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CLONING, EXPRESSION AND HYDROLYTIC CHARACTERIZATION OF THE NEW XYLANASE NSI XYN11A FROM *Neurospora sitophila* BDJ1I

CLONACIÓN, EXPRESIÓN Y CARACTERIZACIÓN HIDROLÍTICA DE LA NUEVA XILANASA NSI XYN11A DE *Neurospora sitophila* BDJ1I

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Abstract

The *Nsi xyn11A* gene, encoding the endo-1,4-*β*-xylanase Nsi Xyn11A was isolated from genomic DNA of *Neurospora sitophila* BDJ1I. The open reading frame of the *Nsi xyn11A* gene was 945 base pairs long and encoded a polypeptide of 314 amino acids with a calculated molecular mass of 33.3 kDa. The *Nsi xyn11A* gene was expressed in *Escherichia coli* and the recombinant enzyme was characterized to evaluate its hydrolytic capacity on lignocellulosic biomass, showing preference on *Agave tequilana* bagasse. The enzyme Nsi Xyn11A showed an optimal activity at pH 5.5 and 55 °C. Nsi Xyn11A is thermostable as it showed half-lives of more than 2h at 70 and 80 °C and more than 4 h at 50 and 60 °C. This is the first report describing the cloning and expression of an endo-1,4-*β*-xylanase encoding gene from *N. sitophila* and the hydrolytic characterization of the new xylanase Nsi Xyn11A. Our study showed that the xylanase Nsi Xyn11A may be suitable for industrial applications in the food and feed industries, in the production of short chain xylooligosaccharides, or in the pretreatment of lignocellulosic biomass required to improve the yields of fermentable sugars for bioethanol production.
Keywords: *Neurospora sitophila*, endo-xylanase, cloning, gene expression, hemicellulase.

Resumen

El gen *Nsi xyn11A* codificante para la endo-1,4-*β*-xilanasa Nsi Xyn11A se aisló de ADN genómico de *Neurospora sitophila* BDJ1I. El marco abierto de lectura del gen *Nsi xyn11A* contiene 945 pares de bases de longitud y codifica para un polipéptido de 314 de aminoácidos con una masa molecular calculada de 33.3 kDa. El gen *Nsi xyn11A* se expresó en *Escherichia coli* y la enzima recombinante se caracterizó para evaluar su capacidad hidrolítica sobre biomasa lignocelulósica, mostrando preferencia sobre bagazo de *Agave tequilana*. La enzima Nsi Xyn11A mostró una actividad óptima a pH 5.5 y 55 ºC. Nsi Xyn11A es termoestable ya que muestra vidas medias de más de 2 h a 70, 80 y 80 ºC y más de 4 h a 50 y 60 ºC. Este es el primer informe que describe la clonación y expresión de un gen que codifica para una endo-1,4-*β*-xilanasa de *N. sitophila* y la caracterización hidrolítica de la nueva xilanasa Nsi Xyn11A. Nuestro estudio mostró que la xilanasa Nsi Xyn11A puede ser adecuada para aplicaciones industriales en las industrias de alimentos y piensos, para la producción de xilooligosacáridos de cadena corta, o en el pretratamiento de biomasa lignocelulósica necesaria para mejorar los rendimientos de azúcares fermentables para la producción de bioetanol.
Palabras clave: *Neurospora sitophila*, endo-xilanasa, clonación, expresión de genes, hemicelulasa.

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1 Introduction

Hemicellulose is the second most common component of biomass on earth, and it is considered an important source of renewable substrate for energy production after cellulose. The complete hemicellulose hydrolysis constitutes the main technological challenge for its use as a renewable substrate. Thus, the enzymatic hydrolysis of this complex polymer is the best option to develop processes considered as green chemistry. Therefore, to transform hemicellulose in a more bio-based economy, a reduction of costs by increasing the hydrolysis efficiency is an important technological trait. Hemicellulase is composed of a complex mixture of different heteropolysaccharides, with xylan being the principal component. Xylan is formed by a linear backbone of β-1,4-linked xyloses residues, which is frequently substituted with a wide variety of chemical moieties side chain residues (Ahmed *et al*., 2009; Pastor *et al*., 2007). The total hydrolysis of xylan requires the cooperative action of several enzymes such as endo-xylanase, β-xylosidase, α-arabinosidase, and acetyl esterase. Among them, the endo-β-1,4-xylanases (EC 3.2.1.8) are essential enzymes since they initiate degradation of xylan into small xylooligosaccharides (Alvarez-Cervantes *et al*., 2016; Pastor *et al*., 2007).

Based on amino acid sequence similarities and hydrophobic cluster analysis, xylanases have been classified into two broad families 10 (GH10) and 11 (GH11) of glycoside hydrolases. In the case of the sequences of family 11 xylanases, computer analysis revealed that this group of enzymes shows a single domain and a clear amino acid similarity, all having a common hold resembling a partly closed right hand. The catalytic residues are two conserved glutamate residues, which are located opposite to each other in an open active site cleft (Pastor *et al*., 2007). Usually, microbial xylanases have attracted considerable research interest due to their potential industrial applications in food, animal feed, textile and paper and pulp industries, waste treatment for separation of cellulose and in bioethanol production (Arrizon *et al*., 2012; Juodeikiene *et al*., 2012; Ramírez-Carmona and Muñoz-Blandón, 2016). Recently, xylanases have found an opportunity to potential application thanks to their capacity to break down xylan into short chain xylooligosaccharides (XOS). The importance of XOS has increased in the recent years, mainly because of their prebiotic properties and their potential use in food and health industries (Faryar *et al*., 2015; Sun *et al*., 2015).

