

Comparative study for the kinetics of extracellular xylanases from *Trichoderma harzianum* and *Chaetomium thermophilum*

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Abstract Xylanases assume special importance in the paper and pulp industry as they replace toxic chemicals such as chlorine and chlorine dioxide for developing eco-friendly processes. This study evaluated xylanases produced by two fungi, the mesophilic fungus *Trichoderma harzianum* and a thermophilic fungus *Chaetomium thermophilum*. Among the polymeric substrates studied for xylanase production by both the fungi, birch wood xylan was found to be the best inducer of xylanases. Xylanases induction was subject to glucose repression. Partially purified xylanases preparation from *T. harzianum* and *C. thermophilum* exhibited optimal activities at pH 5 and 6 and at 60°C and 70°C, respectively. The apparent K_m and V_{max} values for the partially purified xylanase from *T. harzianum* using oat spelt xylan as a substrate were 4.8 mg mL⁻¹ and 0.526 μmol min⁻¹ mg⁻¹, respectively. Whereas values of the partially purified xylanase from *C. thermophilum* were 2.96 mg mL⁻¹ and 0.25 μmol min⁻¹ mg⁻¹, respectively. These findings in this study have great implications for the future applications of xylanases.

Keywords: *C. thermophilum*, kinetics, *T. harzianum*, xylanase

INTRODUCTION

Biomass from plant material is the most abundant and widespread renewable raw material for sustainable production of clean and affordable biofuels, biopower, and high-value bioproducts (Michelin et al. 2011). Bioconversion of the agricultural wastes through microbial fermentation is the natural way to recover resources (Rajoka et al. 2011). Biomass is an alternative natural source for chemical and feedstock with a replacement cycle short enough to meet the demands of the world fuel market (Ahmed et al. 2010). The most abundant hemicellulosic polymer xylan constitutes about 20-40% of total plant biomass, and are made up of β-1,4-linked xylose units (Ninawe et al. 2008). Hydrolysis of xylan requires action of different enzymes. Two enzymes are responsible for the main chain cleavage; endo-β-1-4-xylanases (E.C.3.2.1.8) cleaves the backbone to xylooligosaacharides and β-xylosidases (E.C.3.2.1.17) hydrolysis them to D-Xylose (Subramaniyan and Prema, 2002). Xylan has a high potential for degradation to useful end products.

In recent decades, the interest in cellulases and hemicellulases has increased due to the ethanol production from lignocellulosic residues (de Almeida et al. 2011). Xylanases have been extensively studied for their usage in the production of hydrolysate from agro-industrial wastes, in nutritional improvements of lignocellulosic feeds, processing of food, in increasing animal feed digestibility, biobleaching of paper pulp, clarification of fruit juices and wine, the extraction of plant oil, coffee and starch (Ahmed et al. 2009a). Xylanases have also been studied for the production of xylooligosaccharides, which are used as moisturizing agents for food, sweeteners, and specific health

food (Teixeira et al. 2010). Xylanases in conjunction with other enzymes are used for the generation of biological fuels such as ethanol (Beg et al. 2001). Xylanolytic enzymes have also opened new possibilities for the bioconversion of agricultural wastes into easy fermentable sugars (Romdhane et al. 2010).

Filamentous fungi have been used for decades as major producers in the pharmaceutical, food, and food processing industries (Sharma et al. 2012). Xylanases have been produced by a variety of microorganisms, including filamentous fungi and bacteria (Knob and Carmona, 2010). Fungal xylanases are more interesting from an industrial point of view because their extracellular activities are much higher than those of yeast and bacteria (Polizeli et al. 2005). A number of fungal species are known for the production of xylanases such as *Aspergillus niger*, *Chaetomium thermophilum*, *Humicola lanuginosa*, and *Trichoderma harzianum* (Ahmed et al. 2005; Irshad et al. 2008; Ahmed et al. 2009a).

Since high thermostability is required for industrial applications of enzymes (Sriyapai et al. 2011), therefore thermophilic fungi have attracted growing attention for xylanases. Xylanases produced by thermophilic fungi are usually more stable than those from mesophilic fungi (Haltrich et al. 1996). *Chaetomium thermophilum*, a thermophilic fungus is also well known for the production of xylanase (Ghaffar et al. 2011), however very little information is available on kinetics of xylanases from *C. thermophilum*.

