

PRODUCTION OF XYLANASES AND CELLULASES BY *ASPERGILLUS FUMIGATUS* MS16 USING CRUDE LIGNOCELLULOSIC SUBSTRATES

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Abstract

Xylanolytic and cellulolytic potential of a soil isolate, *Aspergillus fumigatus* (MS16) was studied by growing it on a variety of lignocellulosics, purified cellulose and xylan supplemented media. It was noted that carboxymethyl cellulose, salicin and xylan induce the production of endoglucanase, β -glucosidase and xylanase, respectively. The study revealed that *Aspergillus fumigatus* (MS16) co-secretes xylanase and cellulase in the presence of xylan; the ratio of the two enzymes was influenced by the initial pH of the medium. The maximum titers of xylanase and cellulase were noted at initial pH of 5.0. Relatively higher titers of both the enzymes were obtained when the fungus was cultivated at 35°C. Whereas, cellulase production was not detected when the fungus was cultivated at 40°C. The volumetric productivity (Q_p) of xylanase was much higher than cellulases. The organism produced 2-3 folds higher titers of xylanase when grown on lignocellulosic materials in submerged cultivation than under solid-state cultivation, suggesting a different pattern of enzyme production in presence and in absence of free water. The partial characterization of enzymes showed that xylanase from this organism has higher melting temperature than endoglucanase and β -glucosidase. The optimum temperature for activity was higher for xylanases than cellulases, whereas the optimum pH differed slightly i.e. in the range of 4.0-5.0. Enzyme preparation from this organism was loaded on some crude substrates and it showed that the enzyme preparation can be used to hydrolyze a variety of vegetable and agricultural waste materials.

Key words: Xylanases, Cellulases, *Aspergillus fumigatus* MS16, Lignocellulosic, β -Glucosidase

Introduction

Lignocellulosics are complex and heterogeneous mixtures, predominately comprised of cellulose and hemicellulose. Cellulose is an abundant biopolymer that constitutes most of the plant cell-wall. This homopolymer can be degraded into simple sugars by cellulases produced by a number of microorganisms, especially fungi (Dashban *et al.*, 2009; Lynd *et al.*, 2002). Fungal cellulolytic enzymes including endoglucanase, exoglucanase and β -glucosidase act synergistically to degrade cellulosic biomass completely, hence, are applied in different commercial processes like production of biofuels and fabric manufacture (Bhat, 2000).

In nature, cellulose is present in association with hemicelluloses e.g. xylan which is regarded as the second most abundant component of plant cell-wall. It is composed of xylopyranose residues linked by β -1,4-glycosidic bond that is hydrolyzed by xylanases into xylooligosaccharides (Polizeli *et al.*, 2005; Viikari *et al.*, 2001). In the recent years, this enzyme has gained an increased attention due to its potential applications in bioconversion of hemicelluloses to sugars, ethanol and other useful substances, clarification of juices and wines, improving the nutritional quality of silage and green-feed, de-inking processes of waste paper, pharmaceuticals, paper and pulp industry and agricultural-waste treatment processes (Kulkarni *et al.*, 1999).

Xylanases are produced by fungi, bacteria, yeast, seaweed, protozoa, gastropod and arthropods. Fungi are traditionally employed for industrial production of various enzymes (Iftikhar *et al.*, 2010; Malik *et al.*, 2013; Iftikhar *et al.*, 2014; Abdullah *et al.*, 2014). Species of *Aspergillus* are mainly used to produce xylanases, including *A. niger*, *A. ochraceus*, *A. oryzae*, *A. awamori*, *A. tamari* and *A. fumigatus* (Haltrich *et al.*, 1995).

Though the demand of cellulase-free xylanase in paper and pulp-industry is increasing, a number of fungi reportedly co-produce cellulase and xylanase and such preparations find applications in many biotechnological processes (Heidorne *et al.*, 2006; Sachlehner *et al.*, 1998). Studies on *A. fumigatus* have also shown to possess this ability (Gupte & Madamware, 1997; Kitpreechavanich *et al.*, 1986; Wase *et al.*, 1985). This organism is well known for its pathogenicity (Latge, 2003); however, it has recently gained more attention after the completion of its genome sequence which deciphered a fully functional sexual cycle (O'Gorman *et al.*, 2008) and its endophytic mode (Kusari *et al.*, 2009).

