# Xylanase from *Trichoderma harzianum*: Enzyme Characterization and Gene Isolation

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**Summary**: Xylan is the major component of hemicellulose and should be fully utilized to improve the efficiencies of a biobased economy. Xylanases have received great attention in the development of environment friendly technologies in the paper and pulp industry. The use of xylanases could greatly improve the overall lignocellulosic materials for the generation of liquid fuels and chemicals. *Trichoderma harzianum* E-58 was grown in Vogel's medium with different carbon sources such as glucose, maltose, carboxymethylcellulose (CMC), wheat bran and xylan at 28 °C with constant shaking at 150 rpm to check the induction of xylanase. Expression of xylanase was induced maximally by xylan and repressed by glucose. The pH and temperature optima of the crude xylanase were 5 and 60 °C, respectively. The apparent $K_m$ and $V_{max}$ values of the crude xylanase using Birchwood xylan as a substrate were 2 mg m/ $L^{-1}$ and 3.35 µmol $min^{-1}$ $mg^{-1}$, respectively. RNA was isolated from mycelia of *T. harzianum* by using Trizol reagent. cDNA was synthesized by using cDNA synthesis kit. The subsequent PCR products were purified through agarose gel electrophoresis and ligated into pUC18. The recombinant vectors containing the xylanase gene were transformed into *E. coli* DH10B for characterization.

## Introduction

Hemicelluloses represent about 20-30 % of lignocellulosic biomass. The most abundant hemicellulosic polymers are xylans, made up of β-1,4-linked xylose units. Xylan represents an immense resource of biopolymers for practical applications accounting for 25-30 % of the dry biomass of woody tissues of dicots and lignified tissues of monocots and occurs up to 50 % in some tissues of the cereal grains [1].

Xylan has a high potential for degradation to useful end products. Hydrolysis of xylan requires an action of different enzymes. Mainly two enzymes are responsible for the main chain cleavage i.e. endo-β-1,4 xylanase (E.C.3.2.1.8) cleaves the backbone to xylooligosaccharides, and β-xylosidase E.C.3.2.1.37) hydrolyzes them to D-Xylose [2].

One of the most important large-scale biotechnological applications in recent years is the use of xylanases as bleaching agents in pulp and paper industry [3]. The xylanases assume special importance in the paper and pulp industry as they replace toxic chemicals such as element chlorine and chlorine dioxide for developing eco-friendly processes. Xylanase pretreatment has been reported to lower bleaching chemical consumption and to result in final brightness [4]. Xylanases are also widely used in the manufacture of bread, drinks, textiles and waste treatments [5]. In the animal food industry, xylanases are valuable as a natural means of improving feed utilization and controlling pollution through reducing animal wastes [6]. Currently, xylanases and cellulases together with pectinases account for 20 % of the world's enzyme market [7]. Many different microbial genera, ranging from bacteria to fungi, have been found to produce one or several xylanases [8]. Filamentous fungi have been used for more than 50 years in the production of industrial enzymes [9]. They are particularly interesting producers of xylanases and excrete much higher xylanolytic enzymes into the medium than bacteria or yeast [5].

*Trichoderma* species are reported to produce enzymes involved in the degradation of cellulose, xylan and pectin to fermentable sugars [10]. Among the fungi, the soft rot fungus *Trichoderma* has been shown to be efficient producers of xylan degrading enzyme activity [11-12]. *Trichoderma harzianum* E-58 has a highly active cellulose-xylanase system, which is specific for a wide range of lignocellulosic substrates [12].

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It is difficult to obtain a pure form of a particular enzyme from a fungal preparation. Recombinant DNA technology, that enables to analyze a single gene product, can be applied with more success [13]. Genes encoding xylanases have been cloned in homologous and heterologous hosts with the objectives of overproducing the enzyme and altering its properties to suit commercial applications [14]. Although *Trichoderma harzianum* is an industrially important producer of hemicellulolytic enzymes, little is known about its different endo-xylanases at molecular level.

In this paper, we have reported substrate induction, some properties of crude xylanase and isolation of a xylanase encoding gene from a mesophilic fungus *Trichoderma harzianum* E-58.

