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FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA
CENTRO MULTIDISCIPLINARIO DE ESTUDIOS EN BIOTECNOLOGÍA

# ANÁLISIS BIOQUÍMICO-MOLECULAR DE UNA β-1,4-ENDOXILANASA DE *Colletotrichum lindemuthianum*

TESIS
QUE PRESENTA

**M.C. ULISES CONEJO SAUCEDO**

PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS BIOLÓGICAS
OPCIÓN: BIOTECNOLOGÍA MOLECULAR AGROPECUARIA

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Morelia, Michoacán septiembre de 2016.

El presente trabajo se realizó en el Centro Multidisciplinario de Estudios en Biotecnología de la Facultad de Medicina Veterinaria y Zootecnia de la UMSNH, bajo la asesoría de la Dra. María Guadalupe Zavala Páramo. Para su realización se contó con el apoyo de la Coordinación de la Investigación Científica de la UMSNH y por SEP-CONACyT (Projecto 2012-01, Clave 182755).

Agradezco a CONACyT por la beca (209148) otorgada durante mis estudios de doctorado

**ÍNDICE.**

# I. RESUMEN GENERAL

En este trabajo, se realizó el aislamiento y caracterización de los cDNAs completos de dos genes de endo-β-1,4-xilanasa (*xyl1*) del hongo fitópatogeno *C. lindemuthianum* razas 0 y 1472. Ambas secuencias se depositaron en GenBank (No. de acceso: KF487129, KM587707). El cDNA *xyl1* de la raza 0 tiene un tamaño de 751 pb, con un UTR 5´ de 23 pb y un UTR 3´ de 59 pb. El cDNA *xyl1* de la raza 1472 tiene un tamaño de 905 pb, con un UTR 5´ de 25 pb y un UTR 3´ de 211 pb. Las secuencias de ambas razas mostraron 100% de identidad a nivel de nucleótidos y de aminoácidos. Una comparación a nivel de aminoácidos con secuencias correspondientes en GenBank, mostó 67, 64, 62 and 61% de identidad con una *xylI* de *P. tritici-repentis*, *xil1* de *Cochliobolus carbonum*, *htxyl1* de *Helminthosporium turcicum*, y *Xyn22* de *Magnaporthe grisea*, respectivamente. La proteína putativa tiene un marco de lectura de 222 aminoácidos con un sitio de corte para un péptido señal entre $Ala^{19}$ y $Ser^{20}$, de acuerdo a SignalP 4.1 web server, lo cual es consistente con secuencias previamente reportadas. La proteinna putative madura (residuao 20 a 222) tiene una masa molecular calculade de 21.71 kDa y un pI de 8.94. Se encontró un sitio potencial de *N*-glycosilacion en $Asn^{71}$ de acuerdo a ExPASy Proteomics Server. El análisis de la secuencia *xyl1* y un alineamiento Clustal revelaron los elementos característicos de los genes que codifican para endo-β-1,4-xylanasas de la familia GH11. El crecimiento de las dos razas con glucosa como única fuente de carbono, mostró niveles basales de transcripción de *xyl1* y de actividad endoxilanasa. Cuando la glucosa se sustituyó con xilana o paredes celulares de *Phaseolus vulgaris*, la transcrición de *xyl1* y la actividad enzimática incrementaron significativamente en la raza 1472 comparada con la raza 0. Estableciéndo que la endoxilanasa se expresa de manera diferencial en las dos razas del *C. lindemuthianum*. El análisis filogenético de XYL1 y endo-β-1,4-xylanasas de otros hongos, reveló un proceso de diversificación y separación de las proteínas de la misma especie fúngica en diferentes linajes. Se modeló la estructura tridimensional de XYL1 de *C. lindemuthianum* y se usó para docking con varios compuestos modelo. Los análisis de docking revelaron una estabilidad significativa de XYL1 con el ologómero de xilopentosa. Se encontró que los residuos del sitio activo de la enzima que interactuan con los oligómeros modelo son acordes con su función en la degradación de la xilana. Sin embargo, los análisis de docking de xilanasas

GH11 de especies de Colletotrichun, revelaron diferencias significativas en estrucutran integración del sustrato en el sitio activo y los residuos de glutamato del sitio catalítico que llevan a cabo la hidrólisis del sustrato; de estas proteínas, 36, 60, y 4% integran xilotetraosa, xilopentaosa, y xilohexaosaen el sitio activo, respectivamente. Ya que las endoxilanasas GH11 de especies de Colletotrichum interactuan más eficientemente con xilopentosa y xilotetraosa, y las xilanasas GH11 de diferentes hongos no parecen tener los mismos subsitios de unión a sustrato, proponemos que son enzimas con diferente afinidad a xilooligosacáridos. De acuerdo con esta idea, el análisis filogenético de las xilanasas de especies de Colletotrichum muestra cuatro linajes que sugieren selección diversificante.

**Palabras Clave:** *Colletotrichum lindemuthianum*, endo-β-1,4-xylanasa, expresión, análisis filogenético, docking.

## II. SUMMARY

In this study, the complete cDNAs of two endo-β-1,4-xylanase genes (*xyl1*) from non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* were isolated and characterized. Both sequences were deposited in GenBank (Accessions: KF487129, KM587707). The *C. lindemuthianum xyl1* cDNA of race 1472 has 905 bp, with a 5´ UTR of 25 bp and a 3´ UTR of 211 bp. The *xyl1* cDNA of race 0 has 751 bp, with a 5´ UTR of 23 bp and a 3´ UTR of 59 bp. At nucleotide and amino acid levels, the sequence of both races showed 100% identity. Comparison at amino acid level with corresponding sequences in GenBank showed 67, 64, 62 and 61% identity with a *xylI* of *P. tritici-repentis, xil1 of Cochliobolus carbonum, htxyl1* of *Helminthosporium turcicum, Xyn22 of Magnaporthe grisea,* respectively. The putative protein has an open reading frame of 222 amino acids with a signal peptide cleavage site between $Ala^{19}$ and $Ser^{20}$, according to the SignalP 4.1 web server, which is consistent with previously reported sequences. The putative mature protein (residues 20 to 222) has a calculated molecular mass of 21.71 kDa and a pI of 8.94. A potential *N*-glycosylation site at $Asn^{71}$ was found with the ExPASy Proteomics Server. The *xyl1* sequence analysis and Clustal alignment revealed the characteristic elements of genes coding for endo-β-1,4-xylanases of the GH11 family. The growth of the two races with glucose as the sole carbon source showed both basal transcription levels of *xyl1* and endoxylanase activity. When glucose was substituted with xylan or plant cell walls from *Phaseolus vulgaris*, *xyl1* transcription, and enzyme activity significantly increased in race 1472 as compared to race 0. Establishing that there are a diferential expression of the xylanase between both races of *C. lindemuthianum*. Phylogenetic analyses of XYL1 and endo-β-1, 4-xylanases from other fungi revealed a diversification process and separation of proteins from the same fungal species into different lineages.

The three-dimensional structure XYL1 from *C. lindemuthianum* was modeled and docked with various xylan model compounds. Docking analyses revealed significantly higher stability of XYL1 with the xylopentaose oligomer. Residues interacting with the model oligomers at the respective enzyme active sites were found to be in accord with their role in xylan degradation. Nevertheless, docking analyses of xylanases GH11 from *Colletotrichum* sp, revealed significative differences in structure, integration of the substrate in the active

site and the glutamate residues of the catalytic site that carry out the substrate hydrolysis; of these proteins, 36, 60, and 4% integrate xylotetraose, xylopentaose, and xylohexaose in the active site, respectively. Since endoxylanases GH11 from *Colletotrichum* species interact much more efficiently with xylopentaose and xylotetraose, and xylanases GH11 from different fungi do not seem to have the same substrate binding subsites, we propose that they are enzymes with different affinity to xylooligosaccharides. In agreement with this idea, phylogenetic analyses of xylanases from *Colletotrichum* sp. shows four lineages suggesting diversifying selection.

# III. INTRODUCCIÓN GENERAL

## 1.1 Pared celular vegetal

La pared celular es una cubierta semirrígida y semipermeable que rodea a toda célula vegetal. Sin embargo, algunos estudios recientes han modificado este concepto de "estructura estática" y han puesto en evidencia su dinamismo, elevada complejidad y heterogeneidad estructural. Su presencia es fundamental durante el crecimiento y desarrollo de las plantas debido a sus diversas funciones en estos procesos. La pared celular proporciona soporte mecánico y es responsable de la forma celular y fortaleza de la planta (Humphrey et al., 2007; Sarkar et al., 2009).

Las paredes celulares vegetales están construidas siguiendo una arquitectura común: polímeros resistentes a la tensión, englobados en una matriz resistente a la compresión. Las fibras, formadas por celulosa, se distinguen de la matriz por su relativa homogeneidad en su composición química. Esta estructura es la principal barrera contra patógenos y protege a la planta de condiciones ambientales adversas. Se cree que también está implicada en la transducción de señales en respuesta al ataque de patógenos, estrés ambiental y en los diferentes estados de desarrollo como fuente de señales durante la interacción (Humphrey et al., 2007; Sarkar et al., 2009).

La matriz se compone principalmente de celulosa, hemicelulosas, pectina, glicoproteínas, proteoglicanos, componentes de bajo peso molecular y compuestos de naturaleza fenólica, entre los que se incluyen la lignina (Carrillo, 2003).

La **celulosa** es el compuesto orgánico más abundante sobre la tierra y el principal componente estructural de la pared celular. Desde el punto de vista bioquímico, la celulosa $(C_6H_{10}O_5)_n$ es un polímero natural constituido por una larga cadena de carbohidratos polisacarídicos. La estructura de la celulosa se forma por la unión de moléculas de glucosa a través de enlaces β-1,4-glucosídicos, lo que hace que sea insoluble en agua. La celulosa tiene una estructura lineal o fibrosa, en la que se establecen múltiples puentes de hidrógeno entre los grupos hidroxilo de distintas cadenas yuxtapuestas de glucosa, haciéndolas muy resistentes e insolubles al agua. De esta manera, se originan microfibrillas compactas que constituyen la pared celular de las células vegetales, dándoles

así la rigidez necesaria. Las microfibrillas consisten de más de 250 cadenas de glucosa unidas por la hemicelulosa y dentro de ellas existen regiones no cristalinas o regiones amorfas donde la cantidad relativa de celulosa cristalina y no cristalina varía dependiendo del origen (de Vries and Visser, 2001).

La **pectina** es considerada como el cemento intracelular, forma un gel amorfo que llena los espacios existentes entre las microfibrillas de celulosa y la hemicelulosa (Agrios, 1996). Son complejos de heteropolisacáridos solubles, los cuales contienen característicamente azúcares ácidos tales como el ácido glucurónico y el ácido galacturónico. Algunas pectinas presentan una estructura primaria relativamente simple, tal como la del homogalacturonano el cual es un polímero lineal de ácido galacturónico con enlaces β -1,4, con residuos ocasionales de ramnosa. El ramnogalacturonano I (RG I) tiene subunidades repetidas de disacáridos de ramnosa y galactosa con enlaces α-L-1,2 y α-D-1,2, respectivamente, dentro de las cadenas de arabinanas y arabinogalactanas. El ramnogalacturonano II (RG II), es un carbohidrato altamente complejo de menor abundancia relativa y presenta diversos azúcares que varían en sus enlaces a lo largo de la cadena (Cosgrove, 1997).

La **lignina**, uno de los principales componentes de las plantas vasculares, desempeña las funciones de ayuda estructural, transporte de agua y defensa (Boerjan et al., 2003; Whetten and Sederoff, 1995). La lignina es un polímero fenólico complejo basado en las sub-unidades de cinamato derivadas del metabolismo de los fenilpropanoides y es un producto natural importante de las plantas. La lignina es producida por la polimerización deshidrogenativa de esencialmente tres alcoholes aromáticos (p-cumarilo, coniferilo, y alcohol del sinapilo) que difieren en el grado de metoxilación en las posiciones C3 y C5 del anillo aromático. Cuando están incorporados en la lignina, estos alcoholes se llaman *p*-hidroxifenilo (*h*), guaiacilo (*g*), y las unidades del polímero de siringilo (*s*), respectivamente. Además de los tres alcoholes aromáticos principales, la lignina contiene rastros de unidades de la biosíntesis incompleta e incorpora otras unidades de fenilpropanoides, tales como aldehídos del hidroxicinamil, los acetatos, los *p*-cumaratos y el ferulato de tiramina (Boerjan et al., 2003).

Las **hemicelulosas** son heteropolisacáridos de alta peso molar, los cuales se encuentran constituidos por diferentes unidades de monosacáridos: pentosas, hexosas y ácidos urónicos, enlazados entre sí por enlaces glucosídicos, formando estructuras ramificadas y en general amorfas. Las hemicelulosas son importantes en la madera y su localización es trascendental ya que todas las células contienen de 50-60% de carbohidratos (Saha, 2003).

Después de la celulosa, la **xilana** es el polisacárido más abundante en la naturaleza (Collins et al., 2005). Forma parte de las hemicelulosas presentes en la matriz amorfa de la pared celular secundaria de los tejidos lignificados de las plantas leñosas (Tsoumis, 2013), aunque también se puede encontrar en la matriz de paredes primarias de células en crecimiento y de semillas y bulbos, donde tiene función de reserva.

Es la principal hemicelulosa de las maderas duras (angiospermas), representando un 15-30% del peso seco de la pared vegetal, y es menos abundante en el caso de las maderas blandas (gimnospermas) donde representa del 7-12% del peso seco (Wong et al., 1988).

### 1.2 Degradación de hemicelulosas

Las hemicelulosas son mezclas de polímeros de polisacáridos, cuya composición y frecuencia varían en las distintas especies de plantas y con la etapa de desarrollo. Consisten principalmente de xiloglucana, pero también se pueden encontrar otros compuestos como glucomananas, galactomananas y arabinogalactanas, los cuales se pueden unir a los extremos de los polisacáridos pécticos y a las fibras de celulosa (Agrios, 1996).

Para la degradación enzimática de las hemicelulosas, los hongos requieren de las enzimas llamadas hemicelulasas, las cuales son designadas dependiendo de la región en la que actúan (endo- o exo-) y del monómero liberado del polímero.

### 1.3 Degradación de xilana

La hidrolisis enzimática de la xilana, componente principal de la hemicelulosa, se lleva a cabo principalmente por un consorcio de enzimas microbianas que actúan conjuntamente. La degradación de la xilana es una capacidad que han desarrollado bacterias y hongos a lo largo de millones de años. Sin embargo, la descripción de la degradadción de la xilana a partir de microorganismos data de mediados del siglo XIX, con la primera preparación y

parcial purificación de una xilanasa de *Aspergillus foetidus* (Whistler and Masak, 1955). Estos organismos, poseen sistemas enzimáticos complejos para llevar a cabo la degradación de la xilana a sus componentes más simples (Carrillo, 2003).

Las enzimas degradadoras de xilana se clasifican en dos grupos principales: las endoxilanasas y β-xilosidasas, enzimas que están implicadas en la despolimerización del esqueleto principal de xilosa. Las α-L- arabinofuranosidasas, α-D-glucuronidasas, acetilxilana esterasas, ferulico y *p*- cumarico esterasas, llamadas enzimas desramificantes, se encargan de la eliminación de las cadenas laterales de xilana (Fig. 1). Se ha visto que las enzimas degradadoras de xilana funcionan de manera sinérgica; por un lado, las enzimas desramificantes permiten el acceso de las xilanasas al esqueleto principal de la xilana, mientras que las enzimas accesorias liberan los sustituyentes laterales más fácilmente a partir de fragmentos de xilana (de Vries et al., 2000).