In fact, in order for a xylanase to achieve actual industrial application, it should ideally fulfill a number of specific requirements that are highly desired in the marketplace. Such specifications relate to cost-effectiveness, eco-friendliness, and ease of use (Taibi et al. 2011). In this paper we reported kinetics of partially purified xylanases from *T. harzianum* and *C. thermophilum* ATCC 28076 under optimized culture conditions.

MATERIALS AND METHODS

Chemicals

Oat spelt xylan, birchwood xylan, xylose, glucose and maltose were from Sigma Chemical Co., USA. All the other chemicals used were of analytical grade unless otherwise stated.

Microorganisms

Trichoderma harzianum and *Chaetomium thermophilum* ATCC 28076 used in this study were 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) (Ahmed et al. 2003; Saadia et al. 2008).

Media and culture conditions

For the production of xylanase in liquid state fermentation *T. harzianum* was grown in 500 mL Erlenmeyer flask containing 100 mL of the Vogel's medium 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 $(\text{NH}_4)_2\text{SO}_4$, 0.2 g/L MgSO_4 , 1 g/L peptone and 2 g/L yeast extract (pH 5.5) (Ahmed et al. 2009b).

C. thermophilum was grown in 500 mL Erlenmeyer flask containing 100 mL of Eggins and Pugh medium in which the concentrations of the nutrients were g/L; 1.0 KH_2PO_4 , 0.5 KCl, 0.5 $(\text{NH}_4)_2\text{SO}_4$, 0.2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 L-asparagine, 0.5 yeast extract (Eggins and Pugh, 1962) and the pH of the medium was adjusted to 5 (Saleem et al. 2008). Liquid state cultures were harvested by centrifugation (10,000 g, 20 min).

Xylanase assay

Xylanase activity was assayed using 1% (w/v) of birchwood xylan as a substrate. Reaction mixture contained 1 mL of appropriately diluted enzyme and 1% xylan in citrate phosphate buffer. After predetermined periods the releasing sugars were estimated with 3,5-Dinitrosalicylic acid using xylose

as standard (Miller, 1959). One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol reducing sugars equivalent xylose per min^{-1} .

Preparation of partially purified xylanases

C. thermophilum was grown on different carbon sources such as glucose, maltose, CMC, wheat bran and xylan to find out the effect on xylanase expression and results were compared with production of xylanase from *T. harzianum* on the same substrates as reported in our previous study (Ahmed et al. 2007). Xylanases from both the fungal strains were partially purified by ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G-200 and Sephadex G-50 column. The resulting partially purified xylanases from both the fungal strains were used in further studies of characterization.

Determination of pH and temperature optima

The pH optima of the partially purified xylanases from both fungal strains were estimated by using DNS assay at various pH values between 3.0 and 8.0.

For determination of optimum temperature for the partially purified xylanases, the reactions were carried out at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C at pH 5 for *T. harzianum* and at pH 6 for *C. thermophilum*.

Determination of kinetic parameters

The effect of oat spelt xylan concentration, ranging from 0.5 to 20 mg/mL, on xylanase activities from both the fungal strains were evaluated under optimal assay conditions. The kinetic parameters K_m and V_{max} were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk (1934).

RESULTS AND DISCUSSION

Influence of the carbon sources on xylanase production

It is a well-established fact that culture conditions significantly affect the production of hemicellulases (Ahmed et al. 2009a). Thus carbon source plays an important role in enzyme production; 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 (Haltrich et al. 1996). We are interested in determining how the fungi *T. harzianum* and *C. thermophilum* show xylanase activity on different carbon sources. *C. thermophilum* was grown at pH 5, 45°C for 96 hrs with 1% carbon sources for xylanase production.

C. thermophilum produced xylanase activity (IU/mL) of 0.5 ± 0.003 on glucose, 0.8 ± 0.005 on maltose, 0.9 ± 0.06 on CMC, 1.6 ± 0.002 on wheat bran, 2.7 ± 0.05 on oat spelt xylan and 3.9 ± 0.02 on birch wood xylan. These values of xylanase activities produced by *C. thermophilum* were lower as compared with xylanase production on same substrates from *T. harzianum* (Ahmed et al. 2007).