**Results and Discussion**

*Effect of Various Carbon Sources on Xylanase Production*

Since the cost of the substrate plays a crucial role in the economics of an enzyme production, therefore different lignocellulosic substrates utilized by *Trichoderma harzianum* for xylanase production were compared. *Trichoderma harzianum* was grown in Vogel's medium with various carbon sources including glucose, maltose, carboxymethylcellulose, xylan and wheat bran and the expression of xylanase was studied (Table 1).

Table 1: Xylanase concentrations produced from *T. harzianum* grown on different carbon sources

| S.No | Carbon source (1%) | Xylanase Activity (IU $mL^{-1}$) Mean ± S. D |
|---|---|---|
| 1 | Glucose | 0.05 ± 0.001 |
| 2 | CMC | 3.2 ± 0.04 |
| 3 | Maltose | 0.4 ± 0.008 |
| 4 | Wheat bran | 4.1 ± 0.06 |
| 5 | Oat spelt xylan | 4.2 ± 0.03 |
| 6 | Birch wood xylan | 5.5 ± 0.01 |

Among the lignocellulosic materials tested as carbon source, birch wood xylan was far more effective for xylanase production. Oat spelt xylan and wheat bran exhibited moderate activities, whereas the other carbon sources resulted in lower activities. Xylanase induction was subjected to glucose repression. Effect of different carbon sources on enzyme production was also studied at various time intervals (Fig. 1). When maltose was used as a carbon source, xylanase activity was detected up to 48 hours, after which there was a decreasing trend. Glucose repressed the xylanase activity completely. In the presence of carboxymethylcellulose (CMC), xylanase activity increased significantly up to 48 hours, after which the activity decreased and reached to a base line after 144 hours. Xylanase activity with wheat bran and oat spelt xylan reached maximum at 120 hrs. The highest xylanase activity was also observed at 120 hours with birch wood xylan as a carbon source.

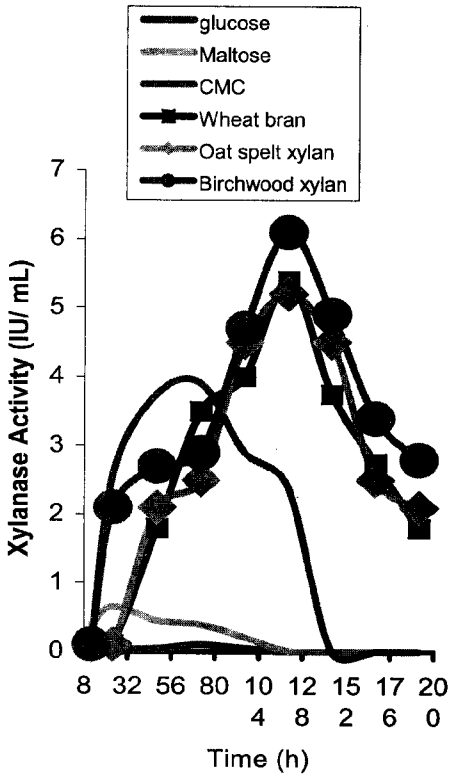


Fig 1: Time course of xylanase production by *T. harzianum* in liquid culture containing 1 % (w/ v) glucose, 1 % (w/ v) maltose, 1 % (w/ v) CMC, 1 % (w/ v) wheat bran, 1 % (w/ v) oat spelt xylan, 1 % (w/ v) birchwood xylan. Values are the mean of three replicates.

The observation that glucose repressed the xylanase production is explained by the earlier reports. It has been reported that glucose represses the synthesis of xylanases from *Trichoderma harzianum* [15]. Xylanase and cellulase genes in *Trichoderma reesei* were repressed in the presence of glucose by the carbon catabolite repressor CREI [16-

17]. It is a well-established fact that culture conditions significantly effect the production of hemicelluloses. Thus, carbon source plays an important role in xylanase production [18]. The choice of an appropriate substrate is of great importance for the successful production of xylanases. The substrate not only serves as a carbon source, but also produces the necessary inducing compounds for the organism [19]. It is therefore concluded that the expression of xylanase is induced by xylan and repressed by glucose. Since the xylanolytic activity observed during *T. harzianum* growth on birch wood xylan was much higher than those observed on the other substrates, birch wood xylan was used as the only carbon source in subsequent experiments.