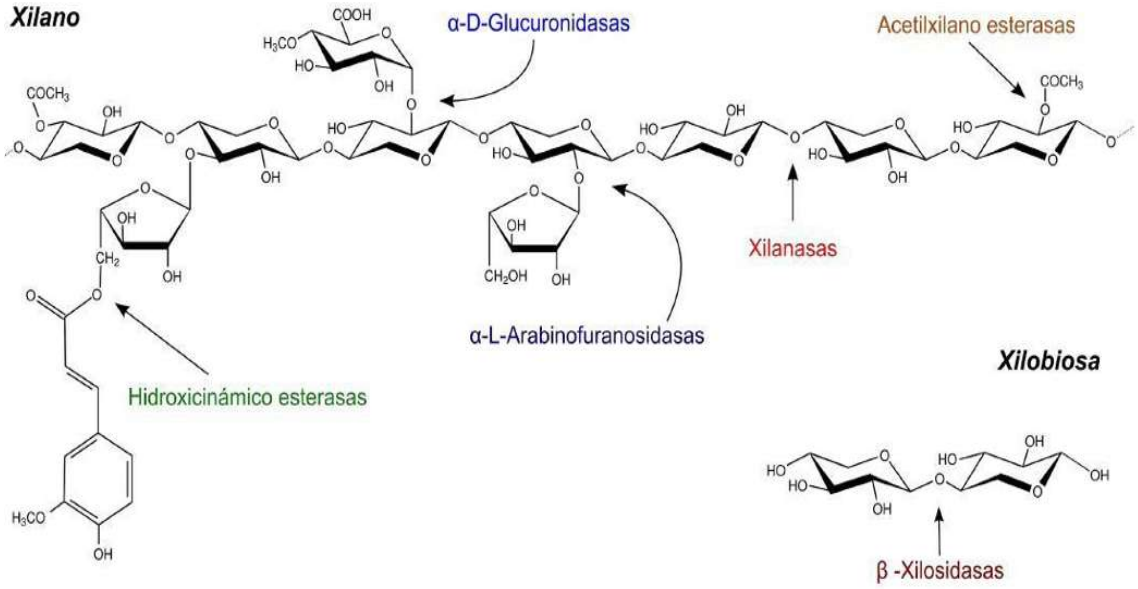


**Figura 1.** Estructura de la molécula de xilana, sus ramificaciones y enzimas que participan en la degradación (Gallardo et al., 2007).

### 1.4 Xilanasas

Las xilanasas (endo-β-1,4-xilanasas; EC 3.2.1.8) son glicosidasas que catalizan la endohidrólisis de los enlaces 1,4-β-D-xilosídicos de la xilana. Actúan sobre la cadena principal del polímero hidrolizando los enlaces internos β(1→4) entre moléculas de xilosa,

dando lugar a una mezcla de xilooligosacaridos de diferentes tamaños (Biely, 1985). Además de xilana pueden hidrolizar xilooligomeros con diferente grado de polimerización (siendo más activas cuanto mayor es el grado de polimerización) pero no hidrolizan a la xilobiosa, lo que permite distinguirlas claramente de las β-xilosidasas.

Las xilanasas son las principales enzimas implicadas en la degradación de la xilana. En los microbios xilanolíticos hay una gran multiplicidad de xilanasas (varios genes distintos los cuales pueden dar lugar a diferentes xilanasas en función del procesamiento post-transcripcional y post-traduccional), que difieren en su especificidad respecto a la xilana (Wong et. al., 1988). Esta gran diversidad de xilanasas está relacionada con el hecho de que la xilana es un polímero muy complejo y se requieren xilanasas distintas para degradar las diferentes regiones del heteropolímero.

Así, muchas xilanasas solo pueden actuar sobre regiones de la xilana no sustituidas, mientras que otras requieren de un determinado tipo de ramificación adyacente al sitio de corte (Coughlan and Hazlewood, 1993). Dado que la xilana es un polímero con elevado grado de polimerización, no puede penetrar dentro de los microorganismos para ser degradada, de manera que las xilanasas han de ser secretadas al medio extracelular, normalmente mediante sistemas de secreción de tipo II ("sec dependientes", es decir, que requieren del sistema de secreción Sec, el *Secpathway*)(Tjalsma et al., 2004).

### 1.5 Clasificación química y molecular de las xilanasas

La diversificación de la xilanasas es muy amplia debido a diferentes factores como: redundancia genética (Wong et al., 1988), modificaciones post-traduccionales, glicosilación (Biely, 1985) y/o presencia de péptido señal (Wong et al., 1988).

Inicialmente la clasificación de las xilanasas se estableció tomando como referencia sus características físico-químicas. Con base en el peso molecular y el punto isoeléctrico, Wong y col. (1988) dividieron a las xilanasas en dos grupos: Un primer grupo de xilanasas de bajo peso molecular (menos de 30 kDa) y pI alcalino; un segundo grupo de xilanasas de mayor peso molecular (más de 30 kDa) y pI ácido. Sin embargo, existían algunas escepciones y no todas las xilanasas se podían agrupar. Posteriormente, las glicosil hidrolasas se clasificaron en 35 familias con base en una comparación de secuencias (Henrissat, 1991) y el análisis de las regiones de hidrofobicidad de las mismas (Henrissat

and Bairoch, 1993, 1996).

Algunos estudios posteriores han corroborado que, tal como sugerían éstos y otros autores en estudios previos (Chothia and Lesk, 1986), existe una correlación entre la secuencia primaria de una proteína y su conformación tridimensional. Actualmente, en la base de datos de enzimas activas sobre carbohidratos, existen más de 100 familias de glicosil hidrolasas (http://www.cazy.org/; (Cantarel et al., 2009), las cuales se encuentran agrupadas en diferentes clanes o superfamilias (grupos de familias que comparten un motivo de plegamiento terciario, aminoácidos catalíticos conservados y mecanismo catalítico similar.

Con base en esta clasificación, las xilanasas quedan distribuidas en las familias 10 y 11, que se corresponden con las familias F y G de Gilkes y col. (1991). La diferencia entre estas dos familias de xilanasas se encuentra principalmente a nivel de secuencia y estructura tridimensional, no existiendo diferencias importantes a nivel de sus características catalíticas. No obstante, las diferencias estructurales influyen de manera determinante en algunas características fisicoquímicas de estas enzimas. Así, la estructura tridimensional del centro activo de las xilanasas de la familia 10 hace que estas sean menos estrictas en cuanto al sustrato y más activas sobre xilooligosacaridos de bajo grado de polimerización que las xilanasas de la familia 11 (Biely et al., 1997; Leggio et al., 2000; Sabini et al., 2001).

### 1.6 Mecanismo catalítico

El mecanismo catalítico de la mayoría de glicosil hidrolasas consiste en un desplazamiento simple que produce la inversión de la configuración anomérica o bien en un doble desplazamiento que conlleva la retención de la configuración anomérica (Rye and Withers, 2000; Yip and Withers, 2004). En el caso de las xilanasas de las familias 10 y 11 el mecanismo utilizado en la hidrólisis del sustrato es el de doble desplazamiento con retención de la configuración anomérica (β→β) (Fig. 2). En la hidrólisis del enlace glucosídico por este mecanismo intervienen dos residuos de glutamato conservados en el centro activo de la xilanasa, separados entre sí 5.4 – 5.5 A, actuando uno de ellos como catalizador ácido/base y el otro como residuo nucleófilo (Davies and Henrissat, 1995).

Una vez que el esqueleto de xilosas ha sido posicionado correctamente entre los dos ácidos glutámicos catalíticos, uno de ellos (el catalizador ácido/base) realiza un ataque ácido sobre

el enlace glucosídico, protonando el oxígeno de dicho enlace, mientras el otro glutamato realiza un ataque nucleofílico sobre el carbono anomérico del enlace (Fig. 2A). El resultado de este primer paso es la liberación de uno de los productos de reacción y la formación de un intermediario α-glicosilo-enzima. A continuación, el glutamato ácido/base pasa a actuar como base, sustrayendo un protón a una molécula de agua, lo que permite que ésta ataque el enlace entre el glutamato nucleofílico y el carbono anomérico (Fig. 2B), produciendo su hidrólisis y dando como resultado un producto cuyo carbono anomérico vuelve a la misma configuración que en el sustrato (β→β), liberándose la enzima de su unión al sustrato para poder iniciar un nuevo proceso de catálisis (Fig. 2C) (Collins et al., 2005; Davies and Henrissat, 1995).

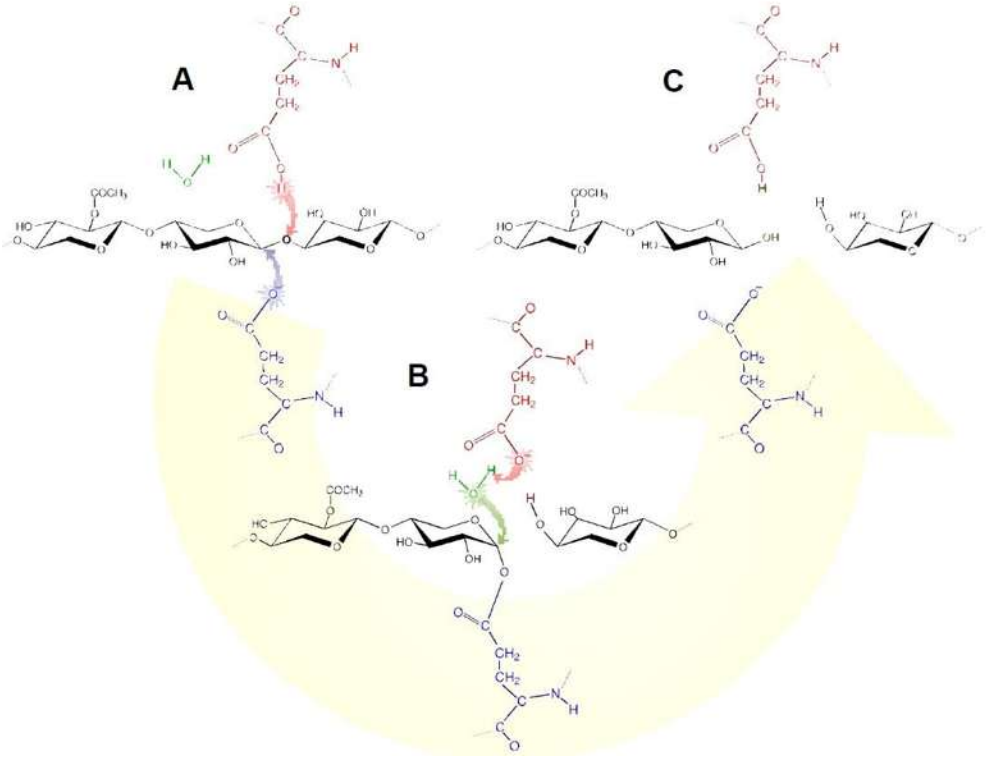


**Figura 2**. Mecanismo catalítico de las xilanasas (Tomado de Gallardo, 2007).

### 1.7 Aislamiento y caracterización bioquímica y molecular de xilanasas

En general, las endoxilanasas secretadas por diversos organismos han sido de gran interés en la industria. Esto motivó que durante las dos décadas pasadas se realizaran extensas investigaciones sobre el aislamiento y caracterización de sus genes y proteínas, determinando sus propiedades bioquímicas y estructuras tri- dimensionales (3-D) (Kulkarni et al., 1999). Entre las mejor estudiadas se encuentran las endoxilanasas de las diversas especies mostradas en la Tabla 1, pero sólo pocos trabajos han investigado en detalle los mecanismos involucrados en su expresión y regulación en hongos fitopatógenos. Entre las primeras enzimas hemicelulolíticas reportadas en hongos fitopatógenos se encuentran las estudiadas por Riou y col. (1991), quienes demostraron que en cultivos del hongo patógeno del tomate *Sclerotinia sclerotiorum*, en presencia de diversos polisacáridos, se secretan enzimas que convierten sustratos hemicelulósicos a formas asimilables. En presencia de xilana, se detectaron altos niveles de actividad xilanolítica y el análisis por SDS-PAGE reveló numerosos polipéptidos, sugiriendo la presencia de diferentes formas enzimáticas.

Debido al conocimiento de que los hongos filamentosos son productores eficientes de enzimas xilanolíticas y que la degradación natural de la xilana por diversos microorganismos requiere la acción coordinada de varias enzimas, incluyendo endoxilanasas y β-xilosidasas, éstas últimas también han sido de considerable interés para varias aplicaciones biotecnológicas (Carapito et al., 2009; Conejo-Saucedo et al., 2011; Douaiher et al., 2007).

**Tabla 1.** Estudios de diferentes xilanasas en hongos.

| Especie | Trabajo realizado | Referencia |
|---|---|---|
| *Bipolaris sorokiniana* | Purificación y caracterización de una xilanasa de 30 kDa | (Karjalainen et.al., 1992) |
| *Cochliobolus carbonum* | Clonación del gen *xyl1* | (Apel et al., 1993) |
| *Fusarium oxysporum f.sp. melonis* | Purificación y caracterización de una endoxilanasa | (Alconada and Martínez, 1994) |
| *Magnaporthe grisea* | Clonación y purificación de dos endoxilanasas | (Wu et al., 1995) |
| *Fusarium oxysporum* | Purificaron y caracterizaron por filtración en gel y por cromatografía de intercambio iónico de dos endoxilanasas de 20.8 (xyl I) y 23.5 (xyl II) kDa | (Christakopoulos et al., 1996) |
| *Sclerotinia sclerotiorum* | Regulación de la síntesis del sistema de enzimas que degradan material lignocelulósico | (Sachslehner et al., 1998) |
| *Claviceps purpurea* | Aislamiento de dos genes de xilanasas (*cpxyl1* y *cpxyl2*) con productos de 21.5 kDa y 33.8 kDa | (Giesbert et al., 1998) |
| *Ustilago maydis* | Detección de una actividad xilanolítica presente en dos etapas del ciclo de vida | (Cano-Canchola et al., 2000) |
| *Cochliobolus carbonum* | Clonación, interrupción y expresión de los genes *xil2* y *xil3* (22 kDa) | (Apel-Birkhold and Walton, 1996) |
| *Aspergillus oryzae* | Clonación, sobreexpresión y purificación de una xilanasa | (Kimura et al., 2000) |
| *Helminthosporium turcicum* | Identificación, clonación, y expresión una xilanasa | (Degefu et al., 2001) |
| *Fusarium oxysporum f.sp. lycopersici* | Detección de la expresión de XIL4 | (Gomez-Gomez et al., 2002) |
| *Ascochyta pisi* | Clonación, secuenciación y análisis filogenético del gen *xyl1* | (Lübeck et al., 1997) |
| *Aspergillus kawachii* | Clonación y secuenciación XynA, XynC | (Ito et al., 1992; Ito et al., 1992) |
| *Aspergillus niger IBT-90* | Expresión hererológa del gen *XYN6* en *Pichia pastoris* | (Korona et al., 2006) |
| *Botryotinia fuckeliana B05-10 (Botrytis cinerea)* | Inhibición del gen *XYNBC1* | (Brutus et al., 2005) |
| *Hypocrea jecorina (Trichoderma reesei)* | Caracterización de los genes XYNI, XYNII | (Törrönen et al., 1992) |
| *Chaetomium thermophilum CBS 730.95* | Producción de las genes Xyn11A, Xyn11B, Xyn11C, | (Mantyla et al., 2007) |
| *Penicillium sp. CGMCC 1669* | Clonación, sobreexpresión y caracterización de XYN11F63 | (Liu et al., 2010) |
| *Trichoderma sp. SY* | Clonación, purificción y caracterización de Xyl | (Min Shin Young et al., 2002 ) |
| *Trichoderma inhamatum* | Caracterización y purificación de XylI y XilII | (Silva et al., 2015) |

## IV. HIPÓTESIS

El sistema hemicelulolitico de *C. lindemuthianum* contiene al menos una endoxilanasa para una degradación efectiva de la pared celular de frijol y esta se expresa de manera diferencial.

