African Journal of Biotechnology Vol. 10(10), pp. 1760-1767, 7 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1952
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

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

Amaro-Reyes, A.[1], García-Almendárez, B. E.[1], Vázquez-Mandujano, D. G.[1], Amaya-Llano, S.[1], Castaño-Tostado, E.[1], Guevara-González, R. G.[2], Loera, O.[3] and Regalado, C.[1]*

[1]DIPA, PROPAC. Facultad de Química, Universidad Autónoma de Querétaro, Centro Universitario s/n. Col. Las Campanas. Querétaro, 76010 Qro. México.
[2]C.A Ingeniería de Biosistemas, Facultad de Ingeniería, Universidad Autónoma de Querétaro, Centro Universitario s/n. Col. Las Campanas. Querétaro, 76010 Qro. México.
[3]Dpto. de Biotecnologia, Universidad Autónoma Metropolitana-Iztapalapa, 09340 México, D.F. México.

Accepted 25 January, 2011

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 glyceraldhehyde-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 carbohydrolases, 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-

*Corresponding autor. E-mail: carlosr@uaq.mx, regcarlos@gmail.com. Tel: +52 442 1921307. Fax: +52 442 1921304.

Abbreviations: CTAB, Cetyl trimethylammonium bromide; **DEAE,** diethyl aminoethyl; **NCBI,** National center of biotechnology information; **ORF,** open reading frame; **PCR,** polymerase chain reaction; **PDA,** potato dextrose agar; **PF,** polyurethane fiber; **SmF,** submerged fermentation; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **SSF,** solid-state fermentation; **XE,** xylanase extract.

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 recombinant proteins. The aim of this work was to compare the homologue expression of an endo-1,4-β-D-xylanase under SSF and SmF using a fungal system.

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. This vector contains the constitutive *gpdA* promoter and the terminator region of the *trpC* gene (both from *Aspergillus nidulans*) separated by *Bam*HI and *Nco*I sites. Vector pAB4.1 which contains the *A. nidulans pyrG* gene (van Hartingsveldt et al., 1987) was used as selection marker. Both vectors were kindly provided by Dr. Punt (TNO, The Netherlands).

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´-*CCATGG*ATGAAGGTCA CTGCGGC-3´, XynR (reverse) 5´-*GGATCC*TTAGTGGTGATG GTGATGATGAAGATATCGTGACAC-3´. Bases coding for His_6-tag are shown underlined and those coding for restriction sites (NcoI 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: *Nco*I and *BamH*I), 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 MicroPulser (Bio-Rad, Hercules, CA, USA), adjusting voltage to 7 kV/cm and pulses lasting approximately 4.3 ms. Spores (100 µl/plate) were extended on sorbitol-containing minimal agar (g/l): glucose, 10; sorbitol, 218.64; $NaNO_3$, 6; KCl, 0.52, KH_2PO_4, 1.52; agar, 15; trace elements: $ZnSO_4$•$7H_2O$, 0.022; H_3BO_3, 0.011; $MnCl_2$•$4H_2O$, 0.005; $FeSO_4$•$7H_2O$, 0.005; $CoCl_2$•$6H_2O$, 0.0017; $CuSO_4$•$5H_2O$, 0.0016; Na_2MoO_4•H_2O, 0.0015; Na_2EDTA, 0.05; without uridine. Spores were incubated at 30°C, for 48 h. Uridine prototrophy transformants stability was tested by velvet-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 (2×10^5spores/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 $1.46x10^7$ spores/ml in 10 ml glucose-rich medium (g/l): glucose, 50; yeast extract, 0.5; $NaNO_3$, 7.5; $(NH_4)_2SO_4$, 1.5; KCl, 8.67; $MgSO_4$•$7H_2O$, 8.67; trace elements. The mixture was incubated at 30°C in an orbital shaker (MRC, 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 $1.46x10^7$ 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 extracellular 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 pI 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