*Properties of Crude Xylanase*

A summary of properties of crude xylanase is presented in Table 2.

Table- 2: Properties of crude xylanase from *T.harzianum*

| | |
|---|---|
| $K_m$ | 2 mg $ml^{-1}$ |
| $V_{max}$ | 3.35 µmol $min^{-1}mg^{-1}$ |
| Optimum ph at 60 °c | 5 |
| Optimum temperature at ph 5 | 60 °c |

*Effect of pH and Temperature on Crude Xylanase Activity*

Maximum *T. harzianum* crude xylanase activity was observed at pH 5 (Fig. 2). pH optimum of *T. harzianum* crude xylanase well compared with those reported for other fungal xylanases. Thus, our results for optimum pH values were same as reported for *A. phoenicis* ATCC 13157 [20], *Trichoderma longibrachiatum* [21], *Schizophyllum commune* [22] and *T. harzianum* strain T4 [23].

The temperature optimum for the crude xylanase was found to be 60 °C (Fig 3). The optimal temperature for crude xylanase activity in this study was similar to the xylanases from *Trichoderma koningi* G-39 [24], *Trichoderma reesei* QM9414 [25], *Humicola grisea var. thermoidea* [26] and *Aspergillus fischer* [27]. The xylanase from *T. harzianum* exhibited high optimal temperature that may have attractive industrial applications.

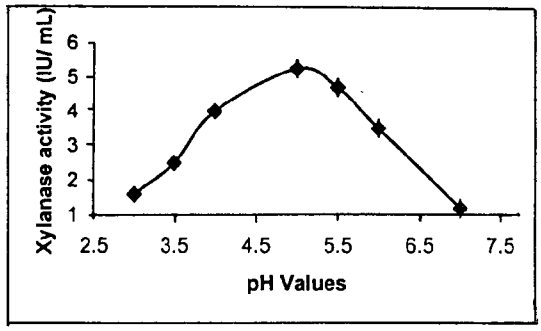


Fig- 2: Influence of pH on xylanase activity from *T. harzianum*. Values are the mean of three replicates.

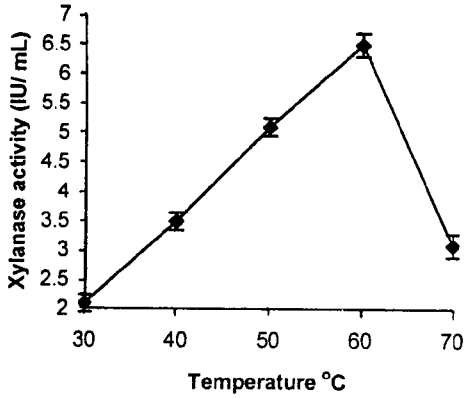


Fig- 3: Effect of temperature on crude xylanase activity from *T. harzianum*. Temperature optimum was determined in the temperature range of 30 - 70 ° C

*Kinetic Parameters $K_m$ and $V_{max}$*

The apparent $K_m$ and $V_{max}$ values of the crude xylanase using Birchwood xylan as a substrate were 2 mg $ml^{-1}$ and 3.35 µmol $min^{-1}$ $mg^{-1}$, respectively. $K_m$ and $V_{max}$ of xylanase from *Aspergillus nidulans* with Birchwood xylan as a substrate were found to be 2.0 mg m/ $L^{-1}$ and 630 µmol $min^{-1}$ $mg^{-1}$, respectively [28]. $K_m$ and $V_{max}$ values of the xylanase from *Trichoderma harzianum* strain T4 were reported to be 1.61 mg m/ $L^{-1}$ and 10.03 IU/m/ L, respectively using soluble Birchwood xylan as a substrate [23]. $K_m$ value for the xylanase from *Paecilomyces themophila* determined toward oat spelt xylan was 2.0 mg m/ $L^{-1}$ [5]. $K_m$ value in this study was in agreement with the already reported fungal xylanases [5-28].

