Homologue expression of a fungal endo-1,4-β-D-xylanase using submerged and solid substrate fermentations

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The xyn5 gene, which encodes an endo-β-1,4-xylanase (Xyn5), in Aspergillus niger GS1 was cloned into an expression cassette under the control of constitutive glyceraldehyde-3-phosphate dehydrogenase gene promoter. The expression system was designed to produce the recombinant enzyme containing a six-histidine peptide fused to the carboxyl end of the protein. The efficiency of Xyn5 production under submerged (SmF) and solid-state (SSF) fermentation was investigated using the homologous co-transformed A. niger AB4.1. A productivity of 17.1 U/(l·h) was estimated for SSF and 3.2 U/(l·h) for SmF calculated at peak value of enzyme titers. Recombinant Xyn5 obtained by SSF on polyurethane fiber, was purified 5.1-fold by anion exchange and immobilized metal affinity chromatography, with 35.7% recovery. The purified recombinant enzyme showed an apparent molecular weight of 30 kDa and optimal activity (522 U/mg protein) at pH 5.5 and 50°C.

Key words: Aspergillus niger GS1, xylanolytic activity, solid-state fermentation, homologue expression, polyurethane fiber.

INTRODUCTION

Hemicellulose is the second source of renewable organic carbon on earth, with a high potential for the recovery of useful end products (Park and Cho, 2010). Xylan constitutes the major component of hemicellulose, while endo-1,4-β-D-xylanases (E.C. 3.2.1.8) and exo-β-xylosidases (E.C. 3.2.1.37) catalyze the hydrolysis of xylan (Polizeli et al., 2011).

In order to obtain xylanolytic enzymes, there is a growing interest in developing both high yield and low cost processes for industrial applications, such as pharmaceutical products, bioconversion of agro-industrial residues, production of prebiotic xylo-oligosaccharides, among others (Dhiman et al., 2008; Antoine et al., 2010). In fact, xylanases are an important group of carbohydrate hydrolases, with a worldwide market of around US $200 million per annum (Mullai et al., 2010). Therefore, the search of strains showing generally recognized as safe status, able to grow in low cost substrates to optimize enzymes production is a highly relevant goal. Among existing technologies, solid-state fermentation (SSF) provides a suitable technique requiring low capital investment and energy supply, while a characteristic decrease in wastewater output as compared with classical sub-
merged fermentation (SmF) (de Castro et al., 2010). Additionally, SSF promotes a relatively low water activity environment favoring growth of fungi inoculated into a liquid medium impregnated in the solid substrate (Kapilan and Arasaratnam, 2011). Many inert materials have been reported for use in SSF, facilitating reproducible and detailed studies, involving perlite and polymeric resins (Gamarra et al., 2010), polyurethane foam or fiber (Montiel-González et al., 2004) and polystyrene (Gautam et al., 2002).

Filamentous fungi are more attractive than bacteria as potential enzyme producers since these microorganisms secrete higher levels of enzymes into the culture medium (Palaniswamy et al., 2008). Aspergillus niger is commonly used in strategies of SSF using inert supports (Rana and Bhat, 2005), to achieve functional and more stable (Palaniswamy et al., 2008).

Ab4.1 (JM109 (Promega, Madison, WI, USA) was used for propagation of fresh sterile potato dextrose agar (PDA; Bioxon, Cuautitlán, Mexico) supplemented with 100 mg/l ampicillin at 37° C. The pGEM-T vector and the termi- 
nator region of the trpC gene (both from Aspergillus nidulans) sepa- 
rated by BamHI and Ncol sites. Vector pAB4.1 which contains the A. nidulans pyrG gene (van Hartingsveldt et al., 1987) was used as the subcloning vector. A. niger AB4.1 (pyrG) strain (van Hartingsveldt et al., 1987) was used for homologous expression of xylanase (xyn5) gene. Vector pAN52.1 was used to construct the constitutive expression vector pANXyl. This vector contains the constitutive gadA promoter and the termi- 

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA), except those indicated.