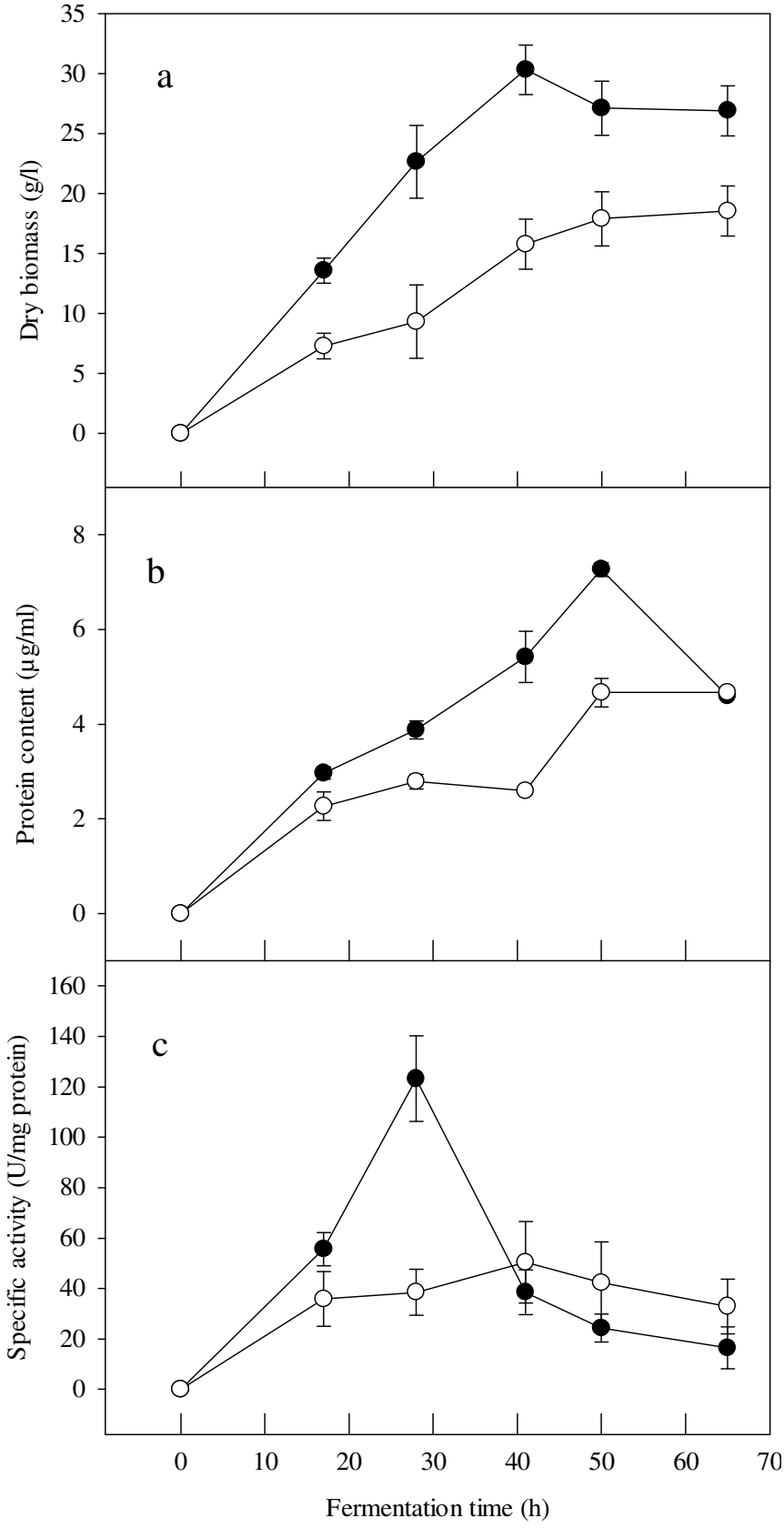

Figure 1. Growth profiles of *A. niger* gpd-Xyl-1 under SmF (○) and SSF (●). a) dry biomass; b) extracellular soluble protein titers; c) endo-xylanase specific activity.

