{o}C

Inoculum size

Inoculum size is defined as the colony-forming units or spores per volume of the medium inoculated as the starting culture. The inoculum size must be sufficient enough to colonize all substrate particles (Sikyta, 1983). The size of an inoculum decides the rate of the fermentation process. The recommended inoculum size for *Aspergillus awamori* was 1.5x108 spores/ml (Smith and Wood, 1991).

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Aeration

The fermentation reaction needs to be carried out strictly according to the behavior of the microbe to be cultured. The industrial production of xylanase needs proper aeration of the medium, that involves the constant supply of oxygen with constant agitation to facilitate uniform mixing of the gas in the liquid medium.

Biotic source of enzyme

The natural sources of xylanases are microbes such as bacteria (Sanghi et al., 2007; Kiddinamoorthy et al., 2008), and fungus (Ninawe et al., 2008; Nair et al., 2008; Sharma and Sharma, 2013) including yeast (Liu et al., 1998). Besides, some marine algae, protozoa, crustaceans, insects, snails, and plants also secrete xylanases (Sunna and Antranikian, 1997). Still, the bacterial and fungal sources are among the most exploited to isolate the xylanases. The use of microbes as a biotic source of xylanase is preferred over the use of the plants and animals because of the availability, structural stability, and easy genetic manipulation of the microbes. The xylanase producing microbes have inherent differences in their ability to produce the enzyme, its activity, and the spectrum of different xylanases (Hinz et al., 2009). The search for more xylanase producing organism is needed in parallel with the development of the methods for the improvement of the existing strains (Narasimha et al., 2006). The naturally occurring strains may or may not become compatible with the fermentation process used in the industries (Ahmed et al., 2009).

Xylanases of fungus origin

The fungi are the organism of choice for the industrial production of the xylanases. *Trichoderma, Streptomyces, Aspergillus, Fusarium, Phanerochaete, Fiberobacter, Clostridium, Bacillus, Pichia,* and *Penicillium* fungi have been reported to secrete xylanases in high yields (Collins et al., 2005; Nair and Shashidhar, 2008). The xylanase producing fungi are summarized in table 2.2. The xylanase has been successively produced from thermophilic fungus, *Myceliophthora thermophila* SH1 (Sharma and Sharma, 2013). *Aspergillus niger* was used to produce xylanase enzyme using various carbon sources (Okafor et al., 2007), and *Trichoderma* using sugar cane bagasse and palm kernel cake (Pang et al., 2006). The optimum activity of the fungal xylanases is reported at less than 50^{o}C temperature and slightly acidic pH (Beg et al., 2000a). The fungal xylanase is reported to have high enzymatic activity. The xylanases of the fungal origin are known to be expressed in various isoforms of the enzyme. These isoforms differ in their structures, productivity, and specificity besides having different physicochemical properties (Ghotora et al., 2006). The loci controlling the expression of the xylanase enzyme are either polycistronic or exist in multiple copies (Collins et al., 2002). The genetic redundancy in the genome coupled with the variable post-translational processing results in the polymorphism of the xylanases exhibited by different fungal strains (Wong et al., 1988). *A. niger* for example is reported to possess 15 isoforms of xylanases while *T. viride* posses 13 isoforms of the enzyme.

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Advantages of the fungal xylanases

Using fungus for xylanase production has its advantage over the bacteria and yeast (Polizeli et al., 2005). Since the fungus secretes the enzyme in the medium, thereby, saves on the cost involved in cell disruption and therefore, proves economic (Sunna and Antranikian, 1997; Polizeli et al., 2005). The cell disruption procedure may compromise with the quantity of the protein obtained. The bacterial and the yeast xylanases, however, remained confined within the protoplasm or the periplasmic boundary (Knob et al., 2010). Moreover, the xylanase enzyme produced by fungi is not subjected to post-translational modifications (Polizeli et al., 2005) and are more specific (Knob et al., 2010).

Fungus in industrial production of xylanases

Among all the fungi known to produce xylanases, *Aspergillus niger* has been the most studied fungus, grown on various substrates, and various types of bioreactors such as submerged and solid-state fermentations. While at higher temperatures, *Thermomyces lanuginosus*, *Thermoascus aurantiacus*, *Talaromyces thermophiles*, and *Myceliophthora thermophila* are widely studied (Maalej et al., 2009; Milagres et al., 2004; Moretti et al., 2012; Yang et al., 2006). Most commercial applications involving the degradation of xylan involves the use of the genetically engineered strains of *Trichoderma* or *Aspergillus* spp. (Mussatto and Teixeira, 2010).