However, such applications prefer a thermostable and cellulase-free xylanase with broad pH spectrum and higher optimal temperature. The pulp and paper industry is a primary target for the use of xylanases. The bio-bleaching of pulps would have environmental benefits, as the use of chlorine could be reduced using xylanases during processing (García-Rivero *et al*., 2015; Kumar and Satyanarayana, 2011). Xylanases are also needed to more fully hydrolyze lignocellulosic biomass into simple sugars that can then be fermented to products, such as liquid fuel and chemical feedstocks. Most of the researches have been focused on developing thermophilic enzymes that can tolerate high temperatures (Lee and Lim 2004; Sun *et al*. 2005). Elevated temperatures, increases the hemicellulose hydrolysis rate; nevertheless, for some bioprocesses, the use of psychrophilic enzymes is preferred (Collins *et al*., 2005; Lee *et al*., 2006). On the other hand, thermostable cellulase-free xylanases are important in many industrial applications; for this reason, recombinant xylanases expressed in mesophilic microorganism are a good option to produce these enzymes and thus avoid the processes of purification.

In Mexico, the biotechnology industries based on hydrolysis of lignocellulosic residues are incipient; this is despite having a significant number of lignocellulosic residues. The sugarcane bagasse (SCB), agave bagasse (ATB) and wheat straw (WS) are three major agro wastes. The ATB is the largest residue from tequila, mezcal, and traditional fermented beverages processing. In 2015 were produced 270 thousand tons of ATB only from tequila industries. On the other hand, the generation of WS was estimated in 4,612 thousand tons per year in Mexico. WS and ATB have not a meaningful use in Mexico; around of 15% of WS is used for food for cattle, soil improver and substrate for mushroom cultivation, the rest of WS is burned in the farmlands. On the other hand, near of 5% of ATB generated is used as a soil improver and the rest have not a real economic value. In contrast, almost all the 8 million tons per year produced of SCB are burned to produce energy in the sugarcane factories; however, this residue could have a better used. For this reason, SCB, WS, and ATB could become an important biomass source for development bio-based economy in Mexico. However, first, it is necessary to found specific enzymes to hydrolysate the different components of these agro wastes.

In this context, most industrial xylanases come from a fungal microorganism. The genus *Neurospora*

has been extensively used to investigate the induction of lignocellulosic enzymes, specifically the fungus *N. crassa*. However, other species of this genus have been little studied to produce these proteins, such as *N. sitophila*. In Mexico, *N. sitophila* can be found living in the three residues before mentioned. Moreover, in previous works of our research group, we observed that *N. sitophila* could produce lignocellulolytic enzymes when it grows on different agro wastes. However, the productivity of this system is low, because the time of xylanase activity induction is long.

For the above mentioned, in this work, we describe for the first time the cloning, expression and hydrolytic characterization of the cellulase-free Nsi Xyn11A xylanase from *N. sitophila* DBJ1I. Nsi Xyn11A belongs to the GH11 family, and this enzyme may be applied in the efficient hydrolysis of hemicellulose at high temperature for the XOS production or in the pretreatment of lignocellulosic biomass to increase the ethanol production.

2 Materials and methods

2.1 Strains and fungus identification

Fungus strain *N. sitophila* BDJ1I was isolated from *Agave tequilana* bagasse (Atotonilco municipality, Jalisco, Mexico). *N. sitophila* BDJ1I was first separated by macro and microscopic morphological characteristics and then was subcultured to purity and examined for xylanolytic activity. *N. sitophila* was maintained on PDA medium at 37 °C from 2 to 3 days. *E. coli* EZ and M15 [pREP4] strains were grown in LB or 2TY medium, supplemented with kanamycin (30 μg ml^{-1}) and ampicillin (100 μg ml^{-1}).

The identification of the *N. sitophila* BDJ1I was performed by sequence analysis of the PCR amplified region ITS1-5.8-ITS4. The genomic DNA was extracted from mycelia of *N. sitophila* using DNeasy Plant Mini Kit (QIAGEN). The genomic DNA concentrations of stock solutions were determined in a spectrophotometer (ND-1000 UV/Vis, Nano Drop Technologies). The ITS1-5.8-ITS4 region was amplified with the primers ITS1 (5′- TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR reactions were performed with HotStar Taq Master Mix (QIAGEN). Sequence reactions of ITS region were performed by Macrogen USA Corp. (Rockville, MD). The sequences obtained was compared with those available in the GenBank database.

2.2 Expression of Nsi xyn11A gene in E. coli

For the isolation of the xylanase gene of *N. sitophila*, degenerate primers were designed based on the sequence of the endo-1,4-β-xylanase B of *N. crassa* (Q871E8). *Nsi xyn11A* gene amplification was performed using primers XYNC-F (GGATCCATGGTTGCTTTCTCCTCCATT with restriction site BamHI) and XYNC-R (AAGCTTCTACACGCAATGCGAATACCACTG with restriction site HindIII) and the genomic DNA of *N. sitophila*. PCR was performed with Taq polymerase Hot Star Master Mix (QIAGEN) using the following program, 1 cycle of 5 min at 95 °C, 35 cycles of 25 s at 95 °C, 45 s at 57 °C and 45 s at 72 °C, 1 cycle of 7 min at 72 °C in a 96-well Veriti thermocycler (Applied Biosystems).

The product of the PCR reaction was cloned into pDrive (QIAGEN) cloning vector; then the *Nsi xyn11A* gene was released from pDrive and purified for subsequent cloning into pQE-30 Xa expression vector, to obtain the construct pQE-30Xa/Nsixyn11A. This plasmid was analyzed by digestion with restriction enzymes and DNA sequencing and then transferred to *E. coli* M15[pREP4] for protein expression assays.

2.3 Production of xylanolytic extract

One positive clone of *E. coli* with xylanase activity was grown in 2TY medium to a cell density of OD600nm = 0.6. IPTG was added to reach a 0.5 mM final concentration; then the culture was incubated for 5 h at 37 °C and 200 rpm. Cells were harvest and then lysed with lysozyme (10 mg ml^{-1}) at 4 °C during 30 min. Cell debris was removed by centrifugation (10,000g at 4 °C for 15 min), and the supernatant was used as a source for crude xylanase, it was analyzed for xylanase activity and was used for further analysis.