# V. OBJETIVOS

## 1.1 General:

Analizar bioquímica y molecularmente una endoxilanasa del sistema xilanolítico de *C. lindemuthianum*

## 1.2 Específicos:

- Analizar la expresión el gen *Clxil1* que codifica una endoxilanasa de *C. lindemuthianum* bajo inducción con pared celular y xilana.
- Establecer las relaciones filogenéticas del gen *Clxil1* de *C. lindemuthianum.*
- Realizar un análisis de docking del modelo tridimensional de XYL1 y las xilanasas de la familia GH11 de especies de *Colletotrichum.*

## VI. RESULTADOS

## CAPÍTULO I

Artículo de revisión **“**Hemicellulases of fungi: A vision of their function in the coordinated degradation of polysaccharides of plant cell walls”. Current Trends in Microbiology, 2011, 7:1-13

# Hemicellulases of fungi: A vision of their function in the coordinated degradation of polysaccharides of plant cell walls

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**Abstract**

Hemicellulases breakdown various polymers of hemicellulose, a major component of plant cell walls. Recent years have witnessed significant advances in the isolation and characterization of the structure and function of bacterial and fungal hemicellulases, as well as in the development of biotechnological applications for the food, paper, textile, drink and juice industries. The application of hemicellulases in industry has typically been restricted to the use of a single enzyme in digestive processes, but current research is directed at the construction of bifunctional enzymes. However, the structural complexity of plant cell walls necessitates the use of a battery of enzymes or lytic complexes that fully breakdown hemicellulose in a coordinated manner. Here we review the functions, properties and regulation of hemicellulolytic complexes in fungi and the potential for integrated use of these enzymes in biotechnological applications.

**Introduction**

Hemicellulases are produced by a wide range of organisms, including Archaea, Bacteria, and Eukarya. The latter includes many types of fungi, protozoans, gastropods, arthropods and higher plants [1][2][3]. Fungi appear to be the most efficient eukaryotic producers of this type of extracellular lytic enzyme [4].
In hemicellulolytic systems, the breakdown of hemicellulose requires the coordinated activity of a number of extracellular enzymes that function synergistically to hydrolyze the polysaccharides into small oligosaccharides and finally to convert them to monomers [5-6]. The complexity of these enzymatic systems is further increased because microorganisms tend to produce different modular enzymes within each class to improve the efficiency of the breakdown of complex and recalcitrant structures, such as plant cell walls [7].
Hemicellulases have a high potential for biotechnological applications in a variety of industrial processes [8]. Breakdown strategies that rely on fungal hemicelluloses have been used in industrial activities including the generation of bioethanol, cattle feed, juices, textiles, paper, beer and table wines. However, the use of multiple enzymes of the hemicellulolytic complex for bioconversion of hemicellulose remains an economic obstacle. Thus, there is a need to reduce the number of enzymes required for industrial applications, as well as to find more efficient enzymes with a lower cost. Here we analyze the functions, properties and regulation of fungal hemicellulolytic complexes and the potential integrated use of these enzymes for the complete utilization of hemicellulose in biotechnology applications.

**Hemicellulose structure and depolymerization**

The main polymers that constitute the plant cell wall are cellulose, pectin, hemicellulose and lignin. Cellulose is the most abundant polymer, but its content depends on the taxonomy group to which the plants belong and the degree of development of the cell wall. Cellulose comprises between 35% and 50% of plant cell walls in terms of dry weight, whereas the content of hemicellulose and lignin ranges from 20% to 35% and 5% to 30% in dry weight, respectively [5, 9]. Hemicellulose, cellulose and lignin form networks of microfibrils with covalent and non-covalent linkages that give cohesion and structure to the plant cell wall, whose composition and frequency varies in different species and development stages [9-10]. Hemicellulose is a complex of

heteropolysaccharides (the second most abundant type of polysaccharide in nature) assembled in structures with lateral and generally amorphous ramifications on a xylose backbone (xylan) or on mannose and glucose backbones (mannan and glucomannan) with galactose, arabinose, and acetic/glucouronic acid ramifications [9, 11]. These complexes are insoluble in water, which is why their enzymatic hydrolysis poses a great challenge for microorganisms [5-6, 12]. Heteropolymers of hemicellulose do not have crystalline arrangements and are found at random inside the cell wall, forming an interface between lignin and cellulose. Microscopic studies indicate that the cellulose microfibrils have a parallel orientation. It has been proposed that due to its cohesion, hemicellulose could act as a lubricant to prevent direct microfibril-microfibril contact both within and on the surface of cellulose [13-14].

The most abundant hemicellulose in cereals and hard woods (angiosperms) is xylan. Rice, for example, contains 46% xylose, 44.9% arabinose, 6.1% galactose and 1.9% glucose. In addition to terrestrial plants, in which xylan is found in heteropolysaccharide complexes with β-1,4 xylopyranosyl linkages in the main chain, marine algae also synthesize xylans with different chemical structures to form a backbone with β-1,3 xylopyranosyl linkages [9]. However, in some Chlorophyceae and Rhodophyta species where cellulose is absent, xylan forms a highly crystalline fiber-like material [15]. In other cases, the main polysaccharide is mannan (β-1,4), which is a structural and reserve component of green algae (siphonaceous) in the Acetabularia, Codium and Halicoryne genera and in some red algae, such as *Porphyra umbilicales* [16].

Galactomannan and galactoglucomannan are the most abundant hemicelluloses in soft woods (gymnosperms) (12-15%), and glucomannan is the most abundant hemicellulose in some hard woods [5, 17]. Galactomannan has a structure of β-1,4-D-mannose residues and α-1,6-D-galactose side chains. It is fairly common in the Leguminosae family, with a content in the seeds of 1-38% of the dry weight, and it has also been identified in Ebenaceae and Palmae. Galactoglucomannan is the main hemicellulose in soft woods and contains a β-1,4-D-mannose backbone that can be substituted with α-1,6-D-galactose or have β-1,4-D-glucose residues. The solubility of galactoglucomannan depends on its high galactose content, but it can be rendered insoluble by the esterification of glucose and/or mannose residues in the main backbone with acetyl groups on C-2 or C-3 [5].

The polydiversity or structural polymolecularity of xylan relies on the nature and content of mono-substituents (neutral or uronic) and short-chain oligosaccharides bound to the main chain of β-1,4 xylopyranosyl linkages, such as glucomannan, galactomannan and arabinogalactan, which can connect to the ends of pectic polysaccharides and to cellulose fibers [1, 18]. The side chains do not affect the geometry of the glycosidic linkage, but they do determine the solubility, physical conformation and reactivity of the xylan molecule with other hemicellulose components, and thus the side chains greatly affect the mode of enzymatic breakdown [1, 12]. The number and nature of the lateral ramifications of hemicellulose vary in different plant species and in different types of tissue [14]. The most common are those formed by the following carbohydrates:

α-L-arabinofuranose, linked to the C3 and, less frequently, to the C2 of β-D-xylopyranose residues in soft wood xylan (13% of xyloses) and the xylan of herbal plants [19];

α-D-glucouronic acid and/or 4-Omethyl-α-D-glucouronic acid, bound to the C2 of β-D-xylopyranose in both hard wood xylan (10% of xyloses) and soft wood xylan (20% xyloses) as well as in the xylan of herbal plants [19];

O-acetyls, bound to the C2, C3 or both carbons of β-D-xylopyranoses in hard wood xylan (70% of acetylated xyloses) and xylan of herbal plants. The xylan of soft woods are not acetylated [19];

Ferulic and *p*-coumaric acids, which are phenolic compounds bound by ester links to the C5 of arabinose residues [19];

Arabinoxylan, the xylan of herbal plants, which are so named due to the large amount of α-L-arabinofuranose side residues and also have side groups of acetyl, glucouronic acid, ferulic acid and *p*-coumaric acid [11, 19];

Glucuronoxylan, the xylan of hard woods that have α-D-glucouronic acid and or 4-O-methyl-α-D-glucouronic acid side chains in addition to O-acetyl groups [11, 19];

Glucuronoarabinoxylan, the xylan of soft woods, which are named for the presence of α-L-arabinofuranose, α-D-glucuronic acid and/or 4-O-methyl-α-D-glucuronic acid, as well as ferulic and coumaric acid side chains [11, 19].

Given their complexity, fungi require several hemicellulases to break down plant hemicelluloses. The most widely studied are those involved in the depolymerization of the main xylan backbone, which are classified into two main groups depending on the site upon which they act, such as endo-1,4-β-D-xylanases (EC 3.2.1.8) (Fig. 1 A, D) and exo-1,4-β-D-xylosidases (EC 3.2.1.37) (Fig. 1 B). In addition, α-L-arabinofuranosidases (EC

3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.6), ferulic and *p*-cumaric esterases and β-galactosidases (EC 3.2.1.23), called debranching enzymes, are responsible for removing the xylan side chains (Fig. 1, A). The debranching enzymes work in a synergistic manner in the breakdown of xylan, allowing access of xylanases to the main backbone, while accessory enzymes (Table 1) free the side substituents more easily from xylan fragments [20]. Depolymerization of the main galactoglucomannan backbone involves endo-1,4-β-D-mannanases (EC 3.2.1.78) (Fig. 1, E), β-mannosidases (EC 3.2.1.25) (Fig. 1, C), α-galactosidases (EC 3.2.1.22) (Fig. 1, E) and β-galactosidases (EC 3.2.1.23) [5, 20]. Endo-1,4-β-D-mannanases break down the main galactoglucomannan backbone to produce mannooligosaccharides, which are further broken down to mannose by β-mannosidases. However, complete breakdown depends on the participation of α- and β-galactosidases. In *Aspergillus niger*, the presence of galactose next to the mannose residues decreases the activity of β-mannosidases by 18% to 43%, depending on the size of the oligosaccharide [5, 16]. It has been shown that *Penicilium funiculosum* synthesizes a complex multienzymatic system that acts in a synergistic manner and includes an endo-1,4-β-D-xylanase, a β-D-xylosidase, an endo-1,3-1,4-β-D-glucanase and an α-L-arabinofuranosidase, in addition to accessory enzymes such as feruloyl esterase, α-D-galactosidase, β-D- mannosidase and endo-1-4-β-mannanase [21-22]. According to physicochemical characterization, fungal hemicellulases are monomeric, with molecular weights that range from 18 to 360 kDa, and function in acidic to neutral pH (2 to 8) at optimal temperatures of 37-85°C (Table 2).

In addition to breakdown activity, there have been reports of transglycosylation activity for endo-1,4-β-D-mannanases in *A. niger* [23], for β-mannosidases in *A. Niger* and *Penicillium wortmanni* [24], α-galactosidases in *A. nidulans* [25] and for β-galactosidases in *A. oryzae* [26]. The *aglA* gene that codes for one α-galactosidase in *A. niger* has also been found to code for a functional α-N-acetylgalactosaminidase [27].

There are currently more than 100 families of glycosyl hydrolases (GH) in the database of active enzymes targeting carbohydrates (http://www.cazy.org/; [28]. These are grouped into four different clans or superfamilies (groups of families that share the same tertiary structure motif, conserved catalytic amino acids and similar catalytic mechanism) (Table 2).

Bacteria and some anaerobic fungi produce multienzymatic complexes called cellulosomes, which are anchored to the cell surface and allow the microorganisms to bind to lignocellulose substrates and increase the breakdown efficiency of cellulose and hemicellulose [29]. Most of the enzymes that make up cellulosomes are cellulases, xylanases, other glycosyl hydrolases and in some cases even esterases are also present [30] [31]. However, in the majority of fungi, hemicellulases are not integrated in cellulosome complexes as in bacteria, and enzymes that act in a synergistic manner are self-induced depending on the substrate that is present, which leads to the breakdown of the plant cell wall and the internalization of the hydrolysis products into the cell.

The diversity of the enzymes and the complexity of substrates suggest that hemicellulases have evolved according to the diversity of hemicelluloses and of the plant cell wall in general, increasing the efficiency of hydrolytic complexes. In fungi involved in white and black rot (*T. versicolor and Penicillium placenta*), enzyme activities fluctuate to establish an interaction between the cellulases and hemicellulases depending on the substrate (lignin, cellulose or hemicelluloses), the incubation time and concentration of the enzymes. However, other factors, such as the anatomy and microstructure of the plant cell wall, have also been observed to affect the integral activity and expression patterns of fungal hemicellulases [32]. In saprophytes, including *Gloeophyllum trabeum* and *P. placenta*, the breakdown process starts with hemicellulose polymers and proceeds with cellulose, initially breaking down galactans and arabinans and then targeting mannan and xylan [33]. On the other hand, in *A. oryzae* the depolymerization of arabinoxylan for activity of arabinofuranosidases involves the synergistic activity of other enzymes such as xylanases and β-xylosidases [34]. The saprophyte fungus *Phanerochaete chrysosporium* has the ability to hydrolyze cellulose, hemicellulose and lignin simultaneously, whereas the basidiomycete *Ceriporiopsis subvermispora* (saprophyte) first breaks down lignin to access cellulose and hemicellulose, thus suggesting an adaptive difference that favors microorganisms that can break down any polymer [35].

Although several genes that encode hemicellulolytic enzymes have been cloned and several of the enzymes have been characterized [5, 36], many have not been identified [36]. To date, many filamentous fungus genomes, including those of species of biotechnological and pathogenic importance, have been sequenced. The analysis of genomes from *A. nidulans* and *A. niger* in particular has demonstrated the great potential of this approach in fungal research. de Vries *et al*. [36] have reported that more than two-thirds of the ORFs putatively involved in plant cell wall polysaccharide breakdown and found in the genome of *A. nidulans* encode novel enzymes. They have also identified ORFs that apparently encode putative intracellular oligosaccharide breakdown enzymes and others with homology to oligosaccharide transporters in other organisms. The genomic

sequencing of *A. niger* and the use of bioinformatic tools have enabled rapid progress in the study of hemicellulolytic genes, illustrating the complexity of hemicellulose and how much we still have to learn about these processes. For example, the secretion of 17 enzymes and the participation of more than 30 genes with apparently differential expression patterns has been described for *Fusarium graminearum* [37] [38].

**Regulation of the hemicellulolytic system of fungi**

Saprophytic and phytopathogenic fungi need to have not only a repertoire of enzymes required to break down cell wall components but also well-coordinated regulation of their gene expression. de Vries *et al.* [39] has proposed that because *Aspergillus* is not able to import polysaccharides, it is likely that monosaccharides or small oligosaccharides generated as products of these enzymatic reactions are the inducers of the regulatory systems. Some evidence in support of this notion includes the observation that ferulic acid, L-arabinose/L-arabitol, D-mannose, GalA or compounds containing GalA induce the expression of feruloyl esterases [40], α-L-arabinofuranosidase [41], β-mannosidase [42] and a large number of genes encoding pectinolytic enzymes [43], respectively.

Studies of the regulation of the expression of genes encoding these enzymes in fungi have been carried out for cellobiohydrolases, endoglucanases, β-glucosidases, endoxylanases and β-xylosidases. Their expression is regulated by three main mechanisms: *i)* regulation by specific activators, *ii)* regulation by universal activators that control the expression of more than one gene, and *iii)* integrated regulation [44].

The expression of hemicellulases in *Aspergillus sp.* and *Trichoderma sp.* is mainly induced by carbon sources. Transcription is suppressed in the presence of D-glucose, whereas transcription is strongly induced in the absence of D-glucose and presence of hemicellulose. Furthermore, cellulolytic and hemicellulolytic enzymes can be co-induced by several mono- and disaccharides, such as sophorose, xylobiose, lactose, D-xylose and L-sorbose [44]. The suppression of gene transcription by glucose in *Trichoderma* and *Aspergillus* species is mediated by the catabolic suppressors Cre1 and CreA, respectively, similar to Mig1 from *Saccharomyces cerevisiae* [45] [46]. This suppressor specifically recognizes the sequence 5'-SYGGRG-3' in the gene promoter regions to negatively regulate transcription [44]. Thus, xylose plays a dual role as a concentration-dependent regulator of xylanase expression. At low xylose concentrations, it acts as an inducer, as it only exerts weak suppression through the CreA system. At high concentrations, xylose acts by suppressing the transcription of xylanolytic genes (through the CreA system) [47].