Microorganisms and plasmid

A. niger GS1 (NCBI no. GU395669) was used as a source of xylanase (UAQ, Querétaro, Mexico). A. niger GS1 was stored in Tween 20 on silica gel at 4 °C. Stock cultures were sub-cultured on fresh sterile potato dextrose agar (PDA; Bioxon, Cuautitlán, Mexico) plates and incubated for 72 to 120 h at 30°C. Escherichia coli JM109 (Promega, Madison, WI, USA) was used for propagation of vectors and was cultured in Luria-Bertani medium (Ausubel et al., 2002) supplemented with 100 mg/l ampicillin at 37°C. The pGEM-T plasmid (Promega) was used as the subcloning vector. A. niger AB4.1 (pyrG) strain (van Hartingsveldt et al., 1987) was used for homologous expression of xylanase (xyn5) gene. Vector pAN52.1 was used to construct the constitutive expression vector pANXyl.

Molecular identification of A. niger GS1 endo-1,4-β-D-xylanase gene

Mycelia from A. niger GS1 grown in PDA slants were employed for genomic DNA (gDNA) extraction and isolation using the CTAB protocol (Ausubel et al., 2002). The endo-xylanase gene was amplified by PCR using gDNA as template. Primers were designed using NCBI reported sequences for A. niger endo-1,4-β-D-xylanase gene (ANU39784): XynF (forward) 5´-CGTTCTGCGGTTAAAGGTCA CTGCCGGC-3´; XynR (reverse) 5´-GGATCTTTAATGTTGATG GTGATGATGAAATCTCGTGACAC-3´. Bases coding for Hisα-tag are shown underlined and those coding for restriction sites (Ncol and BamHI for forward and reverse primers, respectively) are in italics. The amplified DNA was ligated into pGEM-T (Promega) vector and sent for sequencing (MCLab, San Francisco, CA, USA). After sequence confirmation, the DNA open reading frame was then, cloned into the expression vector pAN52.1 (cloning sites: Ncol and BamHI), to obtain pANXyl expression vector.

Aspergillus AB4.1 transformation

A. niger AB4.1 co-transformation was accomplished as previously reported (Sánchez and Aguirre, 1996) developed for A. nidulans, with modifications. All incubation temperatures were performed at 30°C, while growing medium was potato dextrose broth (Difco) supplemented with uridine (2.5 g/l). 2 µg total DNA (pANXyl expression vector plus pAB4.1 vector in a 3:1 volume ratio) was added to 50 µl of ice cold spore suspension. This mixture was electroporated using a MicroPulsar (Bio-Rad, Hercules, CA, USA), adjusting voltage to 7 kV/cm and pulses lasting approximately 4.3 ms. Spores (100 µl/pate) were extended on sorbitol-containing minimal agar (g/l): glucose, 10; sorbitol, 218.64; NaNO3, 6; KCl, 0.52,KH2PO4, 1.52; agar, 15; trace elements: ZnSO4·7H2O, 0.022; H2BO3, 0.011; MnCl2·4H2O, 0.005; FeSO4·7H2O, 0.005; CoCl2·6H2O, 0.0017; CuSO4·5H2O, 0.0016; Na2MoO4·H2O, 0.0015; Na2EDTA, 0.05; without uridine. Spores were incubated at 30°C for 48 h. Uridine prototrophy transformants stability was tested by veltrica replica plating on minimal medium. In addition, a control was transformed with the pyrG gene but without expression vector.

Screening and production of xylanase activity

Co-transformants were placed on minimal agar (without sorbitol) and incubated for 8 days, at 30°C. To screen co-transformants, up to 20 individual clones were inoculated (2×106 spores/ml) into 50 ml minimal medium and checked daily for xylanase activity during 4 days.