2.4 SDS-PAGE and zymogram analysis

Protein analyses were carried out by 10% SDS-PAGE, using the MiniProtean III system (Bio-Rad). Proteins in the gel were stained with Coomassie brilliant blue R-250, and molecular weight was estimated regarding broad range molecular weight protein standards. Protein concentration was measured using the Lowry method with bovine serum albumin as standard. Crude xylanase extracts were used to detect xylanase activity on zymograms. Xylanase zymogram analysis was done on 8% polyacrylamide native gel containing

0.5% of birchwood xylan and 0.5% of Congo Red. Gels were incubated at 50 °C for 1 h.

2.5 Xylanase assay

A 50 μl xylanolytic extract aliquot was added to 1 ml citrate-phosphate buffer (0.1 M, pH 5.5), containing birchwood xylan (0.5% w/v). Xylanase activity was determined from the amount of reducing sugars released during incubation at 55 °C. Reducing sugars were measured with the dinitrosalicylic acid (DNS) method, using xylose as standard. One international unit (IU) of activity was defined as 1 μmol of xylose released per minute under the assay conditions. All tests were carried out in duplicate and the average values were recorded.

2.6 Determination of optimum temperature, pH and thermostability of Nsi Xyn11A

The temperature and pH optima were determined by measuring the xylanase activity with a pH range from 4.0 to 6.5 and a temperature range from 30 to 75 °C. The thermostability of recombinant xylanase was determined by measuring the residual activity of the enzyme, exposed to six different temperatures: 50, 60, 70, 80, 85 and 90 °C, in citrate-phosphate buffer, 0.1 M, pH 5.5 during 5.0 h. At the end of each 30 min time interval, an aliquot of xylanase was removed and assayed to reveal enzymatic activity, as described above. The inactivation rate constants (k_d) were calculated by following a method described by Akgöl *et al.* (2001).

2.7 Evaluation of the hydrolytic capacity of Nsi Xyn11A on lignocellulosic biomass

The hydrolytic capacity of Nsi Xyn11A on lignocellulosic biomass was evaluated, 40 IU ml^{-1} of xylanase activity were added to citrate-phosphate buffer (0.1 M, pH 5.5) containing milling WS or ATB or SCB (20% w/v). Xylan was used as a control. Hydrolytic capacity was determined from the amount of reducing sugars released after incubation (50 ºC and 350 rpm) at 24 and 48 h. Nsi Xyn11A was compared with two commercial xylanases, Novozyme HTec2 (enzymatic complex with endo-1,4-β-xylanase, endo-1,4-β-glucanase, polygalacturonase, and cellobiohydrolase activities) and Sigma X2629, under the same assay conditions.

3 Results and discussion

3.1 Fungus identification

Since the fungus used in this research was isolated from mounds of agave bagasse, it was necessary to apply molecular identification techniques to know the genus and species of the fungus. The analysis of the PCR amplified region ITS1-5.8-ITS4 of the fungus showed a fragment of 587 bp. The ITS region sequence was subjected to BLAST analysis in the GenBank database. According to this result, the fungus isolated from ATB was identified as *N. sitophila* with an identity of 100% (data no shown). For this reason, the fungus was named *N. sitophila* strain BDJ1I. Since the ITS region of *N. sitophila* BDJ1I showed a high identity with *N. crassa*, a pair of primers designed from the endo-1,4-β-xylanase B of *N. crassa* (Q871E8) were used for amplified a xylanase gene in *N. sitophila* BDJ1I.

3.2 In silico analysis of the Nsi xyn11A gene sequence

The nucleotide sequence analysis indicated an open reading frame (ORF) of 945 bp, which encodes a 314-residue polypeptide with a calculated molecular mass of 33.3 kDa (Fig. 1). The new Nsi Xyn11A xylanase was named according to the nomenclature proposed by Henrissat *et al.* 1998.

The in silico analysis of amino acids sequence revealed that Nsi Xyn11A of *N. sitophila* is a modular xylanase containing a catalytic domain belonging to the glycosyl hydrolases family 11 (GH11), which consists of 213 amino acid residues (positions 36 to 249). The catalytic domain shows the typical β-jelly-roll architecture, constituted of two anti-parallel β-sheets named A and B molding a long and deep cleft (Fig. 3). The enzyme shows a carbohydrate binding module (CBM) at the C-terminal with 29 amino acid residues (positions 282 to 310); according to the in silico analysis this domain can be classified as CBM type I. There are several reports that CBM structure potentiates the enzymatic activity in xylanases, and it plays an important role in thermostability of GH11 xylanases (Wang and Xia, 2008; Zhang-Ling *et al.*, 2012, Pavón-Orozco *et al.*, 2012).

Moreover, the multiple alignments of the amino acid sequence of the xylanase Nsi Xyn11A with other xylanases showed the two signature patterns of the GH11 xylanase family, which are highly conserved motifs in this type of xylanases (Fig.

2). Nsi Xyn11A shows a high similarity to several known xylanases including a putative endo-1,4-*β*-xylanase B of *N. crassa* (72%; UniProtKB Q871E8 and Q1K5S8), a putative endo-1,4-*β*-xylanase B from *N. tetrasperma* strain FGSC 2509/P0656 (72%; UniProtKB G4UEW4), a probable endo-1,4-*β*-xylanase B from *N. tetrasperma* strain FGSC 2508/ATCC MYA-4615/P0657 (78%, UniProtKB F8MAI8). However, Nsi Xyn11A varies from most GH11 xylanases, due to its large loop A5-B3, which is 25-residues longer than other xylanases (Fig. 2 and Fig. 3); this loop is related with the thermostability of the family 11 xylanases (Paës *et al*. 2012).

