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Induction of Xylanase and Cellulase Genes from *Trichoderma harzianum* with Different Carbon Sources

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Abstract: Xylanases and cellulases are industrially important enzymes obtained from various microorganisms. *Trichoderma harzianum* is a preferred microorganism for the production of complete xylanases and cellulases. E-58 strain of the fungus was grown in different media including Enzyme Production Medium (EPM) and Vogel's medium with different carbon sources like xylan, glucose and CMC to get the optimal production of xylanases and cellulases. It was found that glucose repressed the synthesis of these enzymes whereas xylan and CMC produced these enzymes in substantial amounts. Maximal activity of cellulases and xylanases was marked at pH 5.5 and at temperature 28°C. The fungus was grown for 5 days at 120 rpm in orbital shaker. Maximum enzyme activity of xylanase was found to be 1.209 IU mL^{-1} whereas maximum activity of cellulases (exoglucanase, endoglucanase and β-glucosidase) was found to be 2.764, 14.4 and 1.1209 IU mL^{-1} respectively.

Key words: Gene induction, xylanase, cellulase, *Trichoderma harzianum*

Introduction

Cellulases and Xylanases are the industrially important enzymes. Celluloses, hemicelluloses and lignin are three major components of plant call wall. Cellulose is the most abundant organic polymer (Goyal *et al*., 1991) on the planet and is an important energy renewable source along with sugars and starches. Cellulase system contains three enzymes that can degrade crystalline cellulose i.e., Exoglucanase (E.C, 3.2.1.9), Endoglucanase (E.C, 3.2.1.4) and β-glucosidase (E.C.3.2.1.4). Xylan is major component of hemicellulose. It is also a major component of all monocots and hard woods, representing up to 35% of dry weight of these plants (Puls and Suchsil *et al*., 1993).

Xylanases have been extensively studied and could potentially be applied for the production of hydrolysates for agro-industrial wastes (Milagres *et al*., 1993), nutritional improvement of lignocellulosic foods, agro fibre (Bailey and Viikari, 1993), bioleaching of kraft pulps (Moreau *et al*., 1994) and in paper industry.

Cellulases are used in industries (Moo-Young 1985); in the preparation of medicines, resins, perfumes, in starch production, waste treatment, baking and in the production of plant protoplasts for genetic manipulation etc (Esterbauer *et al*., 1991).

Considering the industrial potentials of xylanases and cellulases, an important aspect of xylanase and cellulase research is to obtain highly active xylanases and cellulases at low cost.

The use of filamentous fungi may have a number of advantages over yeast and bacteria, the most important of which being their capability to be propagated on a wider variety of substrates generally discarded as wastes from food production and it involves simple harvesting operations (Sinskey, 1978). Some fungal species known for the production of xylanases and cellulases include *Chaetomium thermophile*, *Trichoderma species* and *Aspergillus niger*. Szczodrak (1988) found that *Trichoderma reesei* produces cellulases on wheat straw. Similarly different microbial sources have been investigated for β-xylanase production and strains of fungus *Trichoderma harzianum* have been shown to secrete large amounts of efficient xylan degrading enzymes (Wong and Saddler, 1992). The fungus *Trichoderma harzianum* was grown in Vogel's medium, pH 5.5 and temperature 28°C at 120 rpm using xylan, CMC and glucose as carbon source. Earlier Rajoka *et al.* (1997) used cellulose, cellobiose, xylan and CMC for the enhanced production of cellulases. MacCabe *et al.* (1996) found that *Xyln* C production from *T. reesei* was induced by xylan.

In this paper we have reported the growth of *T. harzianum* on different media and the induction of xylanase and cellulase genes from the fungus by using different carbon sources.

Materials and Methods

Selection of fungi: A fungal strain *Trichoderma harzianum* (E-58) strain was employed to check the induction of xylanase and cellulase genes using different carbon sources. The fungus was obtained from National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad.