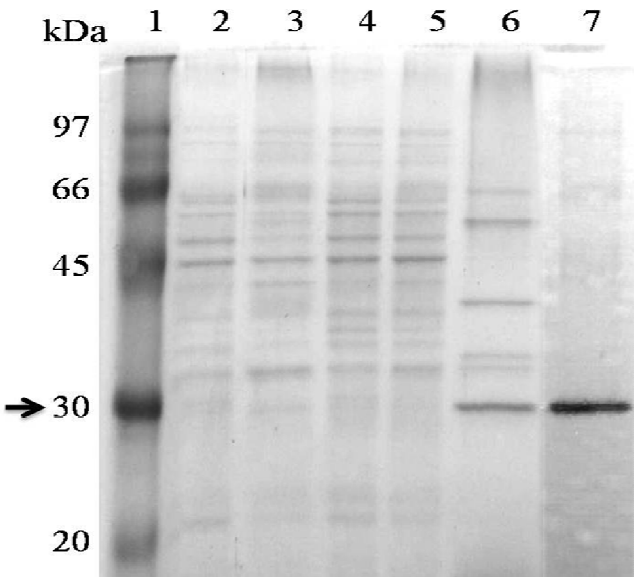

Figure 2. SDS-PAGE of xylanolytic extracts from *A. niger* gpd-Xyl-1 growth under different fermentation systems. Lanes: 1, low molecular weight markers (GE Healthcare); lane 2, extracellular proteins at 28 h of SSF; lane 3, extracellular proteins at 41 h of SmF; lane 4, extracellular proteins at 41 h of SSF; lane 5, extracellular proteins at 50 h of SmF; lane 6, semi-purified endo-xylanase after DEAE-cellulose chromatography; 7, purified endo-xylanase.

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 an easier pass 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