ATG	GTT	GCT	TTC	TCC	TCC	ATT	CTA	CTC	GCT	GCC	TCG	ACA	GCC	ATC	45
M	V	A	F	S	S	I	T	T	A	A	S	T	A	I	15
TCT	GGC	GTC	TTC	GCC	CTC	CCC	GGC	GAG	CTT	CCC	GGT	CAG	GGC	AGC	90
S	G	V	F	A	T	P	G	E	T	P	G	Q	G	S	30
CTC	TTC	AAG	CGC	CAA	ACC	TAC	ACC	TCG	TCG	GCC	ACT	GGC	ACT	CAT	135
T	F	K	R	Q	T	Y	T	S	S	A	T	G	T	H	45
AAC	GGC	TAC	TAC	TTC	TCC	TTC	TGG	ACC	GAC	GGC	CAG	GGC	AGC	GTC	180
N	G	Y	Y	F	S	F	W	T	D	G	Q	G	S	V	60
CGG	TAC	ACC	AAC	GAG	GCT	GGT	GGC	CAA	TAC	ACT	GCC	ACA	TGG	TCT	225
R	Y	T	N	E	A	G	G	Q	Y	T	A	T	W	S	75
GGC	AAC	GGC	AAC	TGG	GTC	GGT	GGA	AAG	GGT	TGG	ATG	CCC	GGT	ACT	270
G	N	G	N	W	V	G	G	K	G	W	M	P	G	T	90
GAT	CGT	ATG	TAT	CTC	TCT	CTC	TCT	CTT	CTC	GCT	TTC	TCA	TGG	CTT	315
D	R	M	Y	T	S	T	S	T	T	A	F	S	W	T	105
GTC	TCA	ACA	ACT	AAC	CCT	TTC	TCA	CCC	ATG	TAC	AGC	ACC	ATC	AAC	360
V	S	T	T	N	P	F	S	P	M	Y	S	T	I	N	120
TAT	ACC	GGC	ACC	TAC	TCG	CCC	AAC	GGC	AAC	TCG	TAC	CTC	GCC	GTC	405
Y	T	G	T	Y	S	P	N	G	N	S	Y	T	A	V	135
TAC	GGC	TGG	ACG	CGC	AAC	CCG	CTC	ATC	GAG	TAC	TAC	GTC	GTC	GAG	450
Y	G	W	T	R	N	**P**	**T**	**I**	**E**	**Y**	**Y**	**V**	**V**	**E**	150
AAC	TTT	GGC	ACT	TAC	AAC	CCC	AGC	ACG	GGC	GCC	ACC	CGT	CTC	GGG	495
N	**F**	G	T	Y	N	P	S	T	G	A	T	R	T	G	165
TCC	GTC	ACC	TCG	GAC	GGC	GGC	GTC	TAC	GAC	ATC	TAC	CGC	ACC	CAG	540
S	V	T	S	D	G	G	V	Y	D	I	Y	R	T	Q	180
CGC	GTC	AAC	CAG	CCC	TCC	ATC	GAC	GGC	ACC	GCC	ACT	TTT	TAC	CAG	585
R	V	N	Q	P	S	I	D	G	T	A	T	F	Y	Q	195
TAC	TGG	TCC	GTC	CGC	CAG	CAG	AAG	CGC	ACG	GGC	GGC	AGT	GTC	AAT	630
Y	W	S	V	R	Q	Q	K	R	T	G	G	S	V	N	210
ATG	AAG	AAC	CAC	TTT	GAC	GCC	TGG	ACT	AGG	TCC	GGC	CTC	ACG	CTC	675
M	K	N	H	F	D	A	W	T	R	S	G	T	T	T	225
GGC	ACC	CAC	AAC	TAC	CAG	ATT	GTT	GCT	ACC	GAG	GGA	TAC	TAT	TCC	720
G	T	H	N	Y	Q	I	**V**	**A**	**T**	**E**	**G**	**Y**	**Y**	**S**	240
AGT	GGC	AGC	AGT	ACT	ATC	AAT	GTT	GGT	GCC	AGC	GGC	GGC	GGT	GAT	765
S	**G**	**S**	**S**	T	I	N	V	G	A	S	G	G	G	D	255
AGC	ACT	ACC	CCC	GAG	GAC	CCG	GGC	ACG	ACT	ACC	ACT	CCT	ACT	AAC	810
S	T	T	P	E	D	P	G	T	T	T	T	P	T	N	270
CCT	GGG	AAT	GGA	GGA	GAC	GGT	AGC	TGC	GCG	GCT	AAG	TGG	GGA	CAG	855
P	G	N	G	G	D	G	S	C	A	A	K	W	G	Q	285
TGC	GGT	GGG	CAG	GGA	TGG	AGT	GGT	GCT	ACT	TGC	TGC	CAG	TCT	GGA	900
C	G	G	Q	G	W	S	G	A	T	C	C	Q	S	G	300
TCG	ACT	TGC	CAG	GCT	GCT	AAC	CAG	TGG	TAT	TCG	CAT	TGC	GTG	TAG	945
S	T	C	Q	A	A	N	Q	W	Y	S	H	C	V	*	314