However, our $K_m$ value for crude *T. harzianum* xylanase was less than that reported earlier [29-30].

$K_m$ values for the xylanase toward Birchwood xylan from *Thermomyces lanuginous* was found to be 4.0 mg m/ $L^{-1}$ [29]. $K_m$ value for xylanase from *Penicillium capsulatum* using Birchwood xylan as a substrate was 4.0 mg m/ $L^{-1}$ [30]. The small $K_m$ value in our study showed that the enzyme has high affinity for the substrate, which makes the enzyme significant for the industrial use, as the substrate to product conversion rate is high for the enzymes with low $K_m$ values.

*Isolation of a Xylanase-Encoding Gene*

RNA isolated from *T. harzianum* was used for *xyn2* gene amplification through RT-PCR. First strand of cDNA was made with the help of cDNA synthesis kit. The amplified products were analyzed by agarose gel electrophoresis. The RT-PCR product was about 750 bp and no other non-specific bands were found. Figure 4 shows RT-PCR from RNA of *T. harzianum* grown on xylan as a substrate. It is clearly demonstrated from the figure that significant amplification was achieved from cDNA. The corresponding bands were excised from the gel by DNA extraction kit and purified for ligation in pUC18. Usually, the size of fungal xylanase genes ranges between 550 bp to 900 bp. Our results were in agreement with earlier work done by La-Grange *et al.,* [31], who amplified a 780 bp *T. reesei xyn*2 gene by PCR. Likewise, a 745 bp xylanase gene was amplified from *A. niger* CGMCC106 [32]. A 633 bp *xyn*6 and 675 bp *xyn*B were amplified by RT-PCR from *A. niger* IBT-90 [33]. A 10,40bp XYL6 gene was amplified by RT-PCR from *Magnaporthe grisea* [34].

*Cloning of Xylanase Gene in E. coli*

Plasmid and PCR products were purified from the gel by DNA extraction kit. The amplified product was inserted into plasmid pUC-18 digested with *Sma*I. The *E. coli* competent cells were transformed with recombinant vector. Colonies of *E. coli* carrying the recombinant vector pUC 18-*xyn* were cultured overnight and used for miniprep analysis. Ligation of xylanase genes in pUC18 was confirmed through restriction digestion. The pUC 18-*xyn* construct was double digested with *Eco*RI and *Hind*III, resulting in the separation of xylanase gene from the vector (vector size, 2686 bp) as shown in the Figure 5. Thus, restriction digestion confirmed the presence of xylanase gene in pUC 18-*xyn*.

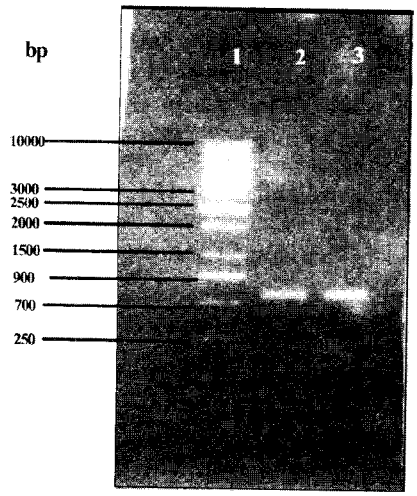


Fig- 4: RT-PCR amplification of xylanase gene from *T. harzianum*. Lane 1: DNA Molecular weight marker, Lane 2 and Lane 3: *xyn 2* gene amplified by RT-PCR