In *A. niger*, the xylanolytic system is under the control of XlnR, which is a central transcriptional regulator of the GAL4 type [6, 48] that is presumed to regulate not only the expression of more than 20 genes involved in the breakdown of xylan but also the expression of cellulose breakdown genes [49]. In *Hypocrea jecorina*, it has been shown that Xyr1 is an ortholog of XlnR and possesses similar functions but acts via different mechanisms [49]. In addition, it was reported that ACE II is an essential universal activator controlling the transcription of cellulases and hemicellulases [50]. XlnR and Xyr1 work in coordination with at least three activators: Ace1, Ace2 and Hap2/3/5 [48, 51]. A coordinated mechanism between XlnR/Xyr1 and the activators has been proposed, in which their combined action regulates the expression of genes such as Xyn2, whose basal expression is mediated by Hap2/3/5 and XlnR. Although Xyn2 expression is regulated by Ace2 under induction conditions, it shows no apparent association with XlnR [48].

**Biotechnology applications**

Some strategies have been used to increase the efficiency of enzyme-mediated breakdown of celluloses and hemicelluloses, including processes of mutation, co-culture and heterologous expression of fungi from the Aspergillus, Trichoderma or Penicillium genera. For example, the activities of cellulases and xylanases have been modified using UV light mutagenesis in *Penicillium verruculosum*, increasing the production of these enzymes by up to three fold in the fermentation stages using wheat bran, yeast extract and crystallized cellulose [52]. In another study, the tolerance of a xylanase to more alkaline pH was increased in *T. ressei* using site-directed mutagenesis, resulting in increased activity during the bleaching of cellulose pulp [53]. The thermostability of a xylanase in *T. ressei* was also increased by a directed mutation that substituted two amino acids (Thr-2 and THr28) with cysteines in the amino terminal region, increasing the stability of the enzyme by 15°C [54]. The integration of two or more (compatible) fungi in lignocellulolytic hydrolysis processes has yielded good results, although there are as yet only a few examples of this strategy. *T. ressei* and *A. wentti* have been used in combination with cellulose and hemicellulose in the culture media [55]. In addition, *T. ressei* and

*A. phoenicis* were combined using chaff treated with ammonia as a substrate. In both cases, the activity of cellulases and hemicellulases increased considerably; however, the main challenge to growing several organisms in the same culture media is to achieve metabolic coordination [56]. Heterologous expression is a powerful tool because it increases not only the activity of the enzyme but also the amount. In response to the need to obtain fungus stocks with greater activities and/or capabilities to generate a functional enzymatic system, many of the fungal cellulase and hemicellulase genes have been cloned and expressed in Aspergillus and bacteria such as *E. coli*; greater success has been achieved in yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* [57]. These strategies have revolutionized the use of fungus hemicellulases in industrial processes. However, the use of only one enzyme in these processes only allows hydrolysis of a single substrate. Adding more enzymes to hydrolyze the hemicellulosic complex is a potential solution to this limitation, but it would increase the cost.

The synergistic activity of hemicellulases has been observed in enzymes from different fungal sources. A feruloyl esterase of A. *niger* has been found to work synergistically with two xylanases, one from the GH10 family (*Thermoascus aurantiacus*) and another from the GH11 family (*T. viride*), in the remains of cereal cell walls [40]. The feruloyl esterase frees more phenolic acids in combination with the GH11 xylanase than in combination with the GH10 xylanase. Studies carried out with pre-treated sugar cane as substrate using a combination of hemicellulolytic enzymes (endo-xylanase, α-arabinofuranosidase, mananase) have shown an increase of up to six times in enzyme activity and an increase in the coordination of enzymes breaking down sugars compared to the untreated substrate. Therefore, pre-treatment is necessary to increase the efficiency of hydrolysis of hemicellulose polymers, but it has no effect on the activity of the enzymes. However, time plays an important role in establishing the cooperative relationships between the enzymes [58].

A biotechnology application of hemicellulases that is becoming increasingly relevant today is the breakdown of lignocellulose waste that is produced in large amounts by different industries, including silviculture, pulp and paper generation, agriculture and viniculture, the production of several urban solid waste (USW) stocks and animal waste. These potentially valuable materials have long been treated as waste all over the world, causing many economic and environmental problems. Important efforts have been made to turn lignocellulolytic waste into added-value products, including biofuels, chemical products and animal feed [59]. However, despite these efforts, 90% of the world production of bioethanol comes from sugarcane in Brazil and cornstarch in the USA, which has generated great controversy because of the use of food sources destined for human consumption and production costs that make bioethanol a low-competitive alternative. In the last decade, research has been focused on developing new fuel sources and products for human use and consumption from lignocellulolytic waste. To partially take advantage of these resources, lignocellulose waste is generally treated with mechanical, physicochemical and biological methods [60]. Some of these treatments have been used in combination. However, these mechanisms do not take full advantage of the available sugars. For example, treatments with concentrated $H_2SO_4$ and HCl (acid hydrolysis) powerfully hydrolyze cellulose but are toxic, corrosive and dangerous and thus require corrosion-resistant reactors. High temperatures and diluted acids are used to hydrolyze hemicellulose into water-soluble sugars, which means that all of this material is wasted. Cellulose and lignin remain in the waste, and the latter is extracted with organic solvents. Pre-treatment with acids improves the hydrolysis of cellulose, but the cost is high when compared to other pre-treatments and requires a neutral pH to prevent the inhibition of fermentation [61].

In the last few years, several strategies that implement the combination of these treatments with lytic enzymes secreted by various microorganisms have been developed. These strategies increase the accessibility of the lignocellulosic material, favoring the hydrolysis of hemicelluloses or lignin for use in several processes, such as the generation of bioethanol [32, 62] [63]. The breakdown of pre-treated substrates such as sugarcane chaff, corn stubble, rice hay and eucalyptus cellulose pulp has been shown to be enhanced by the activity of different enzymes secreted by microorganisms (including fungi and bacteria) that are involved in cellulose and hemicellulose breakdown [64]. The use of the *Coriolus versicolor* fungus in the pre-treatment of bamboo leads to a decrease in the amount of lignin and hemicellulose and an increase of up to 37% in the saccharification rate after treatment [65]. Treatment of pine and fir waste with black rot fungi, *Gloeophyllum trabeum* and *Fomitopsis pinicola*, yields an increase in the saccharification process [66].

With a coordinated process of breakdown by hemicellulases, lignocellulose waste could be transformed into useful products, such as cattle feed, liquid fuels, organic acids, glucose and alcohols (Fig. 2). Recently, several strategies have been developed that involve the breakdown of agricultural waste and other waste sources by fungi capable of hydrolyzing these compounds [67]. A model for study is the filamentous fungus *Humicola var grisea*, for which hemicellulases capable of breaking down the hemicellulose of several sources of carbon have been described. These sources include wheat bran, oat xylan, cellulose (Avicel), oat bran, banana stalks and

coffee spent-ground by the enzymatic activity of xylanases, β-mananases and α-arabinofuranosidases [68]. Similarly, the *Coniochaeta ligniaria* fungus was reported to secrete enzymes capable of hydrolyzing hemicellulose by up to 75%, cellulose by up to 50% and lignin by up to 40% [69].

**Bifunctional enzymes**

The current trend is for the use of enzymatic complexes that capable of completely breaking down the cell walls of any substrate; such use takes full advantage of the material without the need of physicochemical agents or pre-treatments. Thus, microorganisms (mostly bacteria) have been transformed to secrete several bi- or tri-functional enzymes that, in concert, breakdown different hemicellulolytic substrates in a more efficient manner and at lower cost [70] (Table 4). In the search to develop new and efficient strategies, yeasts that have enzymes on the cell surface that are capable of not only breaking down the structures of plant cell walls but also of fermenting these products in a single step are also being designed [71]. Using *S. cerevisiae* as a model, a construct has been generated that contains the fused genes of two enzymes: a *T. reesei* xylanase and an *A. oryzae* β-xylosidase that is expressed on the cell surface [72]. This engineered yeast is capable of hydrolyzing xylan and fermenting the hydrolysis products, showing direct conversion of xylan to ethanol and the potential use of these microorganisms on lignocellulose products. The characteristics of bifunctional enzymes have also revealed new properties. For example, the product of the first reaction is also the substrate of the second reaction. The state of the active site of the first reaction may influence the properties of the active site of the second reaction, and vice versa [73]. The expression of these enzymes is regulated based on where they are located in the enzyme complex; generally the enzyme that acts first (cellulase) is found near the C-terminus of the protein and the accessory enzyme (xylanase) is found near the N-terminus, separated by a link module that binds to carbohydrates [74-75]. This model is known as “end-to-end” fusion. The nature of these bifunctional enzymes makes the integration of these complexes into various microorganisms possible, even though most of them come from bacteria (Table 3).

The breakdown of plant cell walls, and specifically hemicellulose, is a multistep process that requires a multienzymatic complex for efficient bioconversion to sugars. Fungi produce enzymes capable of hydrolyzing and completely breaking down these structures. A coordinated hydrolysis process that uses enzymes from several microorganisms offers an alternative source of energy to offset the depletion of energy resources and also enables the use of hemicellulolytic waste for the production of biofuels and added-value products such as fermentable sugars, organic acids, solvents, resin and feeds for cattle. The generation of new strategies requires the design of genetically modified microorganisms that are capable of carrying out the complete depolymerization of the hemicellulolytic products and secreting more efficient enzymes with low production costs that circumvent the need for solvents or chemical pre-treatment compounds.

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**Table 1**. Synergistic activity of different fungal hemicellulases in the degradation of xylan.

| Microorganism | Enzime | Reference |
|---|---|---|
| ***Thermomonospora fusca BD25*** | B-xylosidase, α-L-arabinofuranosidase, endo-1,4-β-xylanase | [76] |
| ***Humicola insolens*** | α-L-arabinofuranosidases GH43, GH51 | [77] |
| ***Humicola insolens, Meripilus giganteus Trichoderma reesei*** | endo-1,4-β-xylanase, β-xylosidase, α-L-arabinofuranosidase | [78] |
| ***Humicola insolens, Meripilus giganteus*** | α-L-arabinofuranosidase GH 43, α-L-arabinofuranosidase GH 51, endo-1,4-β-xylanase | [63] |
| ***Trichoderma reesei*** | GH10, β-xylosidase | |
| ***Fusarium graminearum*** | endo-1,4-β-xylanase, β-xylosidase, α-L-arabinofuranosidase | [79] |
| ***Penicilium funiculosum*** | Feruloyl esterase, α-D-galactosidase, β-D-mannosidase, endo -1-4-β-mannanase | [22] |

**Table 2.** Biochemical characterization of hemicellulases.

| Enzime | EC | Family | pH optima ~ | Molecular weight kDa~ | Temperature optima °C ~ | Reference |
|---|---|---|---|---|---|---|
| 1,4-β-D-xylanases | 3.2.1.8 | GH 5,8,10,11,43 | 2-7 | 18-39 | 40-70 | [8] [80] [14] |
| 1,4-β-D-xylosidases | 3.2.1.37 | GH 3,39,43,52 | 4- 5 | 60-360 | 40-80 | [8] [80] |
| α-L-arabinofuranosidases | 3.2.1.55 | GH 3,10,43,51,54,62 | 2-6 | 30-80 | 65-85 | [8] [22] |
| α-D-glucuronidases | 3.2.1.139 | GH 4,67 | 4-6 | 90-150 | 40-65 | [81] [82] |
| Acetylxylan esterases | 3.1.1.72 | CE 1,2,3,4,5,6,7 | 4-8 | 28- | 40- 80 | [83] [84] [82] |
| Ferulic esterases | 3.1.1.73 | CE 1 | 5-8 | 31-57 | 37-65 | [85] [86] [87] |
| β-galactosidases | 3.2.1.23 | GH 1,2,35,42,43 | 4-9 | | 37-46 | [88] [26] |
| 1,4-β-D-mannanases | 3.2.1.78 | GH 5,26,113 | 3.5-9 | 30-80 | 40-70 | [16] |
| β-mannosidases | 3.2.1.25 | GH 1,2,5 | 2-7 | 50-130 | 40-70 | [16] |
| α-galactosidases | 3.2.1.22 | GH 4,27,36,57, 97,110 | 5- 6 | 38-108 | 35-70 | [89] [90] [91] |

**Table 3.** Construction of Engineered Multifunctional Enzymes**.**

| Organism | Activity | Reference |
|---|---|---|
| ***Streptomyces chattanoogensis*** | Xylanase-arabinosidase | [92] |
| ***Clostridium papyrosolvens*** | Celulase-xylanase | [93] |
| ***Aspergillus niger*** | Ferulic esterase-CBM-xylanase | [94] |
| ***Aspergillus niger*** | B-glucanase-xylanase | [75] |
| ***Pseudobutyrivibrio xylanivorans*** | Xylanase-deacetylase | [95] |
| ***Bacillus subtilis*** | B-glucanase-xylanase | [96] |
| ***Thermotoga maritima*** | Celulase-xylanase | [97] |
| ***Thermotoga maritima*** | Celulase-β-glucosidase | [98] |
| ***Cytophaga hutchinsonii*** | Xylanase- acetylxylanesterase | [99] |
| ***Clostridium thermocellum*** | (1) Xilanase- β-xylosidase<br>(2) Xylanase- Arabinofuranosidase | [100] |
| ***Escherichia coli*** | Arabinofuranosidase-xylanase- β-xylosidase | [101] |
| ***Clostridium thermocellum*** | Xylanase-arabinofuranosidase-β-xylosidase | [102] |
| ***Thermoanaerobacter ethanolicus*** ***Thermomyces lanuginosus*** | B-xylosidase- α-arabinosidase-Xylanase | [103] |
| ***Thermoanaerobacter ethanolicus*** ***Thermomyces lanuginosus*** | B-xylosidase- α-arabinosidase Xylanase | [104] |