Fig. 1. Nucleotide sequence of the *Nsi xyn11A* gene and its respective predicted amino acid sequence. Signal peptide (- -), glycoside hydrolase signature (underlined amino acids) and CBM type I (amino acids inside the square).
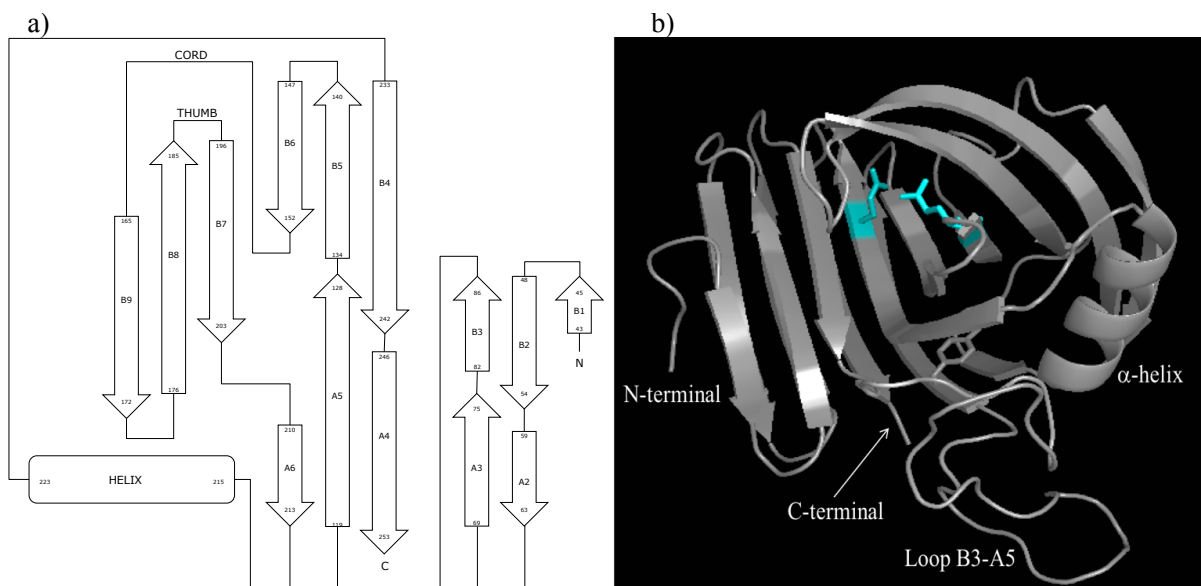
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Nsi Xyn11A    MVAFSSITTAASTAISGVFALPGETPGQGSTFKRQTYTSSATGTHNGYYFSFWTDGQGSV
Q1K5S8        MVAFSSILLAASTAISGVFALPGELPGQGSLFKRQTYTSSATGTHNGYYFSFWTDGQGSV
Q871E8        MVAFSSILLAASTAISGVFALPGELPGQGSLFKRQTYTSSATGTHNGYYFSFWTDGQGSV
G4UEW4        MVAFSSILLAASTVISGVFALPGELPGQGSLFKRQTYTSSATGTHNGYYFSFWTDGQGSV
F8MAI8        MVAFSSILLAASTVISGVFALPGELPGQGSLFKRQTYTSSATGTHNGYYFSFWTDGQGSV
TcXylC        -MKLSLAAIGICTTAAVAFPSGLTQHATGDLSKRQSITTSQTGTNNGYYYSFWTNGGGEV
              : :*       . ..*. : .*.         . *.  ***: *:* ***:****:****:* *.*

Nsi Xyn11A    RYTNEAGGQYTATWSGNGNWVGGKGWMPGTDRMYTSTSTTAFSWTVSTTNPFSPMYSTIN
Q1K5S8        RYTNEAGGQYTATWSGNGNWVGGKGWMPGTD-----------------------RTIN
Q871E8        RYTNEAGGQYTATWSGNGNWVGGKGWMPGTD-----------------------RTIN
G4UEW4        RYTNEAGGQYTATWSGNGNWVGGKGWMPGTD-----------------------RTIN
F8MAI8        RYTNEAGGQYTATWSGNGNWVGGKGWMPGTDR----------------------TIN
TcXylC        TYTNGDNGEYSVTWVNCGDFTSGKGWNPANAQ---------------------TVT
              ***     .*:*:.** .  *:::.**** *..                        *:.

Nsi Xyn11A    YTGTYSPNGNSYTAVYGWTRNPTIEYYVVENFGTYNPSTGATRTGSVTSDGGVYDIYRTQ
Q1K5S8        YTGTYSPNGNSYLAVYGWTRNPLIEYYVVENFGTYNPSTGATRLGSVTSDGGVYDIYRTQ
Q871E8        YTGTYSPNGNSYLAVYGWTRNPLIEYYVVENFGTYNPSTGATRLGSVTSDGGVYDIYRTQ
G4UEW4        YTGTYSPNGNSYLAVYGWTRNPLIEYYVVENFGTYNPSTGATRLGSVTSDGGVYDIYRTQ
F8MAI8        YTGTYSPNGNSYLAVYGWTRNPLIEYYVVENFGTYNPSTGATRLGSVTSDGGVYDIYRTQ
TcXylC        YSGEFNTSGNAYLAVYGWTTDPLVEYYILESYGTYNPSSGLTLLGQVTSDGGTYDIYSTQ
              *:* :...**:* ****** :* :***::*.:*******:* *  *.******.**** **

Nsi Xyn11A    RVNQPSIDGTATFYQYWSVRQQKRTGGSVNMKNHFDAWTRSGTTTGTHNYQIVATEGYYS
Q1K5S8        RVNQPSIDGTATFYQYWSVRQQKRTGGSVNMKNHFDAWTRSGLTLGTHNYQIVATEGYFS
Q871E8        RVNQPSIDGTATFYQYWSVRQQKRTGGSVNMKNHFDAWTRSGLTLGTHNYQIVATEGYFS
G4UEW4        RVNQPSIDGTATFYQYWSVRQQKRTGGSVNMKNHFDAWTRSGLTLGTHNYQIVATEGYFS
F8MAI8        RVNQPSIDGTATFYQYWSVRQQKRTGGSVNMKNHFDAWTRSGLTLGTHNYQIVATEGYFS
TcXylC        RVDQPSIEGTSTFNQYWSVRTEKRVGGTVTTANHFAAWKALGLEMGTYNYMIVSTEGYES
              **:****:**:** ****** :**.**:*.  *** **.   *   **:** **:**** *

Nsi Xyn11A    SGSSTINVGASG----GGDSTTPEDPGTTTTPTNPGNGGDGSCAAKWGQCGGQGWSGATC
Q1K5S8        SGSSTINVGASGGSGGGGGGTTPENPGTTTTPTNPGNGGGGSCAAKWGQCGGQGWSGATC
Q871E8        SGSSTINVGASGGSGGGGGGTTPENPGTTTTPTNPGNGGGGSCAAKWGQCGGQGWSGATC
G4UEW4        SGSSTINVGASS-DGGGGGGTTPENPGTTTTPTNPGNGGGGSCAAKWGQCGGQGWSGATC
F8MAI8        SGSSTINVGASS-DGGGGGGTTPENPVRISGRLSLSRK--------------------
TcXylC        SGSSTITVS-----------------------------------------------
              ******.*.