Both fungal strains showed maximum xylanase production with birch wood xylan as a carbon source. *T. harzianum* seems to have a more efficient battery of hemicellulases as compared to *C. thermophilum* since it presented higher level of xylanolytic activity. Since xylanase activity was higher in *T. harzianum* as compared to *C. thermophilum*, it suggests that further strain improvement in *T. harzianum* will be beneficial for its utilization at industrial scale.

The data indicates differential utilization of the various carbon sources by the two fungi. Among xylan as a carbon source, birch wood xylan is the most effective for xylanase production followed by oat spelt xylan. The use of wheat bran, a nutrient - rich intermediate of the wheat processing industry, resulted in satisfactory appreciable xylanase levels from both the fungi. Other carbon sources used namely carboxymethylcellulase (CMC) and maltose resulted in lower xylanase activities. Glucose repressed the production of xylanases from both the fungal strains. *T. harzianum* and *C. thermophilum* xylanases

synthesis can be affected by carbon catabolite repression, as verified in other fungi. The observation in this study that glucose repressed the xylanase production in both fungal strains is in accordance with the earlier reports (Ahmed et al. 2005; Ahmed et al. 2007).

Our results of maximum xylanases production from both fungi on xylan as a carbon source are in accordance with earlier reports. In *T. inhamatum* (da Silva and Carmona, 2008), *Clostridium absonum* (Rani and Nand, 2000), *Thermomyces lanuginosus* (Damaso et al. 2000) and *A. giganteus* (Coelho and Carmona, 2003), xylan induced highest level of xylanase activity.

It is therefore concluded that the expression of xylanase is induced by xylan and repressed by glucose in both fungal strains.

Properties of extracellular xylanases

Xylanases from both the fungal strains were partially purified by ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G-200 and Sephadex G-50 columns (Purification data not shown). A summary of properties of partially purified xylanases is presented in Table 1.

Table 1. Properties of partially purified xylanases from both fungal strains.

	<i>T. harzianum</i> xylanase	<i>C. thermophilum</i> xylanase
K_m	4.8 mg ml ⁻¹	2.96 mg ml ⁻¹
V_{max}	0.526 $\mu\text{mol min}^{-1} \text{mg}^{-1}$	0.25 $\mu\text{mol min}^{-1} \text{mg}^{-1}$
Optimum pH	5	6
Optimum temperature	60°C	70°C

Effect of pH on xylanase activity

Partially purified xylanase from *T. harzianum* exhibited maximum activity at pH 5, whereas maximum *C. thermophilum* partially purified xylanase activity was observed at pH 6 (Figure 1).

pH optimum of *T. harzianum* xylanase compares well with those reported for other fungal xylanases. Most of the xylanases reported work efficiently in pH optima ranging from 4.5 to 6.5 (Georis et al. 2000). Our results for optimum pH 5 for *T. harzianum* xylanase is same as reported for *A4 Aspergillus phoenicis* ATCC 13157 (Chipeta et al. 2005) and *T. harzianum* strain T4 (Franco et al. 2004). Biotechnological applications of xylanases such as biomass hydrolysis and as animal feed additives requires pH range of 4.8-5.5 (Subramaniyan and Prema, 2002), hence *T. harzianum* xylanase can be used in this process. The optimal pH 5 of *T. harzianum* xylanase makes it suitable for usage in the food industry and starch and bread making industries where optimal pH range between 5 and 5.5 is required (Polizeli et al. 2005).

The optimum pH 6 of *C. thermophilum* xylanase activity found in this study was similar to xylanase from *A. giganteus* (Fialho and Carmona, 2004) and *Marasmius* sp (Ratanachomsri et al. 2006). Microbial xylanases are usually stable over a wide range (3-10) and show optimum in the range 4.0 to 7.0 (Kulkarni et al. 1999). *C. thermophilum* xylanase can be used in paper industry where optimal pH 6 is required (Polizeli et al. 2005).

Effect of temperature on xylanase activity

The temperature optimum for the partially purified xylanase from *T. harzianum* was found to be 60°C, whereas maximum *C. thermophilum* partially purified xylanase activity was observed at 70°C (Figure 2).