Substrates: Different substrates (carboxymethycellulose (CMC), xylan and glucose) were used for the induction of the cellulose xylanase genes from *Trichoderma harzianum.*

Media and culture conditons

A. *Trichoderma harzianum* E-58 was maintained on agar slants (MYJ) containing (g L^{-1}) malt extract 5; yeast extract 2.5; glucose 10; agar 20. Freshly inoculated slants were incubated at 28^{0}C for 5 days and stored at 4°C (Ulho and Peberdy, 1991).
B. Production of Xylanases and cellulases was carried out in one-liter flasks containing 250 mL of EPM (Enzyme Production Medium). EPM comprises (g L^{-1}) glucose 3, bactopeptone 1; urea 0.3; $(NH_4)_2SO_4$ 1.4; $MgSO_4.7H_2O$ 0.3; $CaCl_2.6H_2O$ 0.3: Chitin 5.0; and 0.1% vol vol^{-1} trace elements solution containing (Fe^{2+},Mn^{2+}, $Zn^{2+}$and Co^{2+}).
C. For the induction of xylanases and cellulases genes, the fungus was grown at 28°C with shaking at 120 rpm in Vogel's medium (0.5% Trisodium citrate, 0.5% KH_2PO_4, 0.2% NH_4NO_3 0.4% $(NH_4)_2SO_4$ 0.02% $MgSO_4$, 0.1% peptone, 0.2% yeast extract, pH 5.50) containing 1% glucose or CMC or xylan as a carbon source (Ikram-ul-Haq *et al*., 2000).

Inoculum: Flasks were autoclaved at 121 °C for 15 minutes and inoculated with a loopful of fungal spores suspended in 100 mL of Vogel's medium. The flasks were incubated at 28°C. The incubation time was limited to 24 h with shaking at 120 rpm in orbital shaker.

Enzyme assays

Xylanase assay: Activity of the enzyme was checked by Miller method (1959). One mL citrate phosphate buffer, 0.5 mL Xylan (1%) and 0.5 mL of diluted enzyme mixture were incubated at 50°C for 30 min. After expiry of time 3 mL DNS reagent was added and mixture was placed in boiling water bath for 5 min and the absorbance was noted at 550 nm. The international units of xylanase produced per mL were calculated by factor obtained from standard curve of xylose (Fig. 1).

Exoglucanase assay: Exoglucanase assay is based on the fact that the enzyme releases free cellobiose from avisel. One milliliter of diluted enzyme extract was incubated with 1 mL of 0.1 mL citrate buffer at pH 4.8 at 40°C for 30 min. The reaction was stopped by boiling for 15 minutes and cooling in ice. After cooling, absorbance was measured at 540 nm. The international units of exoglucanase produced mL^{-1} were calculated by factor obtained from the standard curve of cellobiose (Fig. 2).