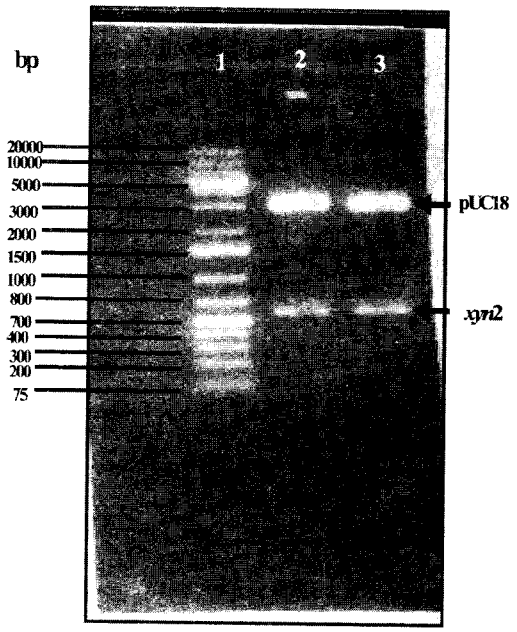


Fig 5: Digestion of pUC18-*xyn* by *Eco* R1 and *Hind* III. Lane 1: DNA Molecular weight marker. Lane 2 and Lane 3: pUC18-*xyn* digested by *Eco*R1 and *Hind* III.

Huang *et al.*, [35], cloned a 642 bp xylanase gene from *Bacillus subtilis* in pUC18 expression vector. Crude recombinant xylanase obtained from

*E. coli* showed high xylanase activity. A 981 bp *xln*C gene from *Aspergillus nidulans* was ligated in plasmid pUC18 and transformed into *E. coli*. [36]. Basran *et al.*, [37] also used pUC18 as expression vector for the cloning and characterization of xylanase gene from *Pichia stipitis*. We therefore, successfully isolated an RT-PCR fragment of 750 bp designated as xylanase gene from *T. harzianum*, cloned into pUC18 and transformed into *E. coli*.

## Experimental

### *Chemicals*

Oat Spelt xylan, Birchwood xylan, xylose, glucose and maltose were obtained from Sigma Chemical Co., USA. cDNA synthesis and DNA extraction kits and restriction enzymes were from Fermentas. All the other chemicals used were of analytical grade unless otherwise stated.

### *Microorganisms and Plasmids*

*Trichoderma harzianum* E-58 was used in this study and was maintained at 4 °C after growing for 7 days in MYG medium (0.2 % malt extract, 0.2 % yeast extract, 2 % glucose and 2 % agar) at 28 °C [38]. *Escherichia coli* DH10B served as host organism and was grown overnight in Luria Bertani (LB) medium containing ampicillin (100μg/m L) at 37 °C. Plasmid pUC-18 (Fermentas) was used for cloning of PCR fragments.

### *Media and Culture Conditions*

For the production of xylanase in liquid state fermentation, the fungus was grown in 500 m/ L Erlenmeyer flask containing 100 m/ L of the Vogel's medium [15] in which the concentrations of the nutrients were 5 g/ L Trisodium citrate, 5 g/ L $KH_2PO_4$, 2 g/ L $NH_4NO_3$, 4 g/ L $(NH_4)_2SO_4$, 0.2 g/ L $MgSO_4$, 1 g/ L peptone and 2 g/ L yeast extract. 1 % glucose was used as a carbon source for inoculum preparations, whereas 1 % xylan was used as a carbon source in cultivation medium. Medium was also supplemented with by 5 m/ L of trace element solution containing g/ L; 5.0 Citric acid. 1 $H_2O$; 5.0 $ZnSO_4$. $7H_2O$, 1.0 Fe $(NH_4)_2SO_4$, 0.25 $CuSO_4$. $5H_2O$, 0.05 $MnSO_4$. $H_2O$, 0.05 $H_3BO_3$. anhydrous and 0.05 $Na_2MoO_4$. $2H_2O$ [39]. pH of the medium was adjusted to 5.5 with 1 M NaOH/1 M HCl before autoclaving. Inoculum preparations were completed by 2 days of cultivation at 28 °C on an orbital shaker (150 rpm). A 10 m/ L of liquid culture from the inoculum was transferred to 1000 mL Erlenmeyer flasks containing 250 m/ L Vogel's medium containing the substrate for 5 days at 28 °C with shaking at 150 rpm [40]. Liquid state cultures were harvested by centrifugation at 10, 000 rpm, for 20 min at 4 °C, and the resulting supernatant was designated as crude enzyme preparation.