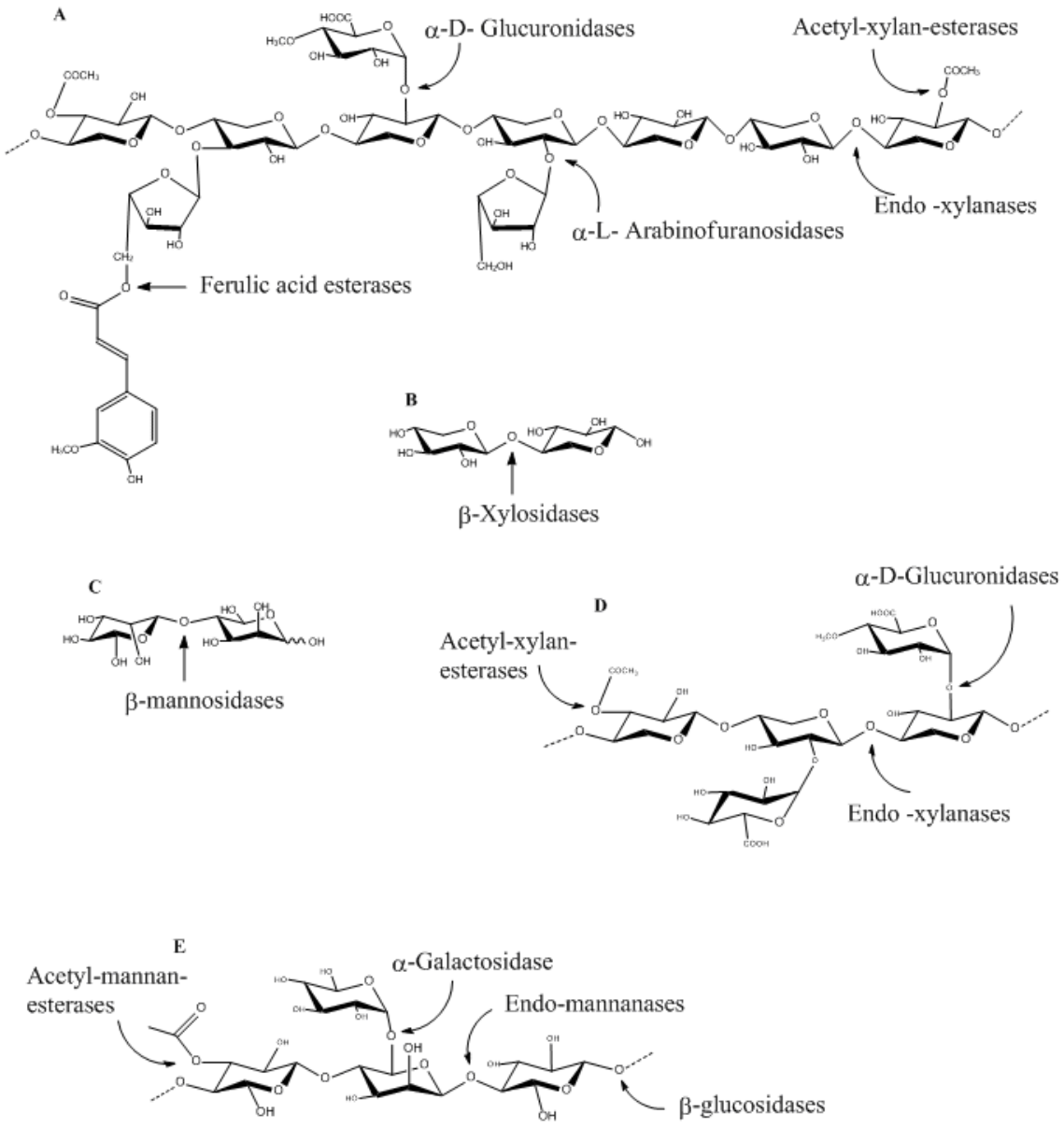


**Figure 1.** Structural components of the hemicelluloses and the main hemicellulases that hydrolyse these polymers. (A) Xylan, major structural component of the hemicellulose formed by a skeleton of molecules in β-D-xylose United together by links β (1→4), except seaweed where these links are also β (1→3). Xilanos soft wood (13% of the xilosas) and herbaceous plants. The α-L-arabinofuranosa is branched C3 and/or C2 (although the C2 less frequently); 4-Ometil-α-D-glucuronic acid and/or α-D-glucuronic acid is branched β-D-xilopiranosas C2 both hardwoods (10% of the xilosas) xilanos and soft Woods (20% of the xilosas) and grasses; the O-acetilos are United C2, C3 or two carbons of β-D-xilopiranosas in hardwoods (70% of xilosas acetiladas) xilanos and herbaceous plants. P-cumárico and ferulic acids are compounds phenolic joined C5 arabinose waste by ester bonds. (B) Xylobiose; (C) Mannobiose; (D) Glucoronoxilano, are the xilanos of hardwoods that have side chains of α-D-glucuronic acid or acid 4-O-methyl-α-D-glucuronic, addition of groups O-acetilo; the β-Mananos as the Xylan are important components of the hemicellulose. Their backbone consists of β-1, 4-Mannose only, or Mannose and some glucose residues distributed randomly; (E) Galacto-glucomanano, contains strings side α-1, 6 of galactose and 2-Ometil and 3-Ometil D-manosa can be replaced with groups acetate.

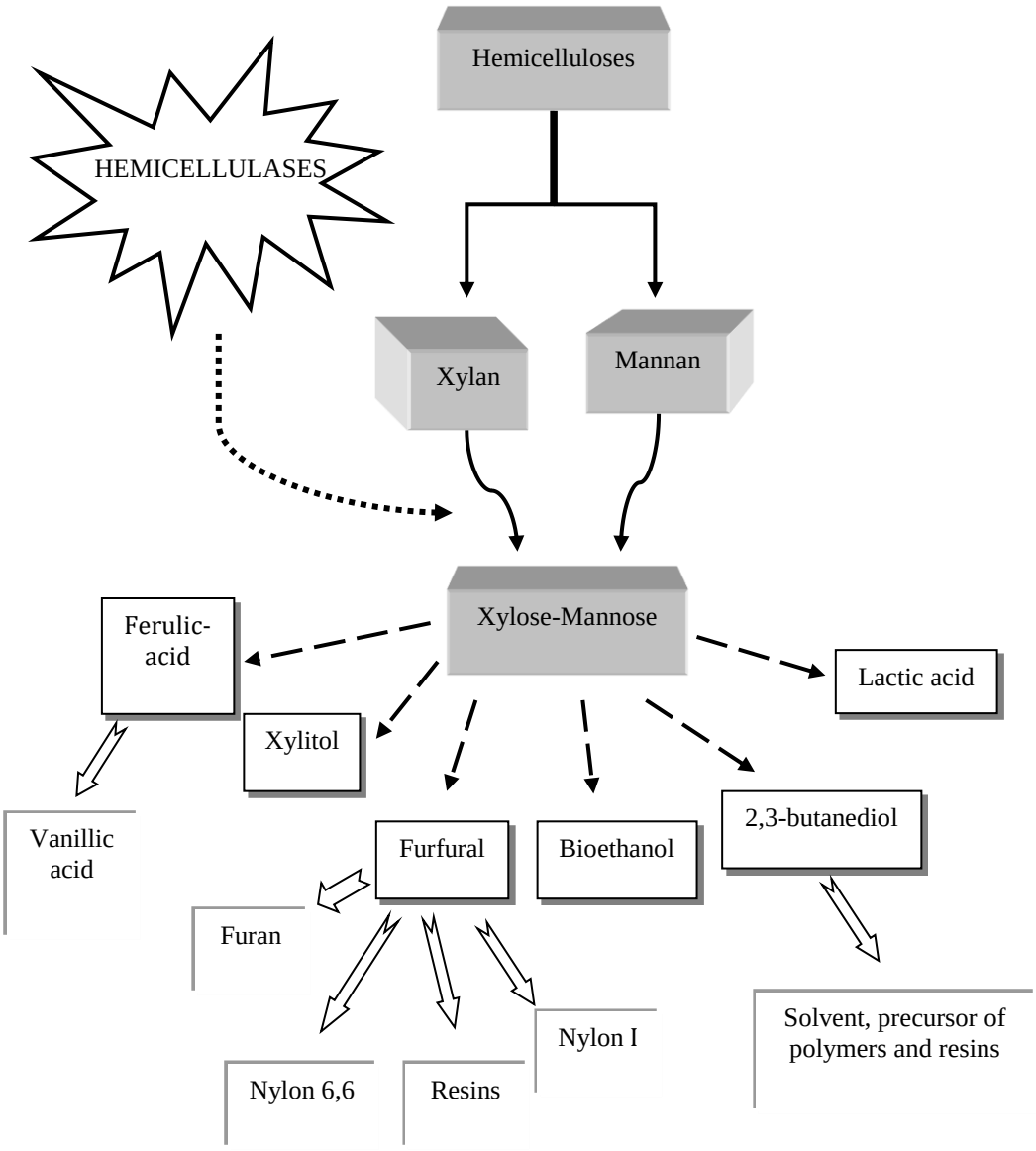


**Figure 2**. Biotechnological applications of hemicellulases and the role of fungal hemicellulases in the generation and production of various compounds.

## CAPÍTULO II

Artículo de investigación, Cloning and characterization of an endo-β-1,4-xylanase gene from *Colletotrichum lindemuthianum* and phylogenetic analysis of similar genes from phytopathogenic fungus. African Journal of Microbiology Research, 2016.

academicJournals

Vol. 10(32), pp. 1292-1305, 28 August, 2016
DOI: 10.5897/AJMR2016.8185
Article Number: A492DF860281 ISSN 1996-0808
**Research**

http://www.academicjournals.org/AJMR

**African Journal of Microbiology**

*Full Length Research Paper*

# Cloning and characterization of an endo-β-1,4-xylanase gene from *Colletotrichum lindemuthianum* and phylogenetic analysis of similar genes from phytopathogenic fungus

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**ABSTRACT**

*Colletotrichum lindemuthianum* is the etiological agent of anthracnose, one of the main diseases of bean (*Phaseolus vulgaris*). In this study, the complete cDNAs of two endo-β-1, 4-xylanase genes (*xyl1*) from non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* were isolated and characterized. To get an insight into the role of endo-β-1, 4-xylanases in their different lifestyles, *xyl1* gene expression and enzyme activity in mycelia of both races grown in the presence of xylan or *P. vulgaris* cell walls were investigated. The *xyl1* sequence analysis and Clustal alignment revealed the characteristic elements of genes coding for endo-β-1, 4-xylanases of the GH11 family. The growth of the two races with glucose as the sole carbon source showed both basal transcription levels of *xyl1* and endoxylanase activity. When glucose was substituted with xylan or plant cell walls, *xyl1* transcription, and enzyme activity significantly increased in race 1472 as compared to race 0. The pathogenic race degraded xylan faster and grew better than the non-pathogenic counterpart. Seemingly, the regulation of xylanolytic gene expression, enzyme production and the nature of the assimilatory carbon substrates processed by these organisms play a determinant role in their lifestyle. Phylogenetic analyses of XYL1 and endo-β-1, 4-xylanases from other fungi revealed a diversification process and separation of proteins from the same fungal species into different lineages.

Keywords: *C. lindemuthianum*, *P. vulgaris*, endo-β-1, 4-xylanase, gene expression, phylogeny.

**INTRODUCTION**

*Colletotrichum lindemuthianum* is an economically important phytopathogen and together with its host *P. vulgaris*, represents a convenient model for studying the physiological and molecular basis of plant-pathogen interactions (Dean et al., 2012; Perfect et al., 1999). This species encompasses different strains or special forms known as races, physiological races or pathotypes identified through the interaction with a group of 12 different cultivars of *P. vulgaris*, a system used worldwide (Rodríguez-Guerra et al., 2006). A non-pathogenic race and more than 100 pathotypes with different virulence levels have been reported around the world. AFLP analyses of 10 out of the 54 *C. lindemuthianum* pathotypes identified in

México have shown high genetic diversity with several lineages (Gonzalez et al., 1998; Sánchez-García et al., 2009). *C. lindemuthianum* is an intracellular hemibiotrophic whose physiological races invade the plant in a manner consistent with the gene-for-gene model interactions (Flor, 1971; Oblessuc et al., 2012). Monogenic dominant resistance in common bean cultivars leads to the appearance of localized necrotic spots that are typical of the hypersensitive response (HR) (O'Connell and Bailey, 1988). After penetrating a host epidermal cell in a susceptible cultivar, pathogenic races of the fungus develop an infection vesicle and extend into adjacent cells by means of large primary hyphae, which invaginate without penetrating the cell membrane and thus persisting as a biotrophic interaction (Münch et al., 2008; O'Connell and Bailey, 1988). Once a large area of the plant tissue has been colonized, necrotrophic hyphae develop. This step closely correlates with the production of a number of host cell wall degrading enzymes that are characteristic of phytopathogenic fungi (Dodds et al., 2009; King et al., 2011; Wijesundera et al., 1989). Currently**,** race 0 is one strain of *C. lindemuthianum* unable to infect 12 different cultivars of *P. vulgaris*, which contrasts with race 1472, one virulent isolated in México (Rodríguez-Guerra et al., 2006). This difference makes the comparison of the two races a convenient approach to investigate the role played by host cell wall degrading enzymes in the pathogenicity of *C. lindemuthianum*.

Analysis of genomic sequences from plant saprophytic and pathogenic fungi has led to the identification of putative genes encoding for carbohydrate-active enzymes (CAZymes) involved in the degradation of plant cell wall. Comparison of these genes has contributed to our understanding of their lifestyle and helped to create infection models (Zhao et al., 2013). For example, biotrophic fungi tend to have fewer CAZymes than necrotrophic and hemibiotrophic fungi. Saprophytic fungi have fewer CAZymes than plant pathogenic fungi, and dicot pathogens often contain more pectinases than monocot pathogens (Zhao et al., 2013). Nevertheless, there have been few studies examining the genetic expression and enzymatic activity of these CAZymes compared to the vast diversity of substrates presented by hosts.

Endoxylanases are CAZymes produced by some saprophytic and pathogenic fungi (Polizeli et al., 2005; Sunna and Antranikian, 1997) and are responsible for the depolymerization of xylan in plant cell wall (Collins et al., 2005; van den Brink and de Vries, 2011). There are

currently more than 100 families of glycoside hydrolases (GHs) in the CAZymes database (Cantarel et al., 2009; Lombard et al., 2014) (http://www.cazy.org/). Endoxylanases are distributed in families GH10 and GH11, which correspond to the F and G families, respectively (Ahmed et al., 2009; Biely et al., 1997; Gilkes et al., 1991). The endo-β-1,4-xylanases (EC 3.2.1.8) belonging to family GH11 hydrolyze the β-1,4 bond of xylan generating xylooligosaccharides, which are further hydrolyzed by β-xylosidase to xylose units (EC 3.2.1.37) (Biely, 1985; Pollet et al., 2010). These endoxylanases fold into β jelly roll sheets that define their secondary structure (Paës et al., 2012). Phylogenetic analysis of endoxylanases of plant saprophytes and pathogens can contribute to the understanding of the evolutionary process in relation to host types and different invasion/nutritional strategies (biotrophic, necrotrophic, or hemibiotrophic).

On this background, here, for the first time the isolation and characterization of *xyl1* cDNA, which encodes an extracellular endo-β-1, 4-xylanase in non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* was reported. Moreover, to understand the role of β-1,4-xylanase in the different fungal lifestyles, *xyl1* gene expression and endoxylanase activity in mycelia of both races grown in the presence of xylan or *P. vulgaris* cell walls were investigated.

Finally, the results of Clustal alignment and phylogenetic analyses of XYL1 from *C. lindemuthianum* and similar enzymes reported in other species of fungi are also presented.

## MATERIALS AND METHODS

### Strains and culture conditions

*C. lindemuthianum* races 1472 and 0 were kindly provided by Dra. June Simpson (CINVESTAV-IPN, Unidad Irapuato, México), which were reported and characterized by interaction with differential varieties of bean (*Phaseolus vulgaris*) and molecular strategies (RAPD and AFLP) as the pathotypes 1472 and 0 by González et al. (1998) and subsequently analyzed by Rodríguez et al. (2006). *C. lindemuthianum* was maintained on potato dextrose agar (PDA) (Difco, México) at 20°C. For expression analysis, 1.6 mg (approximately 5 $cm^2$) of mycelia from both races was inoculated into 250 mL-Erlenmeyer flasks containing 50 mL of PD medium and shaken (150 rpm) at 20°C. After 9 days, mycelia was collected by filtration, washed with water and transferred to 125 mL-

Erlenmeyer flasks containing 50 mL of modified Mathur's medium (Acosta-Rodriguez et al., 2005), supplemented with 2.5% of glucose, xylan (from beechwood; Sigma-Aldrich, St. Louis, MO, USA) or cell walls from *P. vulgaris* (cv. Flor de Mayo). Flasks were shaken (150 rpm) at 20ºC and after various periods of time, mycelia were collected by filtration, washed with water and stored at -80 ºC until use.

For enzyme analysis, 125 mL-Erlenmeyer flasks containing 50 mL of modified Mathur's medium (Acosta-Rodriguez et al., 2005) supplemented with one of the carbon sources described above, were inoculated with 1.6 mg dry weight (approximately 5 $cm^2$) of a 9-day-old colony grown on PDA and incubated at 20°C with continuous shaking (150 rpm). After different periods of time, cultures were centrifuged at low speed and the mycelia and supernatants were saved. Fungal growth was measured as mg of wet mycelia except in those experiments where plant cell walls were used as carbon sources. In these cases, growth was measured as the amount of mycelial protein, as residual undegraded cell walls interfered with weight quantification. A 3-mL aliquot of the cell-free supernatant was filtered through a column (1.5 x 6 cm) of Bio-Gel P-6 (Bio-Rad, Hercules, CA, USA), equilibrated and eluted with 50 mM sodium acetate buffer, pH 5.0 (buffer A), at 4°C to prepare the enzymatic fraction. Fractions corresponding to the void volume ($V_o$) were pooled and the pool, labeled as the filtered extracellular medium (FEM), was used to determine protein and enzyme activity.