After screening a large number of native fungal-flora for the production of numerous hydrolytic enzymes (Sohail *et al.*, 2009a), *Aspergillus fumigatus* MS16, a soil-borne isolate, was selected on the basis of its relatively better cellulolytic and xylanolytic potentials. This study describes the suitability of this organism to produce cellulases and xylanases in submerged as well as in solid-state fermentation, and a comparison was drawn between activities of the two enzymes.

Materials and Methods

Organism, growth conditions and media: *Aspergillus fumigatus* (MS16) was isolated from soil and screened for exo-hydrolase activity, as described previously (Sohail *et al.*, 2009a). It was maintained on Sabouraud's Dextrose agar (SDA) slants at 4°C and subcultured, as and when needed. For the production of enzymes it was cultivated in mineral salt medium containing suitable carbon source at 35°C, pH 5.0, unless otherwise stated (Mandels *et al.*, 1974).

Enzyme assays and protein determination: Enzyme preparation (0.5 ml) was mixed with 0.5 ml of 50 mM sodium citrate buffer, pH 4.5 containing 6 x 1 cm strip of Whatman # 1 filter paper, CMC, salicin or xylan (0.5% w/v, each) to assay filter paperase, endoglucanase, β -glucosidase and xylanase activity, respectively. The reaction mixture was incubated at 50°C for 30 min. and resulting reducing sugars measured by dinitrosalisylic acid (DNS) method using standard curves of xylose or glucose (Miller, 1959). One unit of enzyme was defined as the amount of enzyme that liberates 1 μ mol of xylose or glucose equivalents in 1 minute under standard assay conditions. Protein concentration was estimated by Bradford standard assay (Quickstar[®], Biorad, USA) using bovine serum albumin (BSA) as standard.

Solid-state fermentation (SSF): Different plant materials namely corncob, corn-leaves, sugarcane-bagasse, cabbage-twigs, grass and pea-peels were dried in an oven at 80°C, ground, sized through 100 mesh-size screen and used as substrates for solid-state fermentation. Each substrate (2 g in 500 ml flasks) was autoclaved at 121°C for 30 min., inoculated with 2 ml of fungal spore suspension (5×10^6 /ml) and incubated at 35°C for seven days under static conditions, maintaining the moisture content to ~65% using mineral salt medium without any carbon source. At harvest, 50 ml of 50 mM sodium citrate buffer, pH 4.5 containing 0.2% (w/v) Tween-80 was added and kept in an orbital shaker at 150 rpm for an hour at 35°C to homogenize the mixture. It was followed by the filtration through a thick layer of glass-wool and centrifuged at 6000 rpm for 20 min. The cell-free culture supernatant was used as a source of crude enzyme preparations to determine the enzyme activities.

Optimum temperature and pH and thermal stability: Optimum temperature for the enzyme activity was determined by assaying the enzyme preparation at variable temperatures (ranged 40-80°C). To determine the optimum pH for enzyme activity, the reaction was carried out in the presence of 50 mM buffers of HCl-KCl (pH 1.0-2.0), Glycine-HCl (pH 2.5-3.5), sodium-acetate (pH 4.0-5.5), citrate-phosphate (pH 6.0-7.0), Tris-HCl (pH 7.5-9.0), Glycine-sodium hydroxide (pH 9.5-10.5) containing suitable substrates. Melting temperature (T_m) of enzymes present in crude-extract was investigated by incubating the reaction mixture at temperatures between 50-80°C for 15 minutes and determining the residual enzyme activities under standard assay conditions (Sohail *et al.*, 2013).

Effect of Metal Ions on Enzyme Activity: The effect of metallic-ions on enzyme activity was determined by incubating dialyzed cell-free culture supernatants in the presence of 20 mM metallic salts (AgNO_3 , MnSO_4 , EDTA, KCl, ZnSO_4 , CaCl_2 , NaCl, MgSO_4 , CoCl_2 , BaCl_2 , CuSO_4 and FeSO_4) in the reaction mixture and assayed for enzyme activities (Sohail *et al.*, 2014).

Enzyme hydrolysis kinetic: Enzyme preparations (equilibrated at 10 IU of xylanase) were loaded on 1 gram of crude lignocellulosic substrates (cabbage-twigs, pea-peel and grass) and purified oat-spelt xylan suspended in 50 ml of 50 mM sodium citrate buffer, pH 4.5. Aliquots were withdrawn periodically and amount of reducing sugars determined by DNS method (Miller, 1959).