Nsi Xyn11A    CQSGSTCQAANQWYSHCV
Q1K5S8        CQSGSTCQAANQWYSQCV
Q871E8        CQSGSTCQAANQWYSQCV
G4UEW4        CQSGSTCQAANQWYSQCV
F8MAI8        ------------------
TcXylC        ------------------
```

Fig. 2. Alignment of amino acids sequence of family 11 xylanases. Nsi Xyn11A of *Neurospora sitophila* (this work), *Neurospora crassa* (Q1K5S8), *Neurospora crassa* (Q871E8), (G4UEW4), (F8MAI8), (TcXylC). Conserved and identical amino acids are indicated with asterisk (*) and points (.), respectively. Underline motifs: signal peptide (- - -), glycoside hydrolase signature (–), CBM type I (---), β-sheet A5 ($\sim\!\sim\!\sim$), β-sheet B3 ($=\!=$) and the loop B3-A5 (cursive letter). Gaps are indicated by dashes.

Fig. 3. Structure of Nsi Xyn11A. a) topology diagram (adapted from Sapag et al., 2002) and b) cartoon representation (Swiss-model, Expasy).
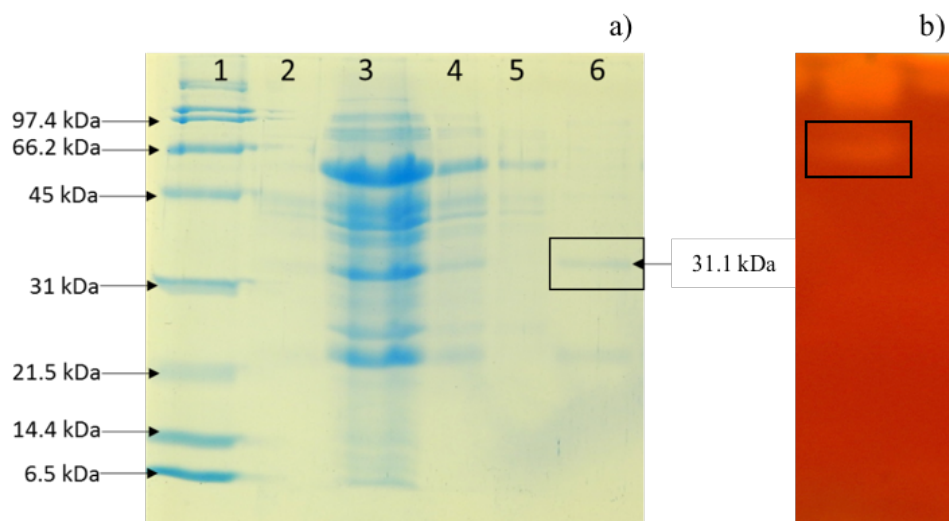

Fig. 4. Protein analysis of recombinant xylanase. a) SDS-page, lane 1, MW protein standards; lane 2, total proteins before induction (negative control); lane 3, total proteins after induction and lane 4-6, partial purification stages of Nsi Xyn11A. b) Zymogram of xylanases induced in *E. coli* after induction. Square show bands with xylanase activity.

3.3 Expression of Nsi xyn11A gene in E. coli

The coding region of the *Nsi xyn11A* gene was expressed from *E. coli* M15[pREP4] for a functional analysis of the recombinant protein. In comparison to non-induced cells, the heterologous protein was expressed by induced cells. After inducing cells, recombinant xylanase was produced intracellularly and partially secreted into the culture supernatant. SDS-PAGE analysis of extracts from the positive clone with xylanase activity revealed a band of approximately 31 kDa, the MW of this band conforms to the theoretical MW of the mature protein without the signal peptide, 31.3 KDa (Fig. 4). Similar results have been reported for other recombinant xylanases expressed in *E. coli* (Amaya-Delgado *et al.* 2010; Zhang *et al.* 2012). The Nsi Xyn11A functionality was

evaluated on zymograms; one band with xylanolytic activity was detected in the crude extracts, which is indicative of *E. coli* synthesized an active xylanase (Fig. 4).

E. coli has been extensively used for cloning and expression of xylanase for different fungi, but this is the first time that a xylanase from *N. sitophila* has been cloned successfully in *E. coli*. *E. coli* is today one of the organisms of choice to produce recombinant enzymes (Table 1). Its use as a cell factory is well-established, and it has become the most popular expression platform; because the time of production of recombinant xylanases is shorter that the production of native fungal enzymes. Moreover, the recombinant enzymes expressed in *E. coli* are easier to purify, and the enzymatic extract can be used for hydrolysis of xylan without previous purification, and this is an advantage when the biotechnological processes are scale-up.

3.4 Temperature, pH optima and thermostability of Nsi Xyn11A

After Nsi Xyn11A was expressed, the influence of pH and temperature on the xylan hydrolysis capacity for the recombinant xylanase was determinate (Fig. 5).

The optimal pH was found at 5.5 (Fig. 5a), and the optimal temperature was observed at 55 ºC (Fig. 5b). Similar results have been observed for other fungal xylanases expressed in *E. coli* (Table 1).

Regarding the thermostability, the Nsi Xyn11A recombinant enzyme retains around 80% of its residual enzymatic activity during 120-150 min at 60 ºC, a closer residual activity was maintained at 80 ºC for 90 min, while a drastic decrease in the residual activity was observed at 85 and 90 ºC (Fig. 6), which makes the Nsi Xyn11A recombinant enzyme more thermostable than other fungal xylanases (Table 1). This positive property can be explained by the length of the loop between the β-strands B3 and A5 of the secondary structure of Nsi Xyn11A (Fig. 2 and Fig. 3). According to secondary and tertiary structure analyses of thermostable xylanases, this loop is longer than other xylanases, and it creates a short α-helix that presses the α-helix and the β-strands B3 and A5 of the enzyme, and new hydrophobic interactions are formed in this region. On the other hand, this loop help to creates new hydrogen bonds with the C-terminal end making the enzyme more stable, since the region around the α-helix and the C-terminal end is considered the initial point of the unfolding process (Fig. 3).