Xylanases of plants origin

Plants are not a good source of xylanases though an endoxylanase has been isolated from Japanese pear fruit at the time of maturation (Yamaura et al., 1997). In addition to the Japanese pear fruit, xylanases have also been reported to occur in immature cucumber seeds and germinating barley (Sizova et al., 2011).

Xylanases of animal origin

Like plants, the animals are not a good source of the xylanases, though some animals like mollusk can also produce xylanases (Yamaura et al., 1997).

Over-expression of xylanase

The over-expression of xylanases involves the expression of a xylanase encoding gene under the influence of a strong promoter in a homologous or a heterologous host.

Media supporting the growth of a microorganism

The choice of medium to grow microorganisms is an important confounding factor that contributes to the over-production of an enzyme. The medium, being a source of various nutrients esp., carbon and nitrogen, is very important for deciding the growth of a microbe at optimum temperature, pH, and oxygen concentration. The carbon, hydrogen, and nitrogen are important elements that contribute to the backbone of the majority of the organic macromolecules. Besides carbon, hydrogen, and nitrogen, the inorganic salts, water, vitamins,

and other growth factors, precursors of fermentation products are other important constituents supplied by the fermentation medium. Metal ions, chelators, detergents, and surfactants are reported to be important for xylanase production. The metal ions are known to induce the production of xylanase (Saxena et al., 1994).

A poor choice of the fermentation medium may cost the cellular growth, multiplication, and thus, the yield of the desired product. The choice of the growth medium depends upon the microbe used for the enzyme production or the fermentation behavior. The agriculture wastes such as wheat bran, wheat straw, corn cobs, sugarcane bagasse, cassava bagasse, barley bran, and rice straw are a cheap medium for the xylanase production that also acts as an inducer for the enzyme synthesis (Beg et al., 2000b; Dhillon and Khanna, 2000).

Nitrogen source is important for protein synthesis (Kulkarni et al. 1999). Xylanase production was reported to increase with a decrease in the protease peptone. In the case of the fungus *Fusarium oxysporum*, modified Mandel and Sternburg's medium containing 0.14% Ammonium sulfate, 0.03% urea, and 0.05% protease peptone supported maximum yield of xylanases (Christakopoulos *et al.*, 1996).

Overexpressing strains

The xylanase production may be increased in microbes employing mutagenesis (Deshpande et al., 2008) or via genetic modification (Otte and Quax, 2005) of a microbe. Various xylanases encoding genes of various microbes are presented in table 2.3. The over-expression of the xylanase via recombinant DNA technology reduces the cost of the production of the enzyme (Juturu and Wu, 2011). The recombinant gene clones have been transformed into homologous as well as the heterologous hosts for the commercial production of xylanases. The expression of recombinant xylanases in the homologous, as well as the heterologous hosts, is thoroughly reviewed by Ahmed et al. (2009). The xylanases of the fungus origin have been successfully transformed into various heterologous hosts such as *Escherichia coli* (Yi et al., 2010; Xie et al., 2012; Le and Wang, 2014), *Pichia pastoris* (Damaso et al., 2003; He et al., 2009; Driss et al., 2012; Fan et al., 2012), and *Saccharomyces cerevisiae* (la Grange et al., 2001; Tian et al., 2013).

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S.No.	Organisms	Cloned genes	Authors
1.	*Streptomyces* sp. S9	xynAS9	Li et al., 2008
2.	*Paenibacillus* sp. strain W-61	xyn1	Watanabe et al., 2008
3.	*Bacillus subtilis*	xynA	Bai et al., 2007
4.	*Bacillus* sp.	Xyl	Lu et al., 2006
5.	*Bipolaris sorghicola*	xyn11A xyn11B	Emami & Hack, 2002
6.	*Cochliobolus sativus*	xyl2	Emami & Hack, 2002
7.	*Cochliobolus- heterotrophus*	xyn11A	Emami & Hack, 2002
8.	*Fusarium oxysporumf*	xyl4 xyl5	Gómez et al., 2001
9.	*Claviceps purpurea*	cpxyl1 cpxyl2	Giesbert et al., 1998
10.	*Magnaporthe grisea*	xyl1	Wu et al., 1995

Table 3. Xylanase encoding genes of different microbes(Source Mendal, 2015)

Substrate for xylanase

Xylanase acts upon hemicellulose, which is a chief component of lignocelluloses. Plants provide the lignocellulosic material that can be used to grow the xylanase producing microbe. The economy of the xylose production is largely controlled by the choice of the source of carbon. The straw of wheat and rice, bagasse of sugarcane, and the cobs of corn provide an excellent substrate for the xylose production. Among these, wheat bran was declared best for bacteria, *Paenibacillus polymyxa* (CKWX1), and *Streptomyces* T-7 (Keskar et al., 1992).