Fermentation systems

SmF was carried out in 50 ml tubes, inoculating 1.46x107 spores/ml in 10 ml glucose-rich medium (g/l): glucose, 50; yeast extract, 0.5; NaNO3, 7.5; (NH4)2SO4, 1.5; KCl, 8.67; MgSO4·7H2O, 8.67; trace elements. The mixture was incubated at 30°C in an orbital shaker (MRG, Hagavish, Holon, Israel) at 200 rpm, for up to 65 h. For SSF, locally produced commercial polyurethane fiber (PF) was washed with boiling water and oven dried for 24 h (WTC Binder, Tuttlingen, Germany) at 70°C. Glucose-rich medium and 1 g of dry PF placed in 250 ml flasks, were sterilized separately at 121 °C for 15 min. 10 ml of medium inoculated with 1.46x107 spores/ml were added to each flask, homogenized and incubated at 30°C using 250 rpm, for up to 65 h.

Biomass and enzyme extracts

Fermentation broth from SmF was filtered through Whatman no. 4 filter ( Maidstone, England). After SSF, extracts containing extra- 
cellular xylanase were obtained by compressing PF in a Buchner funnel lined with a Whatman no. 4 filter. Biomass was determined as the difference between initial and final weights after drying to constant weight at 70°C. Both filtrates were passed through a 0.45 µm pore size membrane (Millipore, Billerica, MA, USA) to remove any insoluble material and were labeled as xylanase extract (XE).

Protein and xylanase activity

Soluble protein content was determined according to Bradford
(1976), using bovine serum albumin as standard. Endo-1,4-β-D-xylanase activity was determined using 5 g/l oats spelt xylan as substrate, dissolved in 50 mM acetate buffer, pH 5.5. The reaction mixture contained 100 µl enzyme solution, 400 µl substrate and was incubated at 50°C for 10 min, followed by immersion in ice cold water. Released reducing sugars were quantified according to Miller (1959), using a xylose standard curve. One activity unit (U) was defined as the amount of enzyme that releases 1 µmol of xylose equivalents/min at 50°C. Specific activity (U/mg protein) was obtained dividing volumetric activity by soluble protein content. Endo-xylanase productivity was calculated as the product of specific activity multiplied by soluble protein content and dividing by fermentation time.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using 12% (w/v) polyacrylamide gels, according to Laemmli (1970) and protein bands were stained with Coomassie brilliant blue R-250 (Ausubel et al., 2002). Endo-xylanase activity was detected in the gel after electrophoresis, by cutting the bands.

Purification of the recombinant endo-1,4-β-D-xylanase

To purify the recombinant Xyn5, co-transformed A. niger was cultivated on SSF in glucose-rich medium. XE from 600 ml culture medium after 30 h fermentation was concentrated using a YM-10 Centricon filter unit (Millipore). Concentrated enzyme was applied into a DEAE-cellulose chromatography column (1.5 x 25 cm) equilibrated and eluted with 50 mM acetate buffer, pH 4.4, at a flow rate of 15 ml/h. Bound proteins were released by using a linear gradient of same elution buffer plus 1 M NaCl. 1 ml fractions were collected and assayed for absorbance at 280 nm and endo-xylanase activity. Active fractions were pooled, concentrated and loaded onto a Ni Sepharose 6 fast flow column (1 ml) (GE Healthcare, Uppsala, Sweden) following manufacturers manual.

Effect of temperature and pH on endo-1,4-β-D-xylanase activity

To determine optimal temperature of xylanase, activity determinations were conducted using 1 µg aliquots of purified recombinant Xyn5 and incubating at 30 to 80°C. Optimal pH was determined using same protein aliquots and total reaction volume. Activity was determined using 50 mM acetate buffer for pH values 3.6 to 5.0, 50 mM phosphate buffer for pH values 6.0 to 8.0 and 50 mM glycine buffer for pH 9.0. All experiments were conducted using three replicates.