Table 1. Temperature and pH optima and thermal stability of fungal xylanases.

| Microorganism	Expression vector	Host	Optimal pH/temperature and thermostability	References
N. sitophila	pQE-30Xa	*E. coli* M15[pREP4]	5.5/55°C. Retained 70% of activity at 70 and 80°C during 100 min.	This work
Aspergillus usami E001	pET-28a(+)	*E. coli* BL21	4.6/50°C. Thermostability at 40°C 60 min.	Zhou *et al.* (2008)
Trichoderma reesei Rut C-30	pET-28a	*E. coli* BL21	5.0/50°C. Thermostability at 60°C 30 min.	He *et al.* (2008)
Aspergillus niger 400264	pET-32a	*E. coli* BL21	XYNB retained 94% of its activity during 10 min at 85ºC and XYNA lost its activity.	Xie *et al.* (2011)
Neocalli-mastigales rumen fungal	pET21a	*E. coli* BL21	7.2/68ºC. XynR8_VNE thermostable mutant retained 65% of its activity during 30 min at 65ºC.	Xue *et al.* (2012)
Trichoderma reesei Rut C-30	pPICZαA	*Pichia pastori* X-33	6.0/60°C. Lost 60% of its activity after 30 min.	He *et al.* (2009)
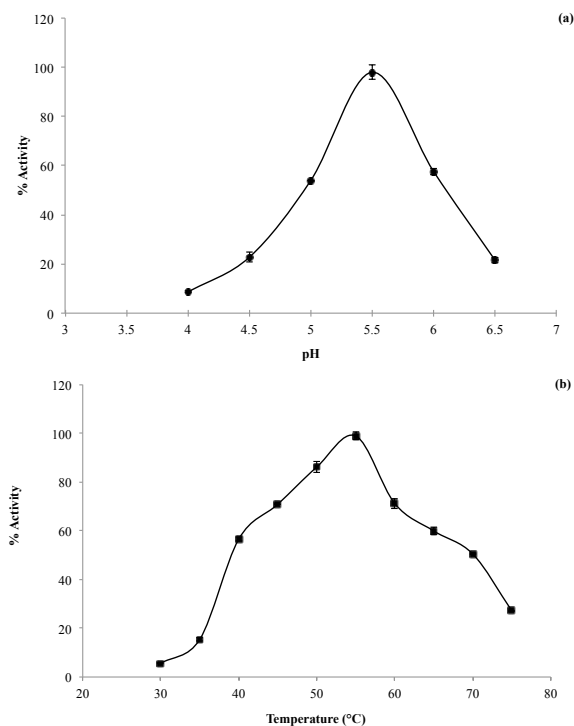
Fig. 5. pH and temperature profiles of Nsi Xyn11A xylanase. (a) Nsi Xyn11A was incubated at pH ranging from 4 to 6.5 (55 °C) for 15 min in citrate phosphate buffer 0.1 M. (b) Nsi Xyn11A was incubated at temperatures ranging from 30 °C to 75 °C for 15 min in 0.1 M citrate-phosphate buffer pH 5.5.
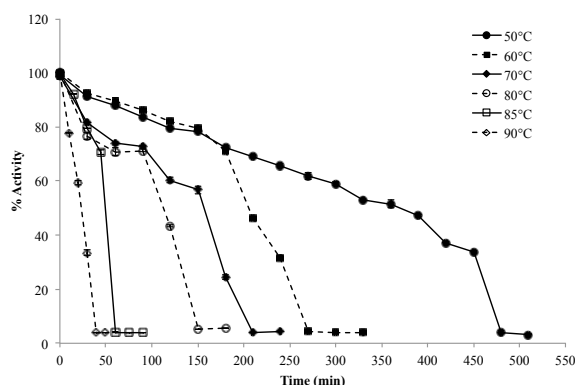

Fig. 6. Thermostability of Nsi Xyn11A xylanase. Nsi Xyn11A was incubated in 0.1 M citrate-phosphate buffer pH 5.5 at 50 °C, 60 °C, 70 °C, 80 °C, 85 °C and 90 °C.

Moreover, some researchers have demonstrated that the longer the N-terminal end is, the higher thermostability is in the xylanases. Therefore, the length of the loop B3-A5 in Nsi Xyn11A may be the reason for its higher thermostability in comparison with other xylanases (Purmonen *et al*., 2007; Paës *et*

al., 2012), even though the loop B3-A5 does not form the little α-helix.

On the other hand, the longer loop B3-A5 in the native protein Nsi Xyn11A of *N. sitophila* could be a region for O-glycosylation, so it is a rich Ser/Thr region. This agrees with the fact that secretory proteins in fungi frequently display Ser/Thr rich regions that could be sites of extensive O-glycosylation, which helps to enhance the stability and solubility of the proteins (Goto, 2007; González *et al*., 2012).

However, it is important to mention that the recombinant Nsi Xyn11A was not purified and that the proteins present in the enzyme extract could help with the stabilization of this enzyme. On the other hand, the modular structure could be involved in the thermostability that is observed at 60, 70 and 80 °C, the CBM type I or the C-terminal region could contribute at this characteristic as suggest by Wang and Xia (2008).

On the other hand, the inactivation rate constants, k_d and half-life ($t_{1/2}$) values for the recombinant xylanase at different temperatures are presented in Table 2. These results indicated that the Nsi Xyn11A xylanase could be classified in the range of thermostable xylanases since the enzyme showed half-lives of more than 2h at 70 and 80 °C and 1 h at 85 °C. Likewise, the recombinant enzyme showed half-lives of more than 4 h at 50 and 60 °C.

These results suggest that the Nsi Xyn11A enzyme may be able to be applied in industrial processes because it is kept active for adequate periods of time under conditions required in industrial processes (Polizeli *et al*., 2005; García-Rivero *et al*., 2015).

3.5 Evaluation of the hydrolytic capacity of Nsi Xyn11A on lignocellulosic biomass

Enzymatic hydrolysis of three agro wastes (WS, ATB, and SCB) was carried out using Nsi Xyn11A to know the catalytic potential of the recombinant enzyme; additionally, two commercial enzymes were used to compare (Table 3). The results show variation in the reducing sugars released depending on the enzyme and substrate. As expected, the reducing sugars obtained were increased as the hydrolysis time augmented, except for ATB for the commercial enzyme HTec2, which is a complex enzymatic extract with other activities different than xylanases; as a result, glucose and galacturonic acid residues are probably released from the agro wastes tested. Therefore, this could have influenced its high hydrolytic capacity on WS and SCB.