The sugarcane bagasse has been reported to induce the xylanase production in marine LAMAI 31 strain of *Aspergillus* cf. *tubingensis* (Dos Santos et al., 2016). In addition, the sugarcane bagasse was also found to the best carbon source for marine NIOCC strain of *A. niger* and LPB 326 strain of *Aspergillus niger* (Raghukumar et al., 2004; Maciel et al., 2008).

Lopes et al. (2018) assayed various enzymes including endo-β-1,4-xylanase and β-xylosidase of six different strains of filamentous fungi grown on sugarcane bagasse. Among the six strains, *Aspergillus japonicus* Saito was found to have maximum endo-β-1,4-xylanase and β-xylosidase activity. Growth on sugarcane bagasse had a positive stimulus on the specific activity of the two enzymes, the specific activity was higher than those reported for the xylanase of *Aspergillus niger* and *A. flavus* on different carbon sources by Guimaraes *et al*. (2013). The high specific activity of β-xylosidase was also reported in *Aspergillus phoenicis* when grown in sugarcane bagasse as a sole carbon source (Rizzatti et al., 2001). The activity was almost double the activity of the same enzyme in the same species at the same conditions when grown

on maize pith as sole carbon source. It may be worth-mentioning over here that the enzymes, endo-β-1,4-xylanase and β-xylosidase of *A. japonicus* Saito exhibit maximum activity at a lower temperature (50°C) when grown on liquid sugarcane bagasse (Lopes et al., 2018) as compared with other carbon sources showing maximum activity at 60 °C - 70 °C (Wakiyama et al. 2008; Semenova et al., 2009). The optimum pH was constant for the enzymes, endo-β-1,4-xylanase (pH 5.5), and β-xylosidase (pH 3.5-4.5) in different substrates (Lopes et al., 2018).

Bioreactors/methods used

The quantity and quality of the xylanase production is a factor of the use of suitable strains of the microbe, the media used and the method adopted for the fermentation. Intelligent choice of the fermentation medium is crucial for decent production of the enzyme since the growth medium supplies nutrients for growth, energy, the building of cell substances, and biosynthesis of fermentation products. The type of bioreactor is another important factor for deciding the output of fermentation since it directly influences the ability of a microbe to utilize the provided substrate.

The biosynthesis of xylanase involves fermentation by submerged fermentation or the solid-state fermentation (Gawande and Kamat, 1999; Kansoh and Gammal, 2001). The microorganisms behave differently in both the systems and the protocol needs to be precisely planned as the metabolism of the particular organism in the particular system (Subramaniyam and Vimala, 2012).

Between the two methods of fermentation, the submerged fermentation provides an added advantage of sufficient nutrient and oxygen availability to the organism plus it is quicker than the solid-state fermentation (Bim and Franco, 2000; Gouda, 2000). The, therefore, is a method of choice for by nearly 90% of the industries (Polizeli et al., 2005). The submerged fermentation involves the submergence of the microbe in the substrate and substrate is never a limiting factor in this type of fermentation (Knob et al., 2014). The process yields a high expression at affordable costs (Krishna, 2005). The limitations of the process are the requirement of large space, more energy, and water inputs (Jain et al., 2013).

There is no dearth of studies that reported the use of solid-state fermentation using cheap substratum like, rice husk, rice straw, corncob, and corn stalk (Yang et al., 2006; Heck et al., 2006). The solid-state fermentor has a solid matrix meant to provide physical support as well as nutrient medium (Pandey et al., 2000; Singhania et al., 2009). The process of solid-state fermentation has the advantage of providing natural conditions for the growth of the microorganisms in addition to having lesser water input. The protein output of the solid-state fermentation is higher than that of the submerged fermentation (Ayyachamy and Vatsala, 2007; Jain et al., 2013; Singhania et al., 2009). The solid-state fermentation procedure, however, it is troublesome to scale up the procedure (Holker and Lenz, 2005; Khanahmadia et al., 2006; Singhania et al., 2009).

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