RESULTS AND DISCUSSION

Molecular identification of endo-1,4-β-D-xylanase gene from A. niger GS1

A single amplification band was obtained from PCR products with an approximate size of 666 bp when primers XynR and XynF were used (data not shown). PCR product was inserted into pGEM-T vector and positive clones were identified by sequencing, showing up to 99% homology with A. niger ATCC 90196 xylanase (xyn5) mRNA complete coding sequence (U39784). Our sequences corresponded to a complete structural gene coding for endo-1,4-β-D-xylanase, plus a His$_6$ tag at the carboxyl end for rapid purification. This sequence obtained the NCBI database accession number GU585574. The ORF (654 bp) was predicted to code for a polypeptide of 217 amino acids with a molecular mass of 23.6 kDa and pl of 5.47 using ProtParam software (Gasteiger et al., 2005). The construction pANXyl contained a sequence coding for 16 amino acids of the own xyn5 signal peptide (AFA-AP), according to Bendtsen et al. (2004).

Expression of endo-1,4-β-D-xylanase gene from the gpdA promoter

From Pyr$^+$ regenerants co-transformed with plasmid pAB4.1 and pANXyl, up to three hundred transformants were identified. Fifty of those transformants were streaked twice on minimal plates and then, screened for endo-xylanase activity by growing in glucose-rich medium that represses Xyn5 synthesis in the wild-type strain. Five morphologically stable strains showed endo-xylanase activity and that showing slightly higher activity was chosen for further studies (gpd-Xyl-1).

Expression of recombinant proteins mediated by gpdA promoter has been reported (Pachlinger et al., 2005; Kainz et al., 2008). This promoter also allows recombinant protein expression by using glucose as carbon source instead of other inducing molecules such as xylan.

Effect of fermentation system on biomass and endo-1,4-β-D-xylanase production

Figure 1a shows biomass production profiles by A. niger gpd-Xyl-1 either under SmF or SSF. Growth curves showed a steady increase up to a maximum value after 41 h in SSF (30.3 g dry biomass/l) and in SmF (15.8 g dry biomass/l). This behavior without apparent presence of lag phase could be attributed to the use of the readily available carbon source (glucose). After about 41 h biomass did not show significant changes (p < 0.05) for both culture systems, probably associated to the exhaustion of some nutrients and/or lack of growth space.

Figure 1b and c, shows the profiles of extracellular soluble protein titers and endo-xylanase specific activity, respectively, obtained during growth of co-transformant A. niger gpd-Xyl-1 on SSF and SmF. Growth of this co-transformed strain in glucose rich medium, produced steadily increased endo-xylanase activity up to 28 and 41 h for SSF and SmF, respectively. After those times, both fermentation systems showed a strong specific activity decrease until the end of culture (65 h) (Figure 1c). This behavior is probably due to the presence of increased proteases release (data not shown), since protein content...
Figure 1. Growth profiles of A. niger gpd-Xyl-1 under SmF (○) and SSF (●). a) dry biomass; b) extracellular soluble protein titers; c) endo-xylanase specific activity.
kept increasing during fermentation time up to 50 h (Figure 1b). An increase in proteins concentration with fermentation time was also noticed by SDS-PAGE (Figure 2, lanes 2 and 4 versus 3 and 5). In the SSF system, high level of endo-xylanase activity (123.2 U/mg protein) was noticed after 28 h, while the highest activity under SmF (50.4 U/mg protein) was observed after 41 h of fermentation (Figure 1c). Endo-xylanase activity was not detected in any of the PyrG transformants. Additionally, our results are in agreement with Oda et al. (2006) who reported that Aspergillus oryzae secreted 4.0- to 6.4-fold more protein per mg mycelium at 32 and 40 h under SSF when compared with SmF.