Results and Discussion

Enzyme production by shake flask method: *A. fumigatus* (MS16) was cultivated in mineral salt medium containing various purified and natural carbon sources such as carboxymethyl cellulose (CMC), cellulose acetate, filter paper, salicin, xylose, beechwood xylan, oat-spelt xylan, pea-peel, grass and cabbage-twigs. The cell-free culture supernatant was prepared and endoglucanase, β -glucosidase and xylanase activities determined. Enzyme activity was noted in the presence of almost all the substrates (data not shown) and the higher titers of endoglucanase, β -glucosidase and xylanase were obtained in CMC, salicin and oat-spelt xylan supplemented medium, respectively. This observation was in line with Sachlehner *et al.* (1998) where a 4-fold increase in endoglucanase production was observed compared to xylanase when *Scletorium rolfssii* was cultivated in CMC containing medium. Similarly, the induction of cellulases and xylanases by the corresponding substrates was noted for *Curvularia inaequalis* (Gomes *et al.*, 1992); indicating that the production of cellulolytic and hemicellulolytic enzyme is dependent on the nature of the substrate; carbon source can enhance the growth of the organism and induce the co-production of certain enzymes (Juhasz *et al.*, 2005).

Cultivation of *A. fumigatus* MS16 at different temperatures in the presence of the inducers (CMC or salicin) depicted that the highest titers of all the enzymes were produced at 35°C (Table 1). The organism was able to elaborate xylanases at temperature as high as 40°C with no cellulase production, though xylanases production was reduced significantly at this temperature. Hence, cultivation temperature can be manipulated to obtain cellulase-free xylanase from the strain or it can be cultivated at 35°C for the co-production of a mixture of two, different substrate specific enzymes. Although, cellulase-free xylanases have many applications, a heterogeneous preparation of cellulase and xylanase is applied in de-inking, waste disposal and to hydrolyze plant-biomass. Consequently, volumetric productivity (Q_p) of cellulase and xylanase by *A. fumigatus* MS16 at 35°C showed a 15-30 folds higher Q_p values for xylanase than endoglucanase and β -glucosidase (Table 2). This finding supports the study conducted by Elisashvili *et al.* (1999) where higher xylanase titers were obtained than cellulases from brown-rot and white-rot fungi even when the organisms were grown on celluloses.

Table 1. Effect of incubation temperature on xylanase and cellulose (endoglucanase and β -glucosidase) production by *Aspergillus fumigatus* MS16 under Submerged Fermentation.

Incubation temperature (°C)	Enzyme activity (IU/ml)		
	Xylanase	Endoglucanase	β -glucosidase
25	1.55	0.020	0.021
30	1.71	0.025	0.044
35	1.9	0.326	0.36
40	0.72	0	0

Table 2. Volumetric productivity (Q_p) of endoglucanase, β -glucosidase and xylanase from *A. fumigatus* MS16.

Enzyme	Q_p (IU.L. $^{-1}$ h $^{-1}$)
Endoglucanase	0.252
β -glucosidase	0.400
Xylanase	6.25

Table 3. Effect of pH on Xylanase and cellulase production by *A. fumigatus* MS16.

pH	Xylanase (IU/ml)	Cellulase (IFPU/ml)	Xylanase/Cellulase ratio
3	0.16	0.155	1.03
4	0.22	0.15	1.466
5	1.2	0.18	6.66
6	1.1	0.16	6.875
7	0.85	0.12	7.083

Studies on pH dependent expression of enzymes in oat-spelt xylan supplemented media suggests the highest titers of xylanases were obtained in a medium having initial pH 5.0, while the levels of cellulases were not significantly influenced by a change in initial pH of the medium (Table 3). The ratio of xylanase to cellulase was 6-7 times higher in a medium having pH 5.0 or above. pH dependent expression of cellulases and xylanases have already been elucidated by Mamma *et al.* (2007) where the higher levels of xylanases and cellulases of *A. niger* BTL were noted at pH 3.5 and 5.0, respectively. On the other hand, xylanase production from *T. reesei* was observed at pH 6.0 while cellulase at 4.0-4.5 in lactose containing medium (Xiong *et al.*, 2004). Recent reports also suggest the influence of initial pH of the medium on the production of cellulases by *A. niger* MS82 (Sohail *et al.*, 2009b) and *Bacillus coagulans* (Angsana *et al.*, 2009).