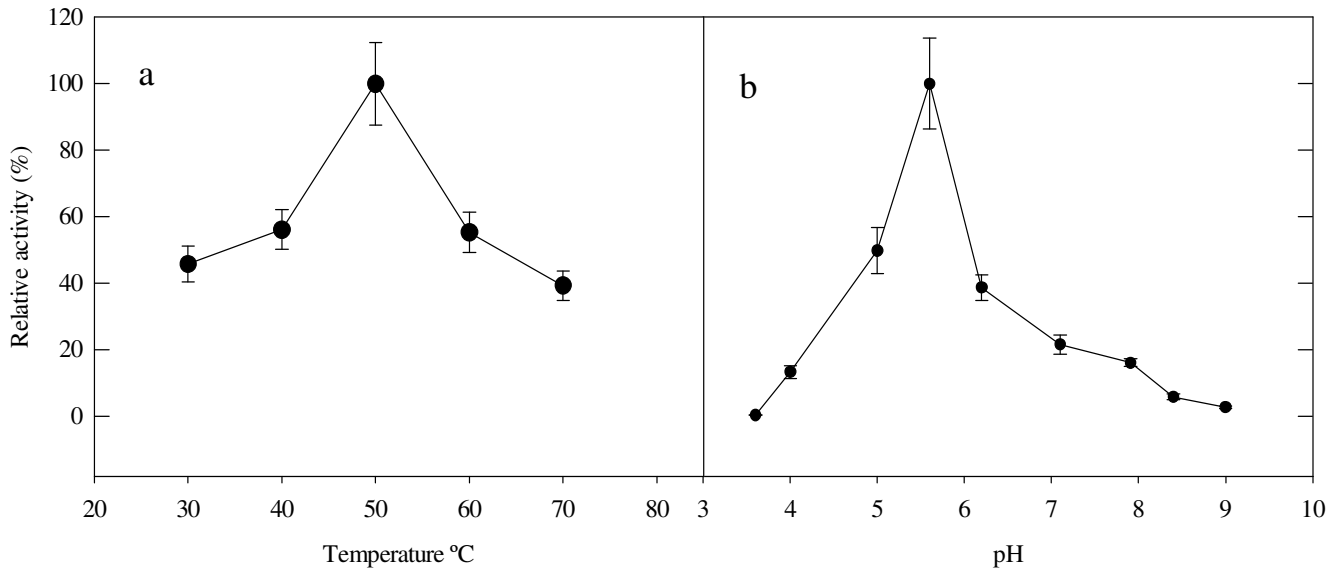

Figure 3. Effect of temperature and pH on purified endo-xylanase activity. (a) Effect of temperature on endo-xylanase activity in 50 mM acetate buffer, pH 5.5 (●). The ordinate represents relative activity that is the ratio of the activity at each tested temperature value to the activity found at optimal temperature 50 ºC (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.

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.

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.

ACKNOWLEDGEMENT

Thanks are given to Consejo Nacional de Ciencia y Tecnologia (Mexico) for PhD fellowship to AAR.

REFERENCES

Antoine AA, Jacqueline D, Thonart P (2010). Xylanase production by *Penicillium canescens* on soya oil cake in solid-state fermentation. Appl. Biochem. Biotechnol. 160(1): 50-62.

Ausubel FM, Brent RE, Kingston R, Moore DD, Seidman JG, Smith JA, Struhl K (2002). Short protocols in molecular biology. Wiley, USA, pp. 2-10, 10-38, 10-39.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004). Improved prediction of signal peptides: signalP 3.0. J. Mol. Biol. 340(4): 783-795.

Betini JHA, Michelin M, Peixoto-Nogueira SC, Jorge JA, Terenzi HF, Polizeli MLTM (2009). Xylanases from *Aspergillus niger, Aspergillus niveus* and *Aspergillus ochraceus* produced under solid-state fermentation and their application in cellulose pulp bleaching. Bioprocess Biosyst. Eng. 32(6): 819-824.

te Biesebeke R, Ruijter G, Rahardjo YSP, Hoogschagen MJ, Heerikhuisen M, Levin A, van Driel KGA, Schutyser MAI, Dijksterhuis J, Zhu Y, Weber FJ, de Vos WM, van den Hondel KAMJJ, Rinzema A, Punt PJ (2002). *Aspergillus oryzae* in solid-state and submerged fermentations. Progress report on a multi-disciplinary project. FEMS Yeast Res. 2(2): 245-248.

te Biesebeke R, Record E, van Biezen N, Heerikhuisen M, Franken A, Punt PJ, van den Hondel CAMJJ (2005). Branching mutants of *Aspergillus oryzae* with improved amylase and protease production on solid substrates. Appl. Microbiol. Biotechnol. 69(1): 44-50.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1-2): 248-254.

de Castro AM, Carvalho DF, Freire DMG, dos Reis Castilho L (2010). Economic analysis of the production of amylases and other hydrolases by *Aspergillus awamori* in solid-state fermentation of babassu cake. Enzyme Res. 2010: 1-9. Article ID 576872.

Chen X, Xu S, Zhu M, Cui L, Zhu H, Liang Y, Zhang Z (2010). Site-directed mutagenesis of an *Aspergillus niger* xylanase B and its expression, purification and enzymatic characterization in *Pichia pastoris*. Process Biochem. 45(1): 75-80.

Dhiman SS, Sharma J, Battana B (2008). Industrial applications and future prospects of microbial xylanases: a review. BioResources, 3(4): 1377-1402.

Díaz-Godínez G, Soriano-Santos J, Augur C, Viniegra-González G (2001). Exopectinases produced by *Aspergillus niger* in solid-state and submerged fermentation: a comparative study. J. Ind. Microbiol. Biotechnol. 26(5): 271-275.

Fang HY, Chang SM, Lan CH, Fang TJ (2008). Purification and characterization of a xylanase from *Aspergillus carneus* M34 and its potential use in photoprotectant preparation. Process Biochem. 43(1): 49-55.