### *Xylanase Assay*

Xylanase activity was assayed using 1 % (w/ v) of Birchwood xylan as a substrate. Reaction mixture contained 1 m/ L of appropriately diluted enzyme and 1 % xylan in citrate phosphate buffer. The mixture was incubated at 50 °C for 30 min. After predetermined periods, the released sugars were estimated with 3,5-dinitrosalysilic acid using xylose as standard [41]. One unit of xylanase activity was defined as the amount of enzyme that released one μmol reducing sugars equivalent (xylose) per $min^{-1}$.

### *Effect of Various Carbon Sources on Growth and Xylanase Induction*

*T. harzianum* was grown in Vogel's medium with different carbon sources glucose, maltose, CMC, wheat bran and xylan to determine their effect on the expression of xylanase. All the samples were analyzed in triplicate and the mean value calculated.

### *Effect of pH and Temperature on Crude Xylanase*

The pH optima of the crude xylanase activity was estimated using DNS assay at various pH values between 3.0 to 8.0. For the determination of optimum temperature for crude xylanase activity, the reactions were carried out at 30 °C, 40 °C, 50 °C, 60 °C and 70 °C at pH 5. Enzyme assays were carried out as described above.

### *Kinetic Determinant*

Kinetic parameters were determined by incubating the crude enzyme under the optimal conditions of temperature and pH. Effect of $K_m$ and $V_{max}$ was determined by using varying concentrations of xylan (0.5 to 20 mg/m/ L) by Lineweaver-Burk plot [42].

*RNA Isolation and cDNA Synthesis*

*T. harzianum* mycelia were frozen in liquid nitrogen before grinding in an ice-cold mortar until powdery. TRI reagent (Molecular Research Center, USA) was used for total RNA extraction from the *T. harzianum* mycelia following the manufacturer's instructions. The RNA was treated with DNase1 and again purified. RNA quality and purity were checked by gel electrophoresis and spectrophotometer. mRNA isolation kit (Molecular Research Center, USA) was used to purify polyA mRNA from the total RNA according to manufacturer's protocols. First strand cDNA from *T. harzianum* was synthesized using RevertAid H Minus cDNA synthesis kit (Fermentas) according to manufacturer's instructions. First strand cDNA was then used as a template for PCR.

*Gene Amplification and Cloning of Xylanase Gene*

Molecular cloning techniques and restriction digestions were performed as described by Sambrook and Russel [43]. Using cDNA as a template, xylanase gene was amplified by PCR technique with *Pfu* polymerase (Fermentas). The following primers were designed for the amplification of *xyn*2 gene of *T. harzianum* based on *T. reesei xyn*2 sequence according to Torronen *et al* [44].

5′-ATGGTTGCCTTTCCCAGCCTC-3′ (Forward).

5′ -GTTGCTGACACTCTATGAGGC- 3′ (Reverse)

The amplification was carried out under the following conditions, 35 cycles of 1 min at 94 °C, 1 min at 49 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. PCR products were separated by electrophoresis on agarose gel and visualized by ethidium bromide staining. The amplicon of expected size was gel purified by DNA extraction kit. The PCR products were inserted into plasmid pUC-18 digested by *Sma*1 and dephosphorylated by calf intestinal alkaline phosphatase. Recombinant plasmids were transformed into *E. coli* DH10B by heat shock method. The resulting construct was called as pUC18-*xyn*. A single colony of *E. coli* (DH10B) cells harboring the recombinant vector was cultured overnight in LB medium and used for minipre, analysis. Small and large-scale plasmid preparations were carried out using the alkaline lysis method [36]. Recombinant vectors were double digested with *Eco*R1 and *Hind* III under optimal buffering conditions at 37 °C for I hour to confirm the presence of insert.

## Conclusion

Birch wood xylan was found to be the best inducer of xylanase from *T. harzianum*. pH and temperature optima for the crude xylanase were found to be 5 and 60 °C, respectively. Successful amplification of the xylanase gene was achieved by RT-PCR. The PCR product (750 bp) was inserted into pUC18 and transformed in *E. coli* DH10B. This study will help in conducting further studies on the characterization of the recombinant enzyme.

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