A commonly cited problem regarding expression of heterologous proteins in fungi are host proteases (Gasser and Mattanovich, 2007) as well as cell morphology and bioreactor environment (Talabardon and Yang, 2007). The A. niger genome encodes 198 proteins involved in proteolytic degradation including a variety of secreted aspartyl endoproteases, serine carboxypeptidases and di- and tripeptidylaminopeptidases (Pel et al., 2007). The extracellular proteases sharply increase when cell growth enters the stationary phase (Talabardon and Yang, 2007). This effect may be correlated with data shown in Figure 1a to c, where protein concentration is rising and when biomass reaches stationary phase the specific activity decreases. Lu et al. (2010) reported that, A. niger shake cultures using xylose and maltose as carbon source are less favorable for recombinant protein production because endoplasmic reticulum-resident chaperones and foldases are present in lower amounts, while vacuolar proteases accumulate to higher levels.

The initial pH of SSF using A. niger gpd-Xyl-1 was 6.5 decreasing to 5.5 at 17 h of fermentation, to end at 5.8 after 65 h. SmF of same recombinant strain decreased pH from 6.5 to 5.6 after 17 h, ending at 6.2 after 65 h of fermentation. Some reports have shown that, fungal proteases production is strictly pH regulated reaching a maximum at pH 5.5 (McKelvey and Murphy, 2010; Sarao et al., 2010).

A productivity of 17.1 U/(l·h) was estimated for SSF and 3.2 U/(l·h) for SmF calculated at times of highest enzyme titers. Thus, productivity from SSF was 5.3 times higher than that from SmF, probably associated to about twice dry biomass. These results may also be explained by an increased number of active tips of growing hyphae during SSF, which are more porous, providing easier access of exoenzymes through the cell wall (Wang et al., 2005). Furthermore, te Biesebeke et al. (2005) suggest that, a higher number of wheat kernels penetrating hyphae per hyphal growth unit may explain the higher secretion of enzyme activities on A. oryzae mutant strains altered in the number of hyphal tips.

A. niger GS1 was not able to grow on SSF when glucose was not present in the medium, indicating that PF alone did not provide any carbon source to sustain growth. It is generally agreed that, enzymatic yields are higher in SSF in comparison to SmF (Ishida et al., 2006; Antoine et al., 2010). This might be related to the fact that, the fungus grows in similar conditions to those found in natural habitats (Dhiman et al., 2008). Díaz-Godinez et al. (2001) and te Biesebeke et al. (2002) hypothesized that, oxygen transfer phenomena, substrates concentration gradients, temperature and water content in solid supports are fundamental factors that account for differences on microbial physiology when SSF is compared with SmF.

**Purification of the recombinant endo-1,4-β-D-xylanase**

A. niger gpd-Xyl-1 produced one endo-xylanase in culture supernatants, which after affinity chromatography was purified 5.1-fold, with 35.7% activity recovery. Purified endo-xylanase showed a specific activity of 522 U/mg protein; showing a single protein band with an apparent molecular weight of 30 kDa (Figure 2). Endo-xylanase activity was confirmed from an isolated fragment of the gel containing this single band. Other Aspergillus spp. endo-xylanases have shown a similar size (6 to 50 kDa) as that of our purified enzyme (Polizeli et al., 2011).