Gamarra NN, Villena GK, Gutiérrez-Correa M (2010). Cellulase production by *Aspergillus niger* in biofilm, solid-state, and submerged fermentations. Appl. Microbiol. Biotechnol. 87(2): 545-551.

Gasser B, Mattanovich D (2007). Review. Antibody production with yeasts and filamentous fungi: on the road to large scale? Biotechnol. Lett. 29(2): 201-212.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005). Protein identification and analysis tools on the ExPASy server. In Walkered JM (ed) The Proteomics Protocols Handbook, protein identification and analysis tools on the ExPASy server, Humana, New Jersey, pp. 571-607.

Gautam P, Sabu A, Pandey A, Szakacs G, Soccol CR (2002). Microbial production of extra-cellular phytase using polystyrene as inert solid support. Bioresource Technol. 83(3): 229-233.

van Hartingsveldt W, Mattern IE, van Zeijl CMJ, Pouwels PH, van den Hondel CAMJJ (1987). Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol. Gen. Genet. 206(1): 71-75.

Ishida H, Hata YY, Kawato A, Abe Y (2006). Improvement of the *glaB* promoter expressed in solid-state fermentation (SSF) of *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. 70(5): 1181-1187.

Kainz E, Gallmetzer A, Hatzl C, Nett JH, Li H, Schinko T, Pachlinger R, Berger H, Reyes-Dominguez Y, Bernreiter A, Gerngross T, Wildt S, Strauss J (2008). N-Glycan modification in *Aspergillus* species. Appl. Environ. Microbiol. 74(4): 1076-1086.

Kapilan R, Arasaratnam V (2011). Paddy husk as support for solid state fermentation to produce xylanase from *Bacillus pumilus*. Rice Sci. 18(1) available online http://www.ricescience.org/qikan/manage/wenzhang/ 2010042.pdf

Krisana A, Rutchadaporn S, Jarupan G, Lily E, Sutipa T, Kanyawim K (2005). Endo-1, 4-β-xylanase B from *Aspergillus* cf. *niger* BCC14405 isolated in Thailand: purification, characterization and gene isolation. J. Biochem. Mol. Biol. 38(1): 17-23.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Levasseur A, Asther M, Record E (2005). Overproduction and characterization of xylanase B from *Aspergillus niger*. Can. J. Microbiol. 51(2): 177-183.

Li Y, Zhang B, Chen X, Chen Y, Cao Y (2010). Improvement of *Aspergillus sulphureus* endo-β-1,4-xylanase expression in *Pichia pastoris* by codon optimization and analysis of the enzymic characterization. Appl. Biochem. Biotechnol. 160(5): 1321-1331.

Lu F, Lu M, Lu Z, Bie X, Zhao H, Wang Y (2008). Purification and characterization of xylanase from *Aspergillus ficuum* AF-98. Bioresour. Technol. 99(13): 5938-5941.

Lu X, Sun J, Nimtz M, Wissing J, Zeng AP, Rinas U (2010). The intra- and extracellular proteome of *Aspergillus niger* growing on defined medium with xylose or maltose as carbon substrate. Microb. Cell Fact. 9(23): 1-13.

Luttig M, Pretorius IS, van Zyl WH (1997). Cloning of two b-xylanase-encoding genes from *Aspergillus niger* and their expression in *Saccharomyces cerevisiae*. Biotechnol. Lett. 19(5): 411-415.

McKelvey SM, Murphy RA (2010). Analysis of wide-domain transcriptional regulation in solid-state cultures of *Aspergillus oryzae*. J. Ind. Microbiol. Biotechnol. 37(5): 455-469.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31(3): 426-428.

Montiel-González AM, Viniegra-González G, Fernández FJ, Loera O (2004). Effect of water activity on invertase production by improved diploids strains of *Aspergillus niger*. Process Biochem. 39(12): 2085-2090.

Mullai P, Fathima NSA, Rene ER (2010). Statistical analysis of main and interaction effects to optimize xylanase production under submerged cultivation conditions. J. Agric. Sci. 2(1): 144-153.