Solid-state fermentation: Enzyme production using crude, natural lignocellulosics showed that the heterogeneity of the substrate led to the synthesis of many hydrolases in varying proportions. In most of the substrates, the levels of xylanases were much higher than cellulases. While, significant titers of pectinase

were also noted (data not shown). The highest titers of xylanase and endoglucanase were obtained when *A. fumigatus* MS16 was grown on pea-peels (Table 4). The use of cabbage-twigs yielded the maximum levels of β -glucosidase. On the other hand, the endoglucanase and β -glucosidase activity was not detected when the organism was cultivated either on sugarcane-bagasse or corn-leaves. Higher xylanase to cellulase ratio in SSF cultivation was also reported from *Penicillium janthinellum* NCIM1171 and *T. viride* NCIM1051 on sugarcane-bagasse (Gawande & Kamat, 1999); *Ceriporiopsis subvermispora* on wood chips (Heidorne *et al.*, 2006) and from *A. niger* KK2 on rice straw (Tsiklauri *et al.*, 1999). Contrary to this study, Gupte & Madamwar (1997) obtained more than 14 U/ml of endoglucanase on sugarcane-bagasse from a strain of *A. fumigatus*.

A comparison for the production of xylanase between submerged (Smf) and solid-state (SSF) cultivation on natural, (crude) lignocellulosic materials suggests that except pea-peel where SSF gave slightly higher titers, Smf yielded 2-3 folds higher specific productivity in comparison to almost all the other substrates (data not shown). A similar observation was made by Gawande & Kamat (1999) for *A. terreus* and *A. niger* where these strains yielded more xylanase under Smf than in SSF of wheat-bran, sugarcane-bagasse, soybean-hull or rice-straw. Likewise, a basidiomycetes species produced more xylanases on vine-cuttings under submerged than solid-state conditions (Tsiklauri *et al.*, 1999).

Effect of temperature and pH for enzyme activity: The optimum temperature for xylanolytic activity (64°C) was higher compared to endoglucanase and β -glucosidase activity (50°C). Furthermore, a significant loss in xylanase activity was not observed when the reaction was carried out at 80°C but only residual activity of endoglucanase and β -glucosidase was noted at this temperature (data not shown). An optimum temperature of 60° and 65°C was reported for xylanase (Anthony *et al.*, 2003) and endoglucanase (Grigorevski-Lima *et al.*, 2009) of *A. fumigatus*, respectively.

Gomes *et al.* (1992) reported that the optimum pH for fungal xylanases activity falls between 5.0-6.0 and pH 4.5 is for xylanase from *A. fumigatus* (Kitpreechavanich *et al.*, 1986). Other authors mentioned that pH 4.8 is optimum for the activity of *Aspergillus* cellulase (De Vries & Visser, 2001; Jahangeer *et al.*, 2005). The results of present study are in agreement to the previous studies as pH 4.0, 4.5 and 5.0 were optimum for β -glucosidase, xylanase and endoglucanase activities, respectively (Table 5). The activity of xylanase and β -glucosidase was decreased drastically when the pH was increased above 5.0. The endoglucanase of *A. fumigatus* MS16 however, behaved differently as it retained more than half of its activity at pH 7.5 and only residual activity was detected when the reaction was carried out at pH 9.0.

Table 4. Xylanase and cellulase production by *A. fumigatus* MS16 on various natural substrates under SSF. (Values in parentheses represent specific activity).

Enzyme activity (IU/ml)	Enzyme activity (IU/ml) on substrates					
	Pea peel	Cabbage twigs	Corn leave	Corn cob	Sugarcane bagasse	Grass
Xylanase	13.971 (0.072)	7.305 (0.041)	4.428 (1.107)	2.463 (0.014)	2.001(0.029)	7.211 (0.028)
Endoglucanase	1.26 (0.006)	0.682 (0.004)	0	0.286 (0.002)	0	0
β -glucosidase	1.556 (0.008)	4.744 (0.0265)	0	0.286 (0.0012)	0	1.186 (0.0047)

Table 5. Melting temperature (T_m), optimum temperature and pH for xylanase, endoglucanase and β -glucosidase activity.

Enzyme	Temp. (°C)	pH	T_m (°C)
Xylanase	60	4.5	66.5
Endoglucanase	50	5.0	60
β -glucosidase	50	4.0	53.5

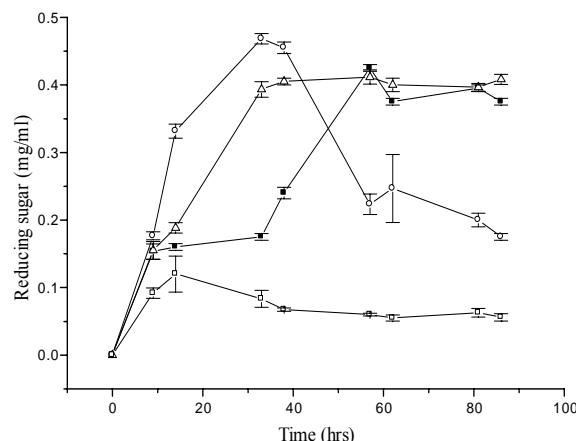


Fig. 1. Kinetics of hydrolysis of pea-peel (-■-), cabbage (-Δ-), grass (□) and oat-spelt xylan (-○-).