**Preparation of plant cell walls**

*P. vulgaris* seedlings (cv. Flor de Mayo) were grown for seven days, and cell walls were extracted and purified from hypocotyls by washing in organic solvents as described elsewhere (Fry, 2006).

**RNA isolation**

Total RNA was purified from mycelia using the Sokolovsky method (Sokolovsky and V.E.A., 1990). RNA samples were treated with DNAse I according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA) to eliminate DNA. The quality and concentration of total RNA were assessed using a Biophotometer Plus system (Eppendorf, Barkhausenweg, Hamburg, Germany).

**cDNA isolation, sequencing, and analysis**

A cDNA fragment (223 bp) of the endo-β-1,4-xylanase gene (xyl1) from race 1472 of C. lindemuthianum was amplified using the reported primers XYNG2-F [5´-GA(A/G)TA(T/C)TA(T/C)AT(T/C/A)GT(A/T/G/C)GA(A/G)(A/T)(G/C) (A/C/G/T)TA-3´] and XYNG2-R [5´-GCCCA(A/C/G/T)GC(A/G)TT(A/G)AA(A/G)TG(A/G) TT-3´] according to Kimura (Kimura et al., 2000). Total RNA was isolated from mycelia induced with xylan for 24 h. This fragment was sequenced (data not shown), the specific primers Xyl-D110 (5'-GCGTGAACCAGCCCAGCATC-3') and Xyl-GSP1 (5'-CAGCGTTGAAGTGGTTG-3´) were designed. The cDNA of xyl1 was amplified by 3´and 5´RACE as specified by the manufacturer using the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) using total RNA isolated from mycelia of race 1472 induced with xylan for 24 h. Finally, the complete cDNAs of xyl1 were amplified with the specific primers designed on the 5´ UTR, Clxil1-F (5´-ACTTATCATCGTCCGCTTCAACCA-3´) and 3´ UTR Clxil1-R (5´-GCAATCCTCGGAGTTCCAATCTGA-3´), using total RNA of mycelia from both races induced with xylan for 24 h. The PCR incubation mixture was heated at 96°C for 3 min in a Thermocycler (Eppendorf Master Cycler Gradient, Brinkmann, Westbury, NY), followed by 30 cycles of denaturation for 30 s at 96°C, annealing for 35 s at 60°C, extension for 1 min at 72°C and then by a final extension for 10 min at 72°C. All PCR products obtained from both races were ligated into the pCR 2.1 vector (Invitrogen).

The sequences of both strands of cDNA were determined by automatized sequencing using the dideoxy-chain termination method by the commercial service of Macrogen USA. Nucleotide sequences were analyzed using DNAsis (Hitachi), Mega6 (Tamura et al., 2013) and 4Peaks v 1.7.2 software (Griekspoor A., 2012). The sequence of the N-terminal secretion signal was identified with SignalP 4.1 Server (Bendtsen et al., 2004). The protein molecular masses, pI values and *N*-glycosylation sites were calculated using ExPASy Proteomics Server (Wilkins et al., 1999). The nucleotide sequences of 12 endo-β-1,4-xylanases from fungal species were obtained from the NCBI GenBank (Table 1) and were numbered when more than one gene was present in a genome. Multiple sequence alignments were performed with Clustal X software (Larkin et al., 2007) using the default

parameters. The signal peptide sequences and N-and C-terminal extensions were excluded.

**Expression analysis of *xyl1***

Relative quantification of gene expression (RT-qPCR) was performed using the comparative Ct method (DDCt) on a Step One Plus Real-Time PCR System (Applied Biosystems Carlsbad, CA, USA) according to the manufacturer's instructions. Reactions were carried out with an SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). A fragment of cDNA (208 bp) was amplified with the designed specific primers TRxil2-F (5´TGTCGGAGGCAAGGGCTGGAATC 3´) and TRxil2-R (5´ ATAAGTGCCGCCGTCGCTGGTGA 3´) using total RNA obtained from mycelia induced with 2.5% glucose for 8 h, or xylan or CW from *P. vulgaris* for 0, 2, 4, 6, 12, 24 and 48 h and 3, 4, 5, 7 and 9 days. Fragments of cDNA (372 and 339 bp for races 1472 and 0, respectively,) from the *C. lindemuthianum* □-tubulin gene (*btub*) were amplified with the primers □□□F and □□2R (5´ CACCCACTCCCTCGGTGGTG 3´ and 5´CATGAAGAAGTGAAGACGCGGGAA 3´, respectively) (Thon and Royse, 1999) using total RNA obtained from mycelia grown with glucose. The □-tubulin fragments were sequenced and deposited in GenBank (Accessions: KF487130 and KM587706). The specific primers TR-βtub2-D and TR-βtub2-R (5´ GAATTCCCCGACC GTATGATG 3´ and 5´ CGGAGAGGGTGGCGTTGTA 3´, respectively) were designed and used as an internal control (endogenous gene).

Data were obtained from three independent experiments performed in triplicate, and analysis of variance (ANOVA) was carried out. The results are reported as the mean and standard errors (SE). *P* values <0.05 were considered significant.

**Assay of endoxylanase activity**

Endoxylanase catalyzes the hydrolysis of the 1,4-β-D-xylosidic linkages present in xylan, releasing β-D-xylopyranosyl oligomers and smaller molecules such as β-D-xylopyranosyl mono-, di- and tri-saccharides (Collins et al., 2005; Polizeli et al., 2005). These products can be quantified as reducing sugars using colorimetric methods. Accordingly, reaction mixtures containing 0.5 mL of 0.5% xylan (Sigma), 0.5 mL FEM and buffer A in a final volume of 1 mL were incubated at 30°C. After 10 min, the reaction was terminated by

heating the samples in boiling water for 5 min and, after cooling, the amount of reducing sugars was measured using the Nelson-Somogyi method (Nelson, 1944). Activity was expressed, as µg of reducing sugars released in one min. Specific activity was referred to one milligram of protein.

**Statistical analysis**

Statistical analysis of data was performed using ANOVA with a GLM using a 2 x 3 factorial design (two races and three carbon sources) at each time point. The mean values of the enzyme activity of the fungus and its substrate and the amount of protein and mycelial protein were compared and grouped using the Tukey's test. All analyses were performed with STATISTICA v 10 software (Inc., 2013).

**Phylogenetic analyses**

Phylogenetic analyses were performed on the deduced amino acid sequence for *C. lindemuthianum* XYL1 and 11 endo-β-1,4-xylanases characterized from other ascomycetes (Table 1) and one sequence from the basidiomycete *Lentinula edodes* as an outgroup. Deduced amino acid sequences were aligned with Clustal X software (Larkin et al., 2007) using the default parameters. Before phylogenetic analyses, the signal peptide sequences, and N- and C-terminal extensions were excluded. Phylogenetic analysis was performed under Neighbor-Joining and Maximum likelihood criteria using Mega6 (Tamura et al., 2013). The JTT substitution model and gamma correction were used, 1000 bootstrap replicates were performed. The amino WAG evolution model with gamma correction was utilized for Maximum likelihood analysis, and the most parsimonious trees were estimated using the heuristic search option (Nearest-Neighbor-Interchange-NNI) with random sequence addition (five random replicates). A WAG+G substitution model was used, and 1000 bootstrap replicates were performed.

**RESULTS**

**Isolation and sequence analysis of *xyl1***

cDNA encoding an endo-β-1,4-xylanase was isolated from each race and deposited in GenBank (Accessions: KF487129, KM587707). The *C. lindemuthianum xyl1* cDNA of

race 1472 has 905 bp, with a 5´ UTR of 25 bp and a 3´ UTR of 211 bp (Fig. 1). The *xyl1* cDNA of race 0 has 751 bp, with a 5´ UTR of 23 bp and a 3´ UTR of 59 bp (Fig. 2). At nucleotide and amino acid levels, the sequence of both races showed 100% identity. Comparison at amino acid level with corresponding sequences in GenBank showed 67%, 64%, 62% and 61% identity with a *xylI* of *P. tritici-repentis, xil1 of Cochliobolus carbonum, htxyl1* of *Helminthosporium turcicum, Xyn22 of Magnaporthe grisea*, respectively. The putative protein has an open reading frame of 222 amino acids with a signal peptide cleavage site between $Ala^{19}$ and $Ser^{20}$ (Figs. 1 and 2), according to the SignalP 4.1 web server (Bendtsen et al., 2004), which is consistent with previously reported sequences (Apel-Birkhold and Walton, 1996; Kimura et al., 2000). The putative mature protein (residues 20 to 222) has a calculated molecular mass of 21.71 kDa and a pI of 8.94. A potential *N*-glycosylation site at $Asn^{71}$ was found with the ExPASy Proteomics Server (Ellouze et al., 2011; Wilkins et al., 1999).

The multiple sequence alignment of the deduced amino acid sequences of *C. lindemuthianum* XYL1 with the endo-β-1, 4-xylanases of other fungi revealed the conserved motif EYY where a residue corresponding to the catalytic site is found (Fig. 3). In various bacteria and fungi, this motif was reported to be the key segment for enzyme catalytic activity or substrate binding (Apel et al., 1993; Degefu et al., 2001; Li and Ljungdahl, 1994). The deduced amino acid sequence of *C. lindemuthianum* XYL1 revealed two Glu residues ($E^{96}$, $E^{187}$) that are highly conserved in xylanases of family GH11 and are likely to be involved in hydrolysis of the glycosidic bond (Figs. 1, 2 and 3) (Degefu et al., 2001; Kimura et al., 2000; Tanaka et al., 2005), with one acting as an acid catalyst/base and the other as a nucleophilic residue (Davies and Henrissat, 1995; Sapag et al., 2002). Additionally, XYL1 has an Asp in position 55 that may be necessary for maintaining the optimum pH of these enzymes (Ellouze et al., 2011; Lübeck et al., 1997b).

**Analysis of *xyl1* expression and production of endoxylanase activity**

When used as the principal carbon source, glucose sustained growth of both fungal races but the maximum growth of race 0 (501.67 mg after 10 days) was delayed by approximately two days and was 29% lower compared with race 1472 (708 mg after 8 days) ($P<0.0001$) (Fig. 4A). Extracellular endoxylanase (XYL) production by the two races

was very low, with values of specific activity in the range of 6.4 and 10.5 reducing sugars/min/mg protein after 2 and 12 days of growth, respectively, for the race 1472 (Fig. 4A). Corresponding values for the race 0 were 2.5 and 11.83 (Fig. 4A). However, even under these conditions, significant differences in XYL production were observed with an increase for race 1472, particularly during early days of growth. For both races, a peak was observed after seven days ($P$<0.0001). The *C. lindemuthianum xyl1* transcript exhibited basal levels of expression (1-fold) in both races (Figs. 5A and B).

As described earlier (Hernández-Silva et al., 2007), both races utilized xylan as a carbon substrate, but growth of race 1472 (416.67 mg of mycelia after 8 days) was over 5.5-fold higher and substantially faster than that of race 0 (75 mg of mycelia after 8 days) ($P$<0.0001) (Fig. 4B). Values of 416.67 mg and 75 mg represent 59% and 15% of the maxima obtained in glucose-supplemented cultures, respectively. Xylan stimulated the production of XYL activity, which was increased and peaked at 90 µg reducing sugars/min/mg protein after seven days of growth and was faster in the pathogenic race ($P$<0.0001)(Fig. 4B). This result was by far the highest activity detected in this study and was 1.5-fold higher than that of race 0, which reached a maximum of 59.67 µg reducing sugars/min/mg protein one day later (Fig. 4B). The pathogenic race strongly expressed the *xyl1* transcript after 6 to 48 h and three days (16.5-fold) and decreased over the following 4-9 days (Fig. 5A). In contrast, the non-pathogenic race weakly expressed the *xyl1* transcript between 0 h and three days, increased and peaked after four days (6.6-fold) and decreased over the following 5-9 days (Fig. 5A).

The ability of cell walls fractions from *P. vulgaris* to sustain the growth of *C. lindemuthianum* and induce endoxylanase was also tested. As observed with other carbon sources, the pathogenic race grew faster and to a greater extent than the non-pathogenic race. This difference was maintained up to approximately 8-9 days of incubation ($P$<0.0001) (Fig. 4C). After that, both races grew with similar rates producing comparable amounts of mycelium protein after 12 days of growth ($P$>0.05). Due to technical restrictions to measure growth on cell walls, values obtained with this substrate could not be compared to those obtained in soluble carbon substrates. The pathogenic race exhibited low expression levels of the *xyl1* transcript during early time points, then a weak peak was observed after 48 h (3.3-fold), and this increased over the following 5-9 days (9.3-fold)

(Fig. 5B). In contrast, the non-pathogenic race weakly expressed the *xyl1* transcript between 0-48 h, then it increased, peaked after 3 and five days (~2-fold) and finally decreased over the following 7-9 days (Fig. 5B). Interestingly, after eight days of incubation, the pathogenic race produced an amount of XYL activity (80 µg reducing sugars/min/mg protein) equivalent to 89% of the maximum observed after seven days of growth on xylan (Figs. 4C and B). The non-pathogenic race produced a 1.6-fold lower amount of enzymatic activity (50 µg reducing sugars/min/mg protein) that corresponded to 67% of the maximum induced by xylan (Fig. 4C and B). It should be noted that in this case, the maximum XYL activity was produced by the non-pathogenic race two days after the maximum produced by the pathogenic race.

The regulation of lytic enzymes in some fungi (St. Leger et al., 1988) and gene expression in several species of *Colletotrichum* can be modulated by ambient pH (Prusky et al., 2001) and this mechanism of regulation operates in a vast diversity of organisms (Denison, 2000). Therefore, we considered it necessary to determine whether the pH of the extracellular medium varied during the period of incubation under the different culture conditions. Throughout this study, the initial pH of all culture media was adjusted to 5.5. The growth of the pathogenic race in the presence of glucose, xylan or cell walls resulted in a rather irregular profile of pH variation. After 12 days, the pH reached values of 6.0, 6.5 and 7.2, respectively (Table 2). On cell walls, an abrupt acidification of the medium was observed after two days and then it became increasingly alkaline. The growth of the non-pathogenic race on glucose or xylan resulted in a steadier pattern of pH alkalinization to final corresponding values of 6.2 and 6.5. Alkalinization of the medium to pH 7.0 by race 0 grown on plant cell walls followed a more irregular profile.

**Phylogenetic analyses**

Comparison of amino acid sequences of endoxylanases showed 40% to 67% identity suggesting a diversification process that gave rise to proteins that shared identity mainly in sequence and the structure of the catalytic site. Clustal alignment identified the location of amino acids in endoxylanases expected to have a catalytic role (Fig. 3) (Ellouze et al., 2011; Sapag et al., 2002). The phylogenetic analyses revealed to xyl3 of *Cochliobolus carbonum* and xyl1 of *Claviceps purpurea* in a basal clade (Fig. 6). Next, xyn11A of *Botrytis cinerea*

was separated as the next version of these enzymes. Later in an evolutionary progression, the rest of enzymes were grouped into a widely diversified clade with XYL1 of *C. lindemuthianum* as basal of two sub-clades or lineages. In one of these sub-clades, other protein (xyl1) of *C. carbonum* grouped with htxyl1 of *H. turcicum*, the enzyme of *Didymella pisi* and xyl5 of *Fusarium oxysporum* was found. In the other sub-clade, htxyl2 of *Helminthosporium turcicum* grouped with xyl2 of *Cochliobolus sativus*, xyl4 of *Fusarium oxysporum* and xyn22 of *Magnaporthe grisea* were found (Fig. 6).

## DISCUSSION

No differences were found in the coding region of the endo-β-1, 4-xylanase of the non-pathogen and pathogen races of *C. lindemuthianum.* The *xyl1* sequence analysis and Clustal alignment with xylanases reported for other fungi revealed the characteristic elements of genes coding for endo-β-1, 4-xylanases of family GH11.