Several endo-xylanases have been purified from Aspergillus spp., where the most active was one induced with wheat bran and xylan (5,870 U/mg protein) (Krisana et al., 2005), which is 11 times more active than that obtained here. Results similar to our study were found by...
Wakiyama et al. (2010) for an extracellular endo-1,4-β-xylanase with specific activity of 566 U/mg protein, purified from *Aspergillus japonicus* MU-2 grown on oat spelt xylan. However, about half the activity of the one showed here (288.7 U/mg protein) was reported for an endo-xylanase isolated from *Aspergillus ficuum* AF-98 on SSF of wheat bran and bagasse (Lu et al., 2008). On the other hand, using *Aspergillus carneus* M34 a xylanase obtained by SmF supplemented with oats spelt xylan showed 245.9 U/mg protein (Fang et al., 2008). In addition, Yang et al. (2010) purified and characterized an extracellular xylanase from *A. niger* C3486 grown under SmF showing activity of 123.4 U/mg protein. In relation to *Aspergillus* recombinant xylanases, one of the first reports for heterologous expression of fungal xylanase genes was done by Luttig et al. (1997), who successfully cloned xyn4 and xyn5 genes from *A. niger* ATCC 90196 in *Saccharomyces cerevisiae*. Moreover, high activity (3,330 U/mg protein) was achieved by heterologous expression of the xylanase gene from *A. niger* F19 in *Pichia pastoris* using SmF growth (Chen et al., 2010). Yi et al. (2010) reported a heterologous expression of a XYNA1 and XYNB in *E. coli* BL21 showing specific activities of 16.58 and 1201.7 U/mg protein, respectively. An endo-xylanase gene from *Aspergillus usamii* E001 was expressed in *E. coli* BL21 and the purified enzyme showed only 49.6 U/mg protein (Zhou et al., 2008). The use of a protease-negative strain of *A. niger* BRFM281 allowed xynB gene expression by SmF, obtaining 691 U/mg protein (Levasseur et al., 2005), which is about 1.3 times more active than the one reported here.

**Figure 3.** Effect of temperature and pH on purified endo-xylanase activity. (a) Effect of temperature on endo-xylanase activity in 50 mM acetate buffer, pH 5.5 (●). The ordinate represents relative activity that is the ratio of the activity at each tested temperature value to the activity found at optimal temperature 50 °C (530 U/mg protein), expressed as percentage; (b), effect of pH on endo-xylanase activity (●). The ordinate represents relative activity that is the ratio of the activity at each tested pH value to the activity found at optimal pH (480 U/mg protein), expressed as percentage. Each data point represents the mean of three independent experiments ± standard deviation.

**Effect of temperature and pH on recombinant endo-1,4-β-D-xylanase activity**

The optimum temperature for activity by our purified endo-xylanase was 50°C, while at 40 and 60°C, the enzyme exhibited 56.2 and 55.3% relative activity, respectively (Figure 3a). Optimum pH of purified endo-xylanase was 5.5 (Figure 3b). A relative activity of 49.7 and 21.6% was observed for pH values 5.0 and 7.1, respectively. These results are within the range of optimal temperature (40 to 70°C) and pH (4.0 to 6.0) of previously reported *Aspergillus* spp. endo-xylanases (Dhiman et al., 2008; Polizeli et al., 2011). Endo-xylanases produced by *A. niger*, *A. niveus*, and *A. ochraceus*, using SSF, wheat bran and corn cob as substrates showed same optimal temperature of 55°C, while optimum pH values were 6.0, 5.0 and 5.5, respectively (Betini et al., 2009). A recombinant xylanase reported by Li et al. (2010) showed similar values of optimal activity which were 50°C and pH 5.0.

A recombinant XynB showed an optimal activity at 50°C decreasing rapidly to 25% initial activity at 70°C. The pH of optimal activity of this recombinant protein was 5.5 and only 25% of the maximum was reached at pH below 3.5 and above 7.5 (Levasseur et al., 2005). This behavior is comparable to our recombinant endo-xylanase. A similar profile was observed for *A. niger* BRFM281 recombinant endo-xylanase expressed in *E. coli*. Optimal activity was found at pH 4.6, with 50% activity retention in the pH range 4.2 to 5.3, while optimal temperature at pH 4.6 was 50°C (Zhou et al., 2008).
We conclude that the expression system employed here, using PF as inert support, is a suitable alternative for production of homologous endo-xylanase by SSF, under conditions of selective expression of a single xylanase. This system provided higher enzyme yield, lower risk of contamination and an ecologically friendly process when compared with SmF system. This approach could be used to express individual proteins from gene families which are coordinately regulated. However, other biotechnological aspects such as optimization of bioreactor design and heat and mass transfer should be developed to make SSF a feasible technology to obtain value added products at commercial scale.

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REFERENCES