Determination of melting temperature (T_m): Data obtained during this study show that xylanase had relatively higher T_m of 66.5°C (Table 5), while, β -glucosidase was the most heat-sensitive enzyme with a T_m of 53.5°C and a sharp decrease in its activity when temperature was increased further (data not shown). The T_m for endoglucanase was 60°C with a significant activity loss when enzyme preparation was subjected to 75°C. The earlier reports suggest that T_m values for cellulase from many organisms generally fall within a range of 50-60°C (Rajoka *et al.*, 2004; Rajoka *et al.*, 2003; Singh *et al.*, 1990).

Effect of metal-ions and EDTA on enzyme activity: Studies on the effect of metallic-ions and EDTA revealed that the presence of EDTA slightly decreases enzyme activities, an indication that neither the cellulolytic nor xylanolytic enzymes of *A. fumigatus* MS16 require divalent metallic ions as cofactor (data not shown) as reported previously by for xylanase of *Penicillium citrinum* (Dutta *et al.*, 2007) and for β -glucosidase of *Trichoderma harzianum* (Yun *et al.*, 2001) cellulases from *Alternaria* sp. (Sohail *et al.*, 2011).

The presence of Mg^{2+} enhanced cellulase and xylanase activity, whereas, a slight increase in xylanase activity was observed when assay mixtures contained Ca^{2+} which is in agreement with the studies conducted by Fernandez-Espinosa *et al.* (1993) and Bakare *et al.* (2005). Furthermore, there was a potent inhibition of enzyme activity in presence of Ag^+ which was in line with Singh *et al.* (1990). The presence of cations like Co^{2+} , Cu^{2+} and Fe^{2+} moderately inhibit xylanase activity. There was, however, a complete loss of endoglucanase and β -glucosidase activity in the presence of Fe^{2+} . A similar finding was also made by Kim *et al.* (2005) for cellulase from alkalophilic *Bacillus* sp. where the presence of 1 mM Fe^{3+} or Hg^{2+} significantly inhibited the enzyme activity. An earlier report also suggests about the inhibitory action of Hg^{2+} on β -glucosidase activity of *Rhodotorula glutini* (Oikawa *et al.*, 1998).

Studies on enzymatic hydrolysis: Studies on enzymatic hydrolysis of plant materials (pea-peels, cabbage-twigs and grass) and commercially available oat-spelt xylan (Sigma, USA) by using crude enzyme preparations of *A. fumigatus* MS16 suggests a maximum hydrolysis of commercial as well as natural raw materials within 40 h (Fig. 1). Hydrolysis of oat-spelt xylan yielded 0.45 g l⁻¹ of reducing sugars after 32 hours that was much lower than reported by Gawande & Kamat (1999) where 7.2 g l⁻¹ and 8.2 g l⁻¹ of reducing sugars were released upon hydrolysis of oat-spelt xylan when the enzyme preparations from *A. tereus* and *A. niger*, respectively, were used. The higher yield obtained by these authors can be attributed to the strength of enzyme loadings.

Amongst the lignocellulosic substrates tested during this study, hydrolysis of cabbage-twigs resulted in a maximum of 0.42 mg ml⁻¹ of sugars after 30 hrs and the hydrolysis of pea-peels yielded 0.45 mg ml⁻¹ of reducing sugars. The yield obtained by the hydrolysis of both of these substrates was comparable to a yield from oat-spelt xylan. Grass appeared as the most resistant substrate to hydrolysis as it only gave a maximum of 0.12 mg ml⁻¹ of reducing sugars. Gomes *et al.* (1992) obtained up to 1.2 and 1.7 g of reducing sugars upon hydrolysis of corn-husk or orange-bagasse, respectively, by using enzymes of *Curvularia inaequalis*. A yield of 344.4 mg g⁻¹ of reducing sugar was obtained when orange peels were exposed to the crude enzymes of *A. niger* BTL after 168 h (Mamma *et al.*, 2007).

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