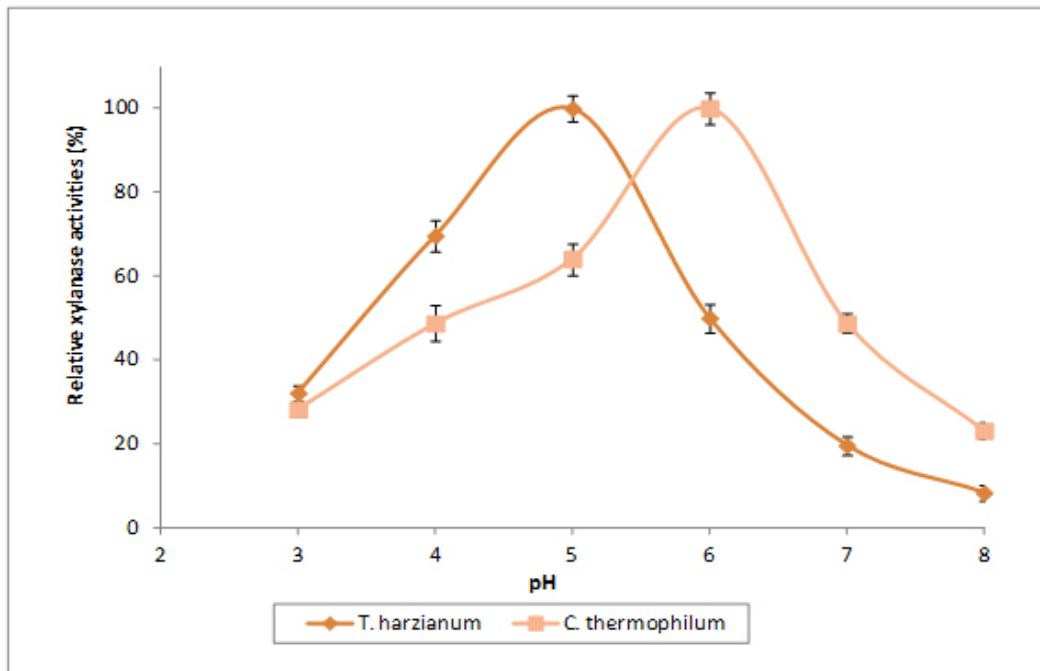


Fig. 1 Influence of pH on partially purified xylanase activities by *T. harzianum* and by *C. thermophilum*.

The optimal temperature of 60°C for xylanase activity from *T. harzianum* found in this study was similar to the xylanases from *Trichoderma koningii* G-39 (Huang et al. 1991), *Humicola grisea* Var. *Thermoidea* (Lucena-Neto and Ferreira-Filho, 2004) and *A. fischeri* (Chandra Raj and Chandra, 1996). The xylanase from *T. harzianum* exhibited higher optimal temperature that may have attractive industrial applications.

The optimal temperature of 70°C for xylanase activity of *C. thermophilum* found in this study compares well with other thermophilic fungal strains. Depending on the optimal temperature, enzymes can be classified as mesophilic (40-60°C), thermophilic (50-80°C) and hyperthermophilic (>80°C) (Polizeli et al. 2005). The xylanase from thermophilic strains like *Talaromyces emersonii*, *Thermomyces lanuginosus* and *Thermoascus aurantiacus* possess optimum temperature between 60°C and 80°C and are very stable in this range (Polizeli et al. 2005). In this study, *C. thermophilum* xylanase exhibits highly appealing and promising features that make it a strong candidate for future industrial applications mainly in pulp bleaching technology since its optimal temperature is 70°C which is suitable for this process.

Kinetic parameters K_m and V_{max}

The apparent K_m and V_{max} values of the *T. harzianum* partially purified xylanase using oat spelt xylan as a substrate were 4.8 mg mL⁻¹ and 0.526 μmol min⁻¹ mg⁻¹, respectively. The apparent K_m and V_{max} values of the *C. thermophilum* partially purified xylanase using oat spelt xylan as a substrate were 2.96 mg mL⁻¹ and 0.25 μmol min⁻¹ mg⁻¹, respectively (Table 1). Thus, both partially purified xylanases have higher catalytic efficiencies for hydrolyzing oat spelt xylan.

The K_m and V_{max} values exhibited by both xylanases is in agreement with the values presented by other fungal xylanases which range from 0.09 to 40.9 mg mL⁻¹ for K_m and from 0.106 to 6300 μmol min⁻¹ mg⁻¹ for V_{max} (Beg et al. 2001).