Table 2. Half-lives ($t_{1/2}$) and inactivation rate constants (k_d) of Nsi Xyn11A.

Temperature (°C)	50	60	70	80	85	90
k_d (min^{-1})[a]	0.0021	0.0028	0.0035	0.0058	0.0108	0.0355
$t_{1/2}$ (min)[b]	330	248	198	120	64	20

[a]k_d was calculated using the equation ln v = ln v_o - k_dt. [b]$t_{1/2}$ = ln $2k_d^{-1}$.

Table 3. Comparison of hydrolytic capacity on lignocellulosic biomass between Nsi Xyn11A and two commercial enzymes.

Xylanase activity (40 UI ml^{-1})	Reducing sugars (g l^{-1})					
	WS		ATB		SCB	
	24 h	48 h	24 h	48 h	24 h	48 h
HTec2	1.42±0.04	8.82±0.23	10.83±0.02	10.94±0.17	5.22±0.06	9.81±0.01
SIGMA X2629	5.73±0.07	7.64±0.14	8.42±0.06	10.92±0.02	5.84±0.06	8.12±0.08
Nsi Xyn11A	3.23±0.02	6.03±0.05	9.02±0.01	12.04±0.11	6.67±0.07	8.03±0.06

Comparing the reducing sugars obtained from the three substrates tested, the maximum quantity was achieved in ATB, followed by SCB and WS, it can be noticed that in the case of ATB the hydrolysis was faster as a higher concentration of reducing sugars was observed at 24 h of hydrolysis compared with the other substrates (Table 3). These differences between the substrates can be explained by the molecular structure of them as well as the industrial origin. WS and SCB were obtained only after a milling process; while ATB came from the tequila elaboration process, where cooking and milling steps are applied (Pinal *et al*. 2009). Thus, the ATB could be considered as a pretreated substrate, which possibly facilitated the access of the hydrolytic enzymes to the xylan. Furthermore, Nsi Xyn11A showed a better catalytic behavior with ATB; this can be explained because *N. sitophila* BDJ1I was isolated from ATB, and it may be possible that this microorganism produces optimal enzymes in order to adapt to the environment in which they are growing (Niladevi *et al*., 2008; Arrizon *et al*., 2012; García-Rivero *et al*., 2015).

Also, when xylan was used as a substrate, Htec2 and Xyn11A presented similar results; Htec2 released 17.82 and 29.31 g/L of reducing sugars at 24 and 48 h, respectively; while Nsi Xyn11A released 19.55 and 26.21 g l^{-1} at the same times.

Consequently, it can be concluded that Nsi Xynl1A shows catalytic and stability properties for the hydrolysis of xylan under acid conditions at high temperature, which are further improved by expression in eukaryote hosts. All these catalytic properties make Nsi Xynl1A a promising candidate enzyme for its development as an industrially viable biocatalyst.

Conclusions

In this study, we report for the first time the cloning of the *Nsi xyn11A* gene from *N. sitophila* encoding for a modular xylanase Nsi Xyn11A belonging to glycoside hydrolase family GH11. We found that Nsi Xyn11A varies from most GH11 xylanases, due to its large loop B3-A5 according to with modular xylanases with the specific carbohydrate binding module type I.

We suggest that Nsi Xyn11A is an attractive enzyme for different industrial applications, as thermostable xylanases are important in many processes such as xylooligosaccharides production, xylitol, paper and liquid fuels industries.

On the other hand, the Nsi Xyn11A enzyme showed a high affinity for the hydrolysis of ATB, this could be possible since *N. sitophila* was isolated from ATB and its enzymes are well adapted to the degradation of this agro waste, consequently an additional specific enzyme for the hydrolysis of ATB has been discovered. This result is important, because in Mexico there is the expectation of using ATB to produce ethanol or other products such as XOS, thus specific xylanases are needed. Moreover, Nsi Xyn11A could be a model xylanase for the study of the functionality of the loop B3-A5 in fungal secretory enzymes and its association with the thermostability.

Accordingly, to these results, the Nsi Xyn11A enzyme may be applied in simultaneous saccharification and fermentation processes to produce

different metabolites from xylose, utilizing agro wastes as substrates, or in the pretreatment of lignocellulosic biomass required to increase the yield of fermentable sugars for bioethanol production.

Acknowledgements

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