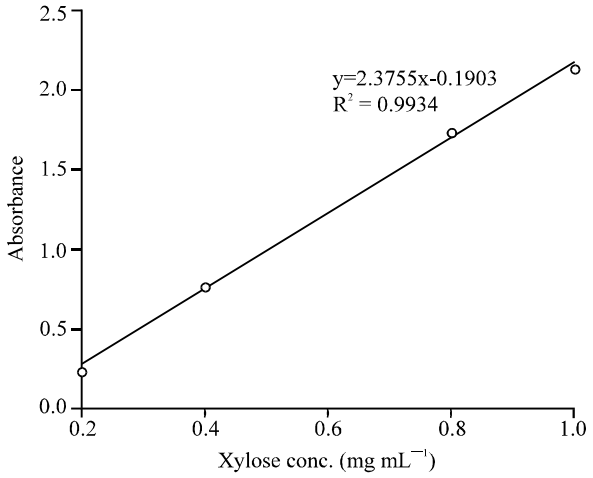

Fig. 1: Standard curve of xylose

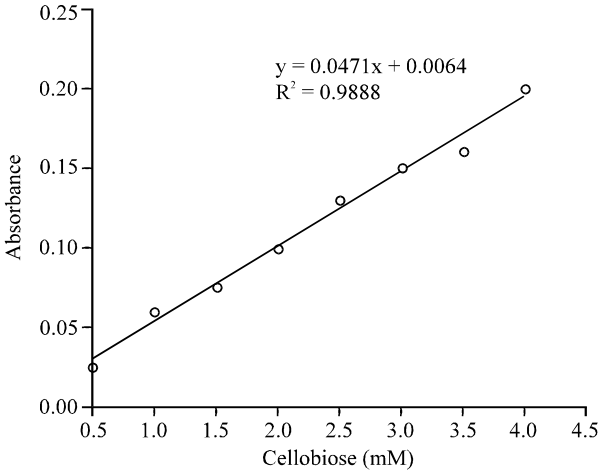

Fig. 2: Cellobiose standard curve

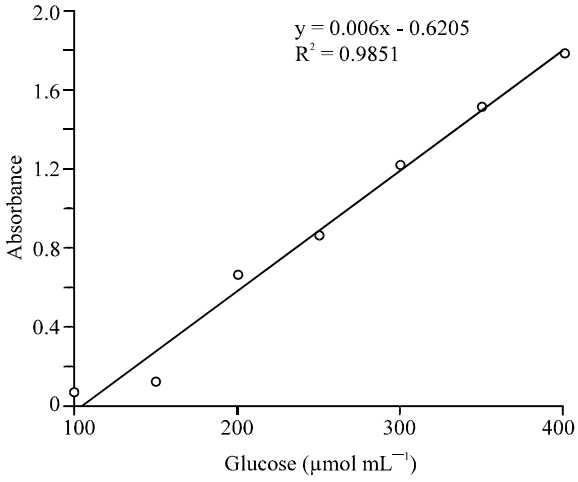

Fig. 3: Standard curve of glucose

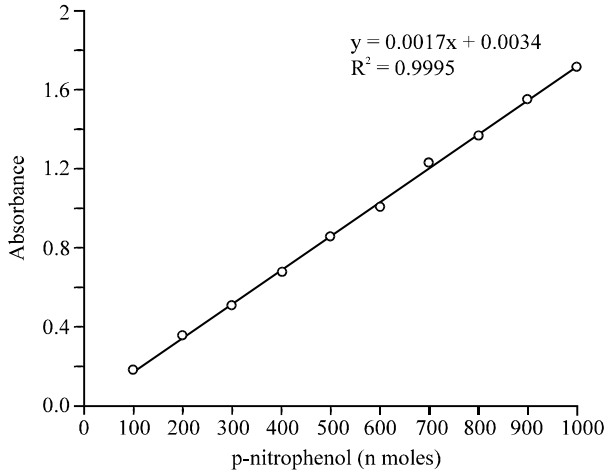

Fig. 4: Standard curve of p-nitrophenol

Endoglucanase assay: It was performed as described by Gadgil (1995). Briefly 1 mL of appropriately diluted enzyme (0.01mL) was incubated for 30 minutes with 1 mL of 1% CMC and 1 mL of 0.1M citrate buffer of pH 4.8 at 50°C. The reaction was trimmed by adding 3 mL of DNS reagent and boiled for 15 min and cooled in ice. The international units of endoglucanase produced mL^{-1} were calculated by factor obtained from standard curve of glucose (Fig. 3).

β-glucosidase assay: It is based on the principal that p-nitrophenyl β-D-glucopyranoside is hydrolyzed by β-glucosidase to yield β-D-glucose and p-nitrophenol. One mL of β-D-glucopyranoside solution (substrate) in buffer and 1 mL of appropriately diluted enzyme were added and incubated at 40°C for 10 min. Three mL of 1M Na_2CO_3 (Stopper) were added and volume was made up to 10 mL with distilled water. Absorbance was measured at 400 nm. The international units of β-glucosidase produced mL^{-1} were calculated by factor obtained from standard curve of p-nitrophenol (Fig. 4).

Results and Discussion

Growth of *Trichoderma harzianum:* The fungus *Trichoderma harzianum* was employed in this research. Earlier Esterbauer *et al.* (1991) found that *Trichoderma* strains are preferred microorganisms for the production of complete cellulases. Wong and Saddler (1992) studied properties and applications of xylanases from *Trichoderma* spp, which produced these enzymes with high xylanolytic activity. They isolated and purified different xylanases form *Trichoderma* species.

EPM (enzyme production medium) was used for the growth of fungus but growth did not come out. However Ulhoa *et al.* (1991) obtained positive results from EPM for the growth of *Trichoderma harzianum* for large-scale production of chitobiase. MYG medium (Heesing *et al.*, 1994) was tried for sporulation of the *Trichoderma harzianum* in agar slants but desirable results could not be achieved. After failing to get the growth of *Trichoderma harzianum* on EPM and MYG media, Vogel's medium was used for the growth of *Trichoderma harzianum.* Spores of *T.harzianum* were found on agar slants containing 0.2% xylan or CMC. The spores were inoculated in shake culture. *T. harzianum* was grown on Vogel's medium for 5 days at 120 rpm.