Induction of cell wall degrading enzymes by different carbon substrates has been studied in some of phytopathogenic fungi such as *Sclerotinia sclerotiorum* (Riou et al., 1991), *Sclerotium rolsfii* (Sachslehner et al., 1998) and *Penicillium* sp. (Rahman et al., 2003), among others. The involvement of some of these enzymes in the development of bean anthracnose by *C. lindemuthianum* race γ was first described by Wijesundera et al. (1989). Results presented here show a clear difference between the non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* regarding growth, induction of *xyl1* transcript expression and production of extracellular endoxylanase activity when they are challenged with different carbon substrates. Accordingly, though maximum growth was obtained on glucose, a readily metabolizable nutrient, basal expression of *xyl1* and only trace amounts of XYL were produced by both races; however, although low, a higher XYL production was observed for race 1472, particularly early during incubation. A similar basal production of xylanase and endoglucanase (Sachslehner et al., 1998; Tuncer et al., 2004), pectinases (Hernández-Silva et al., 2007; Oyeleke et al., 2012; Riou et al., 1991) and cellulases (Acosta-Rodriguez et al., 2005; Carle-Urioste et al., 1997; Sharada et al., 2013) has been described in the fungi *S. rolsfii*, *S. sclerotiorum* and *C. lindemuthianum*, respectively.

In fungi, the expression of extracellular hydrolytic enzymes is coordinately regulated by

transcriptional activators and repressors (Aro et al., 2005; Tani et al., 2014). The expression of genes encoding xylanolytic enzymes is subject to catabolic repression through the action of CreA under a preferred carbon source and the activation through the action of XlnR under carbon limitation (Cho and Choi, 1999; de Vries and Visser, 2001; Tani et al., 2014). In this study, significant levels of the *xyl1* expression and endoxylanase were produced in pathogenic race 1472 only when the enzyme substrate was available in a medium lacking other nutrients of easier assimilation, such as glucose, indicating that expression of *xyl1* can be regulated by the carbon source. It has been proposed that basal levels of endoxylanase commence degradation of xylan generating products that induce further enzymatic activity. Xylose, as the final product of xylan degradation, functions as a regulator of the expression of xylanases, acting as an inducer at low concentrations and as a repressor at high levels (de Vries, 2003; de Vries et al., 1999; Kulkarni et al., 1999; Mach-Aigner et al., 2012; Mach-Aigner et al., 2010; Stulke and Hillen, 2000). These results support this idea as degradation of xylan occurred when basal levels of endoxylanase activity produced low levels of xylose, which then induced expression of higher levels of expression of *xyl1* and enzyme production in the pathogenic race 1472. At later time points, a reduction of activity was observed most likely due to repression by the accumulation of xylose.

The non-pathogenic race of *C. lindemuthianum* used in this work is unable to infect *P. vulgaris*, and thus its lifestyle is closer to that of a saprophytic fungus. Therefore, it is possible that the differences found between the non-pathogenic and pathogenic races of *C. lindemuthianum* are related to the speed of activation of the lytic enzyme genes during the interaction with the host. Additionally, the pathogenic race degrades xylan faster and grows better than the non-pathogenic race, suggesting a different ability in the degradation of this polysaccharide and the use of oligo- or monomeric sugars. As previously described (Hernández-Silva et al., 2007; Lara-Marquez et al., 2011), the expression of *Clpnl2* gene and activity of pectin lyase between the two races were similar to the observed in this study when 92% esterified pectin was utilized as the sole carbon source. In other words, the pathogen requires a rapid and higher level expression of endoxylanase activity and other related lyticases for successful interaction with the live plant tissue, which implies an energy cost that the non-pathogen does not have to invest because it feeds on dead plant tissue.

The response of the fungus to cell walls from *P. vulgaris* is interesting if it is considered that only a certain proportion of the provided substrate corresponds to xylan. A typical primary cell wall contains 9-25% cellulose, 25-50% hemicellulose (whose main structural polymer is xylan), 10-35% pectin and 10% proteins (Cosgrove, 1997). The authors previously demonstrated that *P. vulgaris* cell walls also induce pectin lyase (PNL) activity in the pathogenic race to levels that represent up to 46% of the maximum obtained with 92% esterified pectin, the best PNL inducer. This value was approximately 2.8-fold higher than that produced by race 0 (Hernández-Silva et al., 2007). This difference is close to that observed for endoxylanase activity in this study. These data indicate that polysaccharides present in the cell wall structure cooperate efficiently to induce a range of polysaccharidases specific for the types of glycosidic linkages present in the cell wall components, thus granting the fungus the ability to degrade the wall barrier efficiently.

A number of evidences indicate a role of ambient pH in the regulation of production of pectinolytic enzymes in fungi such as *Penicillium paxilli* (Szajer and Czajer, 1985), *P. itallicum* (Alaña et al., 1989), the avocado-pathogen *C. gloeosporioides* (Drori et al., 2003; Kramer-Haimovich et al., 2006; Yakoby et al., 2000), *Trametes trogii* (Levin and Forchiassin, 1998) and *Aspergillus oryzae* (Fontana and Silveira, 2012). In the latter, alkalinization during fruit infection is necessary for the conversion of the biotrophic stage into the necrotrophic stage (Kramer-Haimovich et al., 2006). Also, ambient pH has been described as a regulatory factor related to the pathogenesis of *S. sclerotiorum* (Rollins and Dickman, 2001), *C. gloesporoides* (Alkan et al., 2013) and *C. acutatum* (You et al., 2007). Contrary to these findings, an effect of the pH on growth, expression of *xyl1* gene and production of XYL activity by *C. lindemuthianum* was not observed, which is consistent with previous results on PNL activity (Hernández-Silva et al., 2007).

The results, allow us to hypothesize that the regulation of enzyme expression and nature of the assimilatory carbon substrates processed by these organisms play a determinant role in their lifestyle. The differences in growth, *xyl1* expression and production of enzymatic activity between the two races of *C. lindemuthianum* suggest an adaptation of race 1472 that results in a rapid degradation of xylan, induction of increased activity and utilization of depolymerization products as carbon nutrients. Race 0 does not seem to prefer xylan as a carbon source but instead grows better with bean cell walls, suggesting that differences

exist in the utilization of mono- or oligosaccharides on race 1472. The authors observed a similar behavior of other enzymes of the complex involved in the degradation of the cell wall suggesting that it may be a general phenomenon (Acosta-Rodriguez et al., 2005; Hernández-Silva et al., 2007; Lara-Marquez et al., 2011). The differences at this level can be part of the general response of fungi to host components. However, future studies comparing the enzymatic complexes of degradation of more fungal species with different lifestyles will be required to confirm this hypothesis.

Finally, phylogenetic analyses showed a diversification of endo-β-1, 4-xylanases and separation of proteins from the same fungal species into various groups or lineages. Similar results were described after a phylogenetic analysis of the nucleotide sequences of the *htxyl1* and *htxl2* xylanase genes from the corn pathogen *H. turcicum.* These genes showed differential expression related to the substrate type (xylan and/or xylose) or stages of infection of maize, suggesting a role in saprophytic or pathogenic phases (Degefu et al., 2004; Ellouze et al., 2011). Here, a phylogenetic separation of other xylanases with differential expression was found; *xyl1* and *xyl3* in *C. carbonum* (Apel-Birkhold and Walton, 1996) and *xyl4* and *xyl5* in *F. oxysporum* (Gomez-Gomez et al., 2001; Gomez-Gomez et al., 2002), suggesting diversifying selection (Brunner et al., 2013). In this context, the differential expression of cutinases, cellulases, hemicellulases and pectinases related to different stages of the life cycle, namely, biotrophic, necrotrophic and saprophytic, has been reported in the hemibiotrophic pathogen *Zymoseptoria tritici* (Brunner et al., 2013). Also, purified selection has been detected in many genes, which can be related to the optimization of enzymatic activity. In some of these genes, diversifying selection has also been detected, which is possibly related to the adaptation to the host and/or the life cycle of the fungus (Brunner et al., 2013).

**ACKNOWLEDGEMENTS**

The authors thank the financial support provided by SEP-CONACyT (Project 2012-01, code 182755 to MGZP), CONACyT for scholarships granted to UCS and MGVR, and UNAM for a postdoctoral fellowship program 2012-14 to ALM.

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**Table 1.** Sequences of endoxylanases used for the analyses.

| Fungi | GenBank Access | In this study | Reference |
|---|---|---|---|
| *Didymella pisi* | CAA93120.1 | *Didymella_pisi* | (Lübeck et al., 1997a) |
| *Botrytis cinérea* | AAZ03776 | *B.cinerea_xyn11A* | (Brito et al., 2006) |
| *Cochliobolus carbonum* | L13596 | *Cochliobolus_carbonum-xyl1* | (Apel-Birkhold and Walton, 1996) |
| *Cochliobolus carbonum* | U58916 | *C.carbonum-xyl3* | (Apel-Birkhold and Walton, 1996) |
| *Cochliobolus sativus* | CAA06151.1 | *C. sativus-xyl2* | (Emami and Hack, 2001) |
| *Claviceps purpurea* | CAA76570 | *Claviceps_purpurea-xyl1* | (Giesbert et al., 1998) |
| *Fusarium oxysporum f. sp. lycopersici.* | AAK27975.1 | *Fusarium_oxysporum-xyl4* | (Gomez-Gomez et al., 2002) |
| *Fusarium oxysporum f. sp. lycopersici.* | AF246830_1 | *F.oxysporum-xyl5* | (Gomez-Gomez et al., 2002) |
| *Helminthosporium turcicum* | CAB52417.1 | *Helminthosporium_turcicum-htxyl1* | (Degefu et al., 2004) |
| *Helminthosporium turcicum* | CAD70174.1 | *H. turcicum-htxyl2* | (Degefu et al., 2004) |
| *Magnaporthe grisea* | L37530 | *Magnaporthe_grisea*-Xyn22 | (Wu et al., 1995) |
| *Lentinula edodes* | AAL04152.1 | *Lentinula_edodes* | (Lee et al., 2005) |

**Table 2.** Kinetic data of pH analysis in races 1472 and 0 of *C. lindemuthianum,* grown with 2.5% of glucose, xylan or *P. vulgaris* cell walls.

| | Race 1472 | | | Race 0 | | |
|---|---|---|---|---|---|---|
| **Time*** | **Glucose** | **Xylan** | **Cell walls** | **Glucose** | **Xylan** | **Cell walls** |
| 0 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |
| 2 | 5.66 | 5.585 | 4.43 | 5.52 | 5.605 | 5.5 |
| 4 | 6.79 | 6.04 | 6.5 | 5.63 | 6.015 | 6.08 |
| 6 | 5.6 | 6.88 | 6.93 | 5.81 | 6.09 | 7.06 |
| 7 | 5.92 | 6.485 | 6.91 | 5.93 | 6.295 | 6.36 |
| 8 | 4.83 | 7.215 | 7.99 | 6.13 | 6.655 | 6.66 |
| 10 | 4.95 | 6.705 | 8.05 | 6.39 | 6.79 | 7.21 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 12 | 6.03 | 6.66 | 7.41 | 6.18 | 6.725 | 7.15 |

* Time expressed in days

**Figure 1.** Nucleotide and deduced amino acid sequences of the *xyl1* gene of *C. lindemuthianum* race 1472. The signal peptide sequence is underlined, the catalytic residues (Glu-96 and 187) are boxed, and an asterisk indicates the stop codon.

**Figure 2.** Nucleotide and deduced amino acid sequences of the *xyl1* gene of *C. lindemuthianum* race 0. The signal peptide sequence is underlined, and the catalytic residues (Glu-96 and 187) are boxed, and an asterisk indicates the stop codon.

**Figure 3.** Clustal alignment of endoxylanases. Identical residues and conserved catalytic residues are marked with an asterisk (*). Dots indicate the change of one amino acid for another of the same group.

**Figure 4.** Growth and production of extracellular endoxylanase activity (XYL) by race 1472 and race 0 cultivated in the presence of glucose (A), xylan (B), or plant cell walls (C) as the sole carbon sources. Diamonds, the growth of race 1472; circles, the growth of race 0; stripped bars, XYL activity of race 1472; gray bars, XYL activity of race 0.

**Figure 5.** Expression analysis of *xyl1* by RT-qPCR induced with xylan (A) or bean cell walls (B). Stripped bars, show *xyl1* expression in race 1472; gray bars, show *xyl1* expression in race 0. Each bar indicates the mean of triplicates ± SE of three independent experiments. The symbol "*" indicates significant changes ($P<0.05$) about the control (glu, glucose).

**Figure 6.** Phylogenetic analyses of endoxylanases from *C. lindemuthianum* and other fungal species. The tree was constructed using the Neighbor-Joining (NJ) and Maximum likelihood methods and includes only the conserved region of the catalytic domain (190 aa) of the proteins used in the analysis. General topology obtained is represented by the 50% of majority rule consensus tree, in which the NJ posterior probabilities and ML bootstrap support are indicated on the branches. The numbers at the nodes indicate bootstrap values

based on 1,000 bootstrap replications.

