PRODUCTION OF Xylanases AND CELLULASES BY ASPERGILLUS FUMIGATUS MS16 USING CRUDE LIGNOCÉLLULOSONIC 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 (Qp) 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 hemicellulosic 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 (Ifikhar et al., 2010; Malik et al., 2013; Ifikhar 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; Sachchlechner 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 dinitrosalicylic 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 μmol 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 inoculated with 2 ml fungal spore suspension (5 x 10⁶/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 (Tm) 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 (AgNO₃, MnSO₄, EDTA, KCl, ZnSO₄, CaCl₂, NaCl, MgSO₄, CoCl₂, BaCl₂, CuSO₄ and FeSO₄) 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 Sachelehner et al. (1998) where a 4-fold increase in endoglucanase production was observed compared to xylanase when Sclerotium rolfsii 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. fumigates 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 deinking, waste disposal and to hydrolyze plant-biomass. Consequently, volumetric productivity (Qp) of cellulase and xylanase by A. fumigates MS16 at 35°C showed a 15-30 folds higher Qp 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.
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, Gupta & 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.
The presence of Mg²⁺ enhanced cellulase and xylanase activity, whereas, a slight increase in xylanase activity was observed when assay mixtures contained Ca²⁺ which is in agreement with the studies conducted by Fernandez-Espinar 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 Co²⁺, Cu²⁺ and Fe²⁺ moderately inhibit xylanase activity. There was, however, a complete loss of endoglucanase and β-glucosidase activity in the presence of Fe²⁺. A similar finding was also made by Kim et al. (2005) for cellulase from alkalophilic Bacillus sp. where the presence of 1 mM Fe³⁺ or Hg²⁺ significantly inhibited the enzyme activity. An earlier report also suggests about the inhibitory action of Hg²⁺ on β-glucosidase activity of Rhodotorula glutini (Okawa 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 1.7 g of reducing sugars upon hydrolysis of corn-husk 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).

Table 4. Xylanase and cellulase production by A. fumigatus MS16 on various natural substrates under SSF.

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

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylanase</td>
<td>60</td>
<td>4.5</td>
<td>66.5</td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>50</td>
<td>5.0</td>
<td>60</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>50</td>
<td>4.0</td>
<td>53.5</td>
</tr>
</tbody>
</table>

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


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