Oda K, Kakizono D, Yamada O, Iefuji H, Akita O, Iwashita K (2006). Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. Appl. Environ. Microbiol. 72(5): 3448-3457.

Pachlinger R, Mitterbauer R, Adam G, Strauss J (2005). Metabolically independent and accurately adjustable *Aspergillus sp*. expression

system. Appl. Environ. Microbiol. 71(2): 672-678.
Park I, Cho J (2010). Partial characterization of extracellular xylanolytic activity derived from *Paenibacillus sp.* KIJ1. Afr. J. Microbiol. Res. 4(12): 1257-1264.
Palaniswamy M, Pradeep BV, Sathya R, Angayarkanni J (2008). Isolation, identification and screening of potential xylanolytic enzyme from litter degrading fungi. Afr. J. Biotechnol. 7(11): 1978-1982.
Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G , Hofmann G, Schaap5 PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JAE, van den Berg M, Breestraat S, Caddick MX, Contreras, R, Cornell M, Coutinho PM, Danchin EGJ, Debets AJM, Dekker P, van Dijck PWM, van Dijk A, Dijkhuizen L, Driessen AJM, d'Enfert C, Geysens S, Goosen C, Groot GSP, de Groot PWJ, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JPTW, van den Hondel CAMJ, van der Heijden RTJM, van der Kaaij, RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJEC, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NNME, Ram AFJ, Rinas U, Roubos JA, Sagt CMJ, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJJ, Wedler H, Wosten HAB, Zeng AP, van Ooyen AJJ, Visser J, Stam H (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat. Biotechnol. 25(2): 221-231.
Polizeli MLTM, Corrêa ECP, Polizeli AM, Jorge JA (2011). Hydrolases from microorganisms used for degradation of plant cell wall and bioenergy. In: Buckeridge MS, Goldman GH (Eds.). Routes to Cellulosic Ethanol. Springer, Dordrecht, Netherlands.
Rana NK, Bhat TK (2005). Effect of fermentation system on the production and properties oftannase of *Aspergillus niger* van Tieghem MTCC 2425. J. Gen. Appl. Microbiol. 51(4): 203-212.
Sánchez O, Aguirre J (1996). Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. Fungal Genet. Newslett. 43: 48-51.
Sarao L, Arora M, Sehgal VK, Bhatia S (2010). Production of protease by submerged fermentation using *Rhizopus microsporus* var *oligospous*. Internet J. Microbiol. 9(1): 1-10.
Talabardon M, Yang ST (2007). Production of GFP and glucoamylase by recombinant *Aspergillus niger*: effects of fermentation conditions on fungal morphology and protein secretion. Biotechnol. Prog. 21(5): 1389-1400.
Wakiyama M, Yoshihara K, Hayashi S, Ohta K (2010). An extracellular endo-1,4-β-xylanase from *Aspergillus japonicus*: purification, properties, and characterization of the encoding gene. J. Biosci. Bioeng. 109(3): 227-229.
Wang L, Ridgway D, Gu T, Moo-Young M (2005). Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. Biotechnol. Adv. 23(2): 115-129.
Yang Y, Zhang W, Huang J, Lin L, Lian H, Lu Y, Wu J, Wang S (2010). Purification and characterization of an extracellular xylanase from *Aspergillus niger* C3486. Afr. J. Microbiol. Res. 4(21): 2249-2256.
Yi X, Shi Y, Xu H, Li W, Xie J, Yu R, Zhu J, Cao Y, Qiao D (2010). Hyperexpression of two *Aspergillus niger* xylanase genes in *Escherichia coli* and characterization of the gene products. Braz. J. Microbiol. 41(3): 778-786.
Zhou C, Bai J, Deng S, Wang J, Zhu J, Wu M, Wang W (2008). Cloning of a xylanase gene from *Aspergillus usamii* and its expression in *Escherichia coli*. Bioresour. Technol. 99(4): 831-838.