Optimum pH for *Trichoderma harzianum*: The pH of the Vogel's medium was adjusted to 5.5 to obtain the maximal production of the xylanases and cellulases from *Trichoderma harzianum.* Earlier Simpson (1991) worked on the production of thermostable endo 1-4 β xylanase from culture supernatant of *Thermogota* spp and noted that the optimum pH was between 5.0 and 5.5 for its activity. Rose and Van (2002) found that xylanase enzyme expresses its highest activity at pH 5-6. Similarly De Paula-Silveira *et al.* (1999) found that xylanase from *T. harzianum* had a maximum activity at pH 5.0. Milagres *et al.* (1993) worked on the production and characterization of xylanase from *Penicillum Janthium* and found that optimal pH for xylanase activity was 5.5.

Srivastava *et al.* (1984) used cellobiose and 4 nitro phenyl β-D glucosidase as substrate for the growth of *Aspergillus Wentii* to study the kinetic characteristics of β-D glucosidase and glucohydrolase. They found that the enzyme activity was maximum over a pH range of 4.5 to 5.5 for both substrates. Similarly it was found earlier that maximum induction of endoglucanase was achieved at pH 5.5 (Ikram-ul-Haq *et al.*, 2000).

Temperature and shaking: Temperature of the medium was kept at 28°C with shaking at 120 rpm, which is in accordance with the previous work conducted (Sexton *et al.*, 2000). Esterbauer *et al.* (1991) found that optimum temperature for cellulase production form *Trichoderma reesei* was 15-28°C and optimal temperature for its growth was 30°C. Similarly Gomes *et al.* (1992) used *Trichoderma viridie* Bt 2169 for the production of cellulases and xylanases. They noted that the optimum temperature for the maximum production of these enzymes was 31.1°C. Smith and Wood (1991) observed that the optimum temperature was 30°C and 35°C for the production of extracellular xylanases and β xylosidase by *Aspergillus awamori.*

Induction of Xylanase and Cellulase genes from different substrates: *Trichoderma harzianum* was grown in Vogel's medium at 28°C for 5 days at 120 rpm for the

Table 1: Xylanase and cellulase concentrations produced from *T. harzianum*

	Enzyme activities (1U mL^{-1})			
Substrate	Xylanase	Exoglucanase	Endoglucanase	β-Glucosidase
1% glucose	0.121	0.293	4.35	0.0635
1% CMC	-	2.764	9.04	0.1209
1% xylan	1.209	1.056	14.4	0.629

maximal production of cellulases and xylanases with different carbon sources like CMC, xylan and glucose. After 5 days the culture mixture was taken out of orbital shaker. The enzyme filtrates obtained were tested for enzyme activities. Enzyme assays were performed as described in materials and methods to check the activity of xylanases and cellulases. The results obtained are summarized in Table 1. The results are in accordance with those of earlier work conducted on *Trichoderma harzianum* (De Paula-Silveira *et al*., 1999, Emilo *et al*., 1996).

Present results suggest that when glucose was used as a carbon source, the production of xylanases and cellulases was inhibited and when xylan or CMC was used as a carbon source the production increased significantly. The results are in agreement with the earlier reports where it was found that *Xyln*C production from *Aspergillus nidulans* was induced by xylan and repressed by glucose (MacCabe *et al*., 1996). Zeilinger *et al*. (1996) found that *xyln*2 production from *T. Reesei* was induced by the presence of xylan and xylose.The fungus *Aureobasidium pullans* Y2311-1 has been reported to produce highest level of xylanase when xylan was used as a carbon source (Li and Ljungdahl, 1994). Two of endo beta –1,4 xylanases were isolated from culture filtrates of *T.reesei* C30 grown on xylan as a carbon source (Torronen *et al*., 1992).

Earlier Rajoka and Malik (1997) used cellulase, cellulosic residues, xylan, cellobiose and CMC as a carbon source for the enhanced production of cellulases by *Cellulomonas strain*. Ximenes *et al*. (1996) found that high glucose repressed the induction of cellulases whereas low glucose potentiated the enzyme production. Previously it was found by Northern analysis that genes of cellobiohydrolases were induced with 1% cellulose or CMC.

The effect of carbon sources on *T.harzianum* was also studied by Malik and co workers (1986). They optimized the fermentation conditions and found that maximum growth was obtained with glucose as a carbon source but negligible cellulases were produced. They suggested that substantial amounts of cellulases were produced from other carbon sources. Esterbauer *et al*. (1991) used CMC and xylan as a carbon source for the maximal production of glucosidase.

We, therefore, conclude that the optimum conditions for the growth of *T.harzianum* were at pH 5.5, temperature 28°C and shaking at 120 rpm in Vogels medium. It was found that the xylanase and cellulase genes were induced when xylan or CMC were used as a carbon source and repressed by glucose.

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