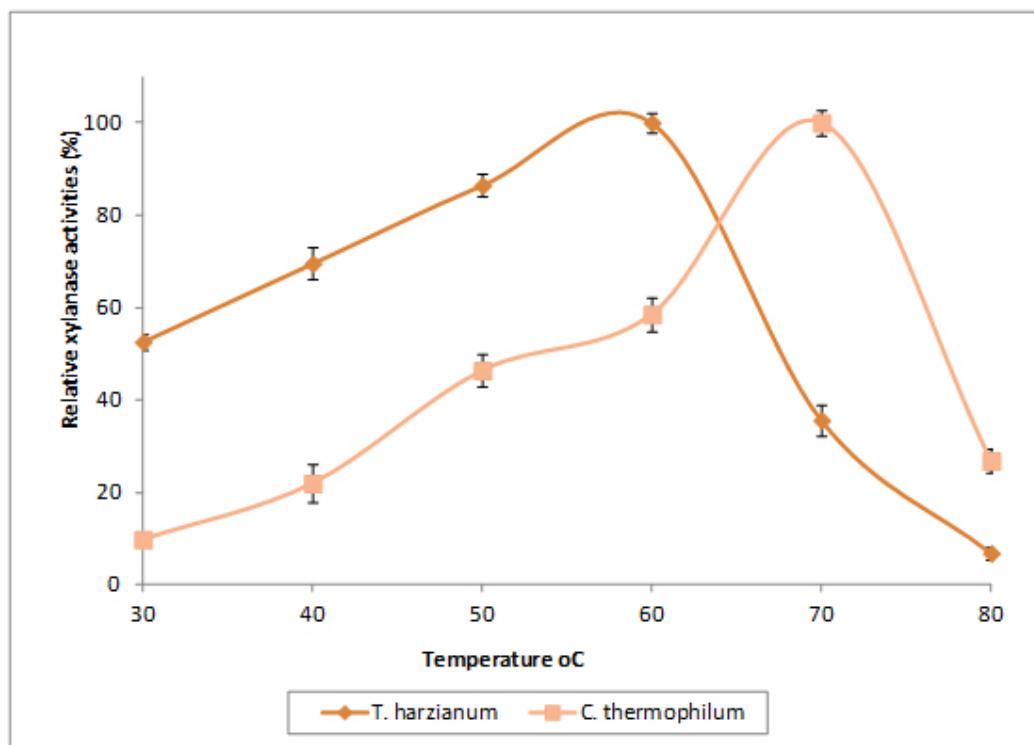


Fig. 2 Effect of temperature on partially purified xylanase activities by *T. harzianum* and by *C. thermophilum*.

K_m value for partially purified xylanase of the *T. harzianum* was higher in comparison with other xylanases such as *A. foetidus* (K_m 3.58 mg mL⁻¹) (Shah and Madamwar, 2005), *A. ficuum* AF-98 (K_m 3.75 mg mL⁻¹) (Lu et al. 2008), *T. harzianum* strain T4 (K_m 1.61 mg mL⁻¹) (Franco et al. 2004).

K_m value for the partially purified xylanase of the *C. thermophilum* was lower in comparison with other thermophilic fungal xylanases such as *Penicillium capsulatum* (K_m 4.0 mg mL⁻¹) (Filho et al. 1993), and *Thermomyces lanuginosus* (K_m 4.0 mg mL⁻¹) (Li et al. 2005).

The small K_m value in our study shows that partially purified xylanases have high affinity for the substrate. This is of significance in industrial use of the enzyme, as substrate to product conversion rate is high for the enzymes with low K_m values.

CONCLUDING REMARKS

The present work surveyed the potentials of xylanases from the mesophilic fungus *T. harzianum* and thermophilic fungus *C. thermophilum*. The results show that both fungal strains produce xylanolytic enzymes, with birch wood xylan being the best inducer and carbon source for the production of xylanases. In terms of xylanases production, *T. harzianum* was found to be better compared to *C. thermophilum*. However, *C. thermophilum* xylanases show higher optimum temperature at 70°C, which encourages for its usage in paper and pulp industry. This study contributes new information and prospects on two xylanase-producing fungal strains in terms of their present and potential biotechnological uses. Further investigations are needed for the production of xylanases at pilot and industrial scale.

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