**Figure 1**

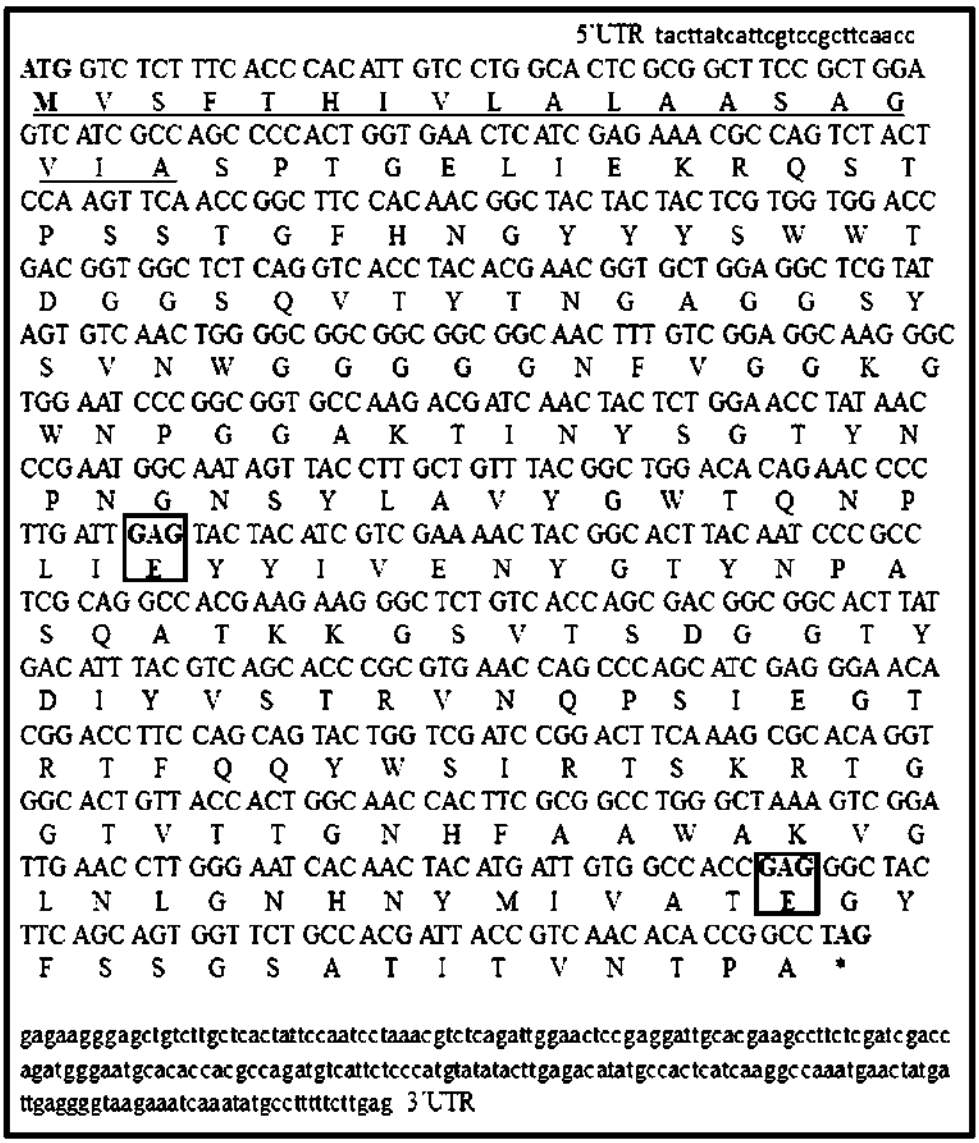
5'UTR tacttatcattcgtccgcttcaacc
ATG GTC TCT TTC ACC CAC ATT GTC CTG GCA CTC GCG GCT TCC GCT GGA
M V S F T H I V L A L A A S A G
GTC ATC GCC AGC CCC ACT GGT GAA CTC ATC GAG AAA CGC CAG TCT ACT
V I A S P T G E L I E K R Q S T
CCA AGT TCA ACC GGC TTC CAC AAC GGC TAC TAC TAC TCG TGG TGG ACC
P S S T G F H N G Y Y Y S W W T
GAC GGT GGC TCT CAG GTC ACC TAC ACG AAC GGT GCT GGA GGC TCG TAT
D G G S Q V T Y T N G A G G S Y
AGT GTC AAC TGG GGC GGC GGC GGC GGC AAC TTT GTC GGA GGC AAG GGC
S V N W G G G G G G N F V G G K G
TGG AAT CCC GGC GGT GCC AAG ACG ATC AAC TAC TCT GGA ACC TAT AAC
W N P G G A K T I N Y S G T Y N
CCG AAT GGC AAT AGT TAC CTT GCT GTT TAC GGC TGG ACA CAG AAC CCC
P N G N S Y L A V Y G W T Q N P
TTG ATT GAG TAC TAC ATC GTC GAA AAC TAC GGC ACT TAC AAT CCC GCC
L I E Y Y I V E N Y G T Y N P A
TCG CAG GCC ACG AAG AAG GGC TCT GTC ACC AGC GAC GGC GGC ACT TAT
S Q A T K K G S V T S D G G T Y
GAC ATT TAC GTC AGC ACC CGC GTG AAC CAG CCC AGC ATC GAG GGA ACA
D I Y V S T R V N Q P S I E G T
CGG ACC TTC CAG CAG TAC TGG TCG ATC CGG ACT TCA AAG CGC ACA GGT
R T F Q Q Y W S I R T S K R T G
GGC ACT GTT ACC ACT GGC AAC CAC TTC GCG GCC TGG GCT AAA GTC GGA
G T V T T G N H F A A W A K V G
TTG AAC CTT GGG AAT CAC AAC TAC ATG ATT GTG GCC ACC GAG GGC TAC
L N L G N H N Y M I V A T E G Y
TTC AGC AGT GGT TCT GCC ACG ATT ACC GTC AAC ACA CCG GCC TAG
F S S G S A T I T V N T P A *
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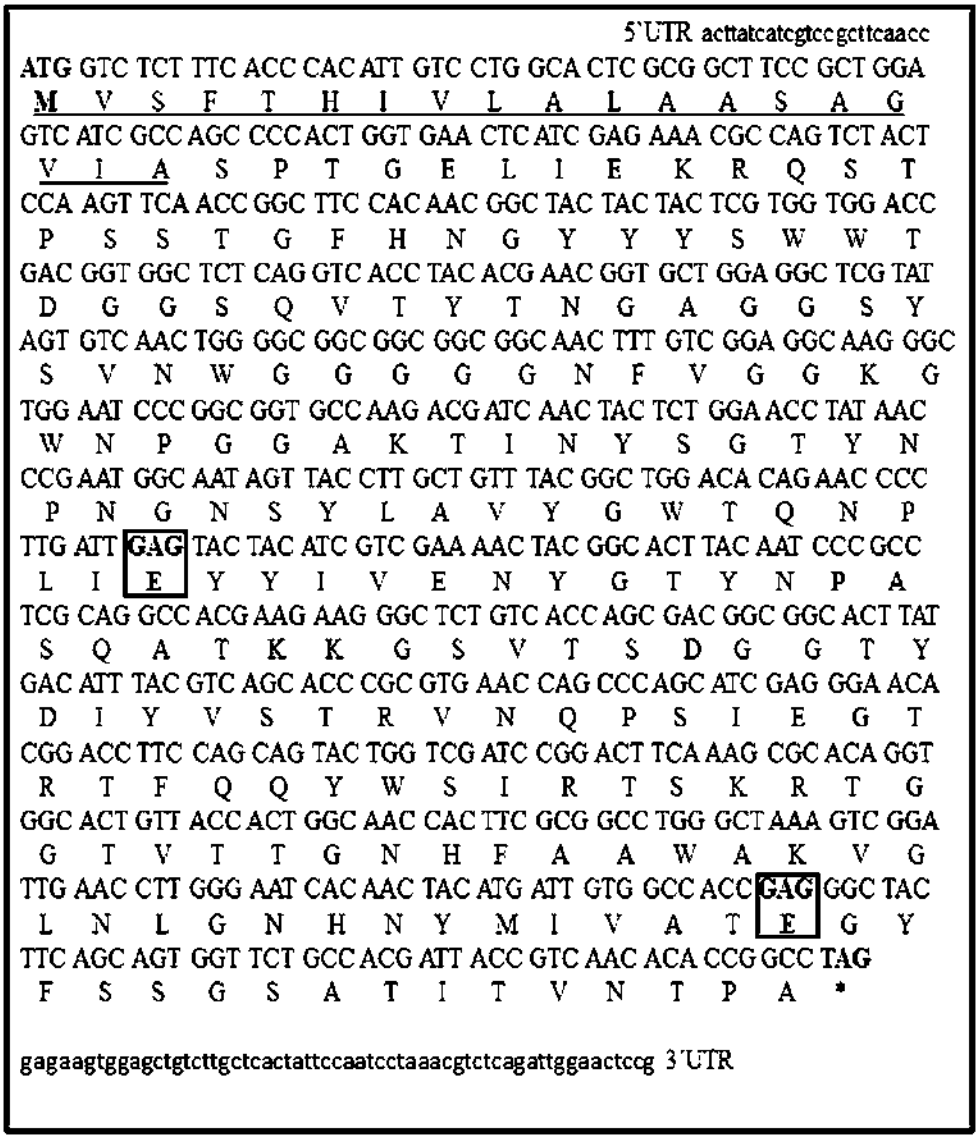
**Figure 2**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V I A S P T G E L I E K R Q S T
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D G G S Q V T Y T N G A G G S Y
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S V N W G G G G G G N F V G G K G
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W N P G G A K T I N Y S G T Y N
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L I E Y Y I V E N Y G T Y N P A
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S Q A T K K G S V T S D G G T Y
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D I Y V S T R V N Q P S I E G T
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R T F Q Q Y W S I R T S K R T G
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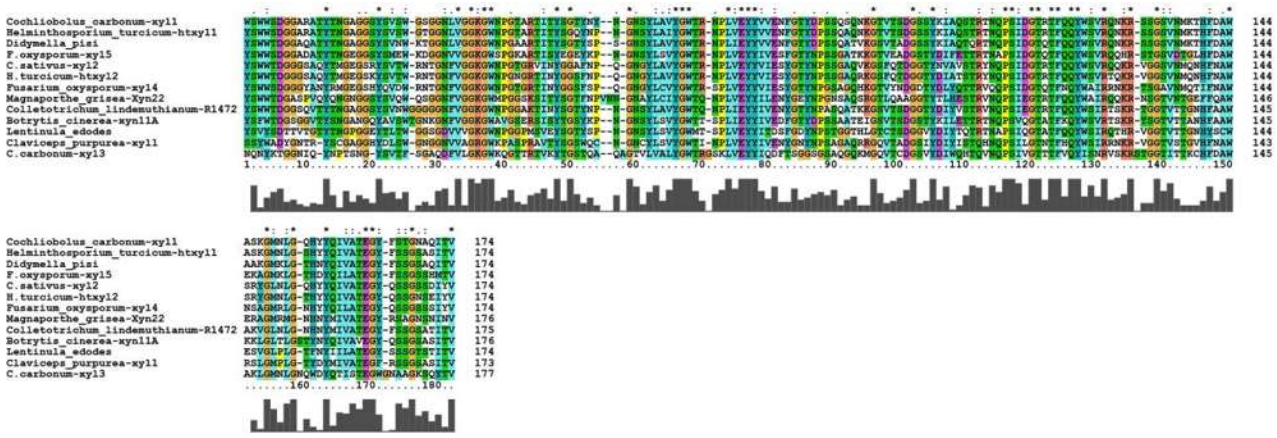
**Figure 3**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Magnaporthe_grisea-Xyn22
Colletotrichum_lindemuthianum-R1472
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Lentinula_edodes
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C.carbonum-xyl3

**Figure 4**

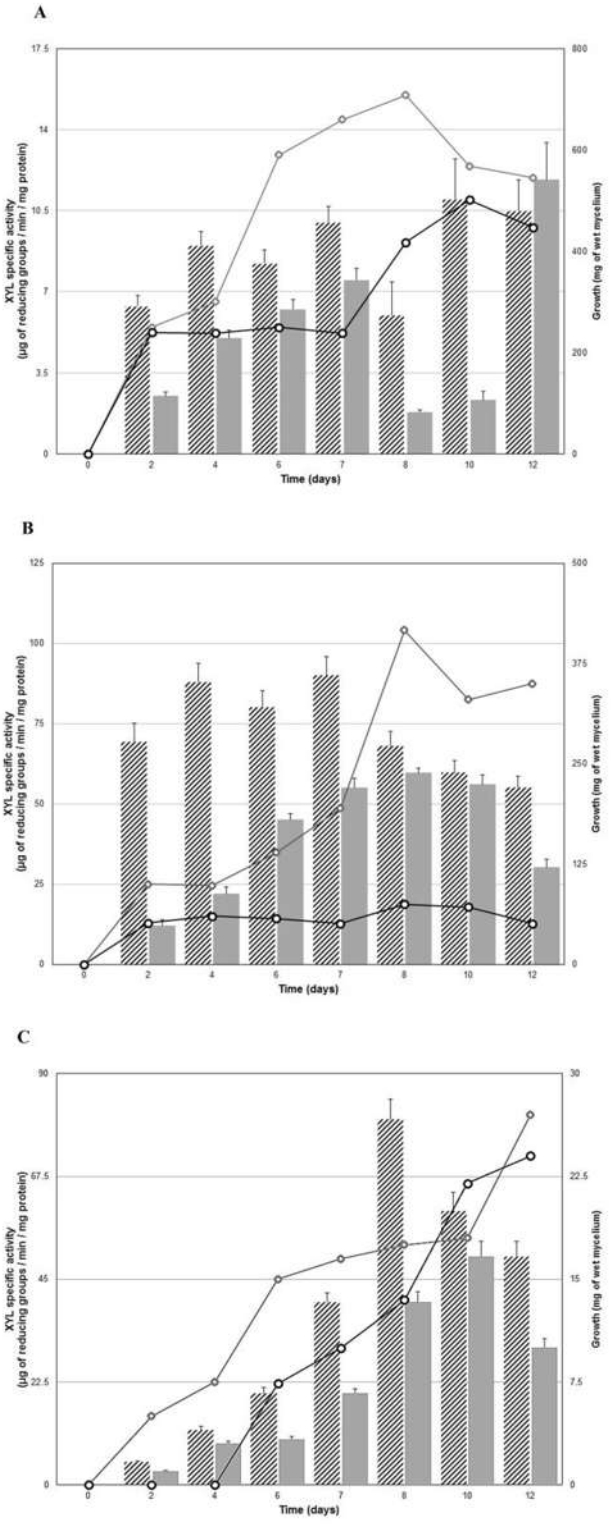
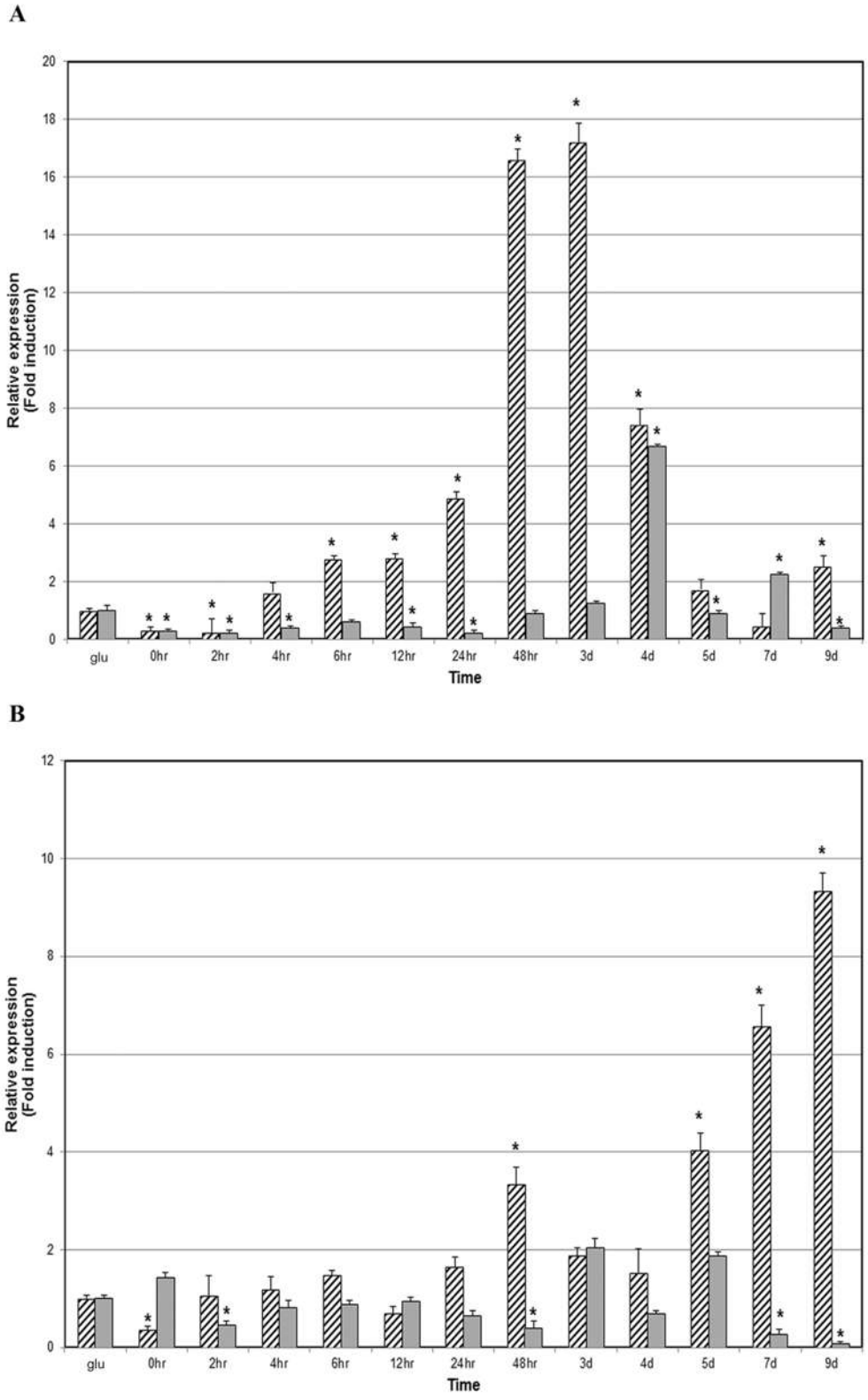

A
XYL specific activity
(µg of reducing groups / min / mg protein)
Growth (mg of wet mycelium)
Time (days)
B
XYL specific activity
(µg of reducing groups / min / mg protein)
Growth (mg of wet mycelium)
Time (days)
C
XYL specific activity
(µg of reducing groups / min / mg protein)
Growth (mg of wet mycelium)
Time (days)

**Figure 5**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Relative expression (Fold induction)
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18
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6hr
12hr
24hr
48hr
3d
4d
5d
7d
9d
Time
B
Relative expression (Fold induction)
12
10
8
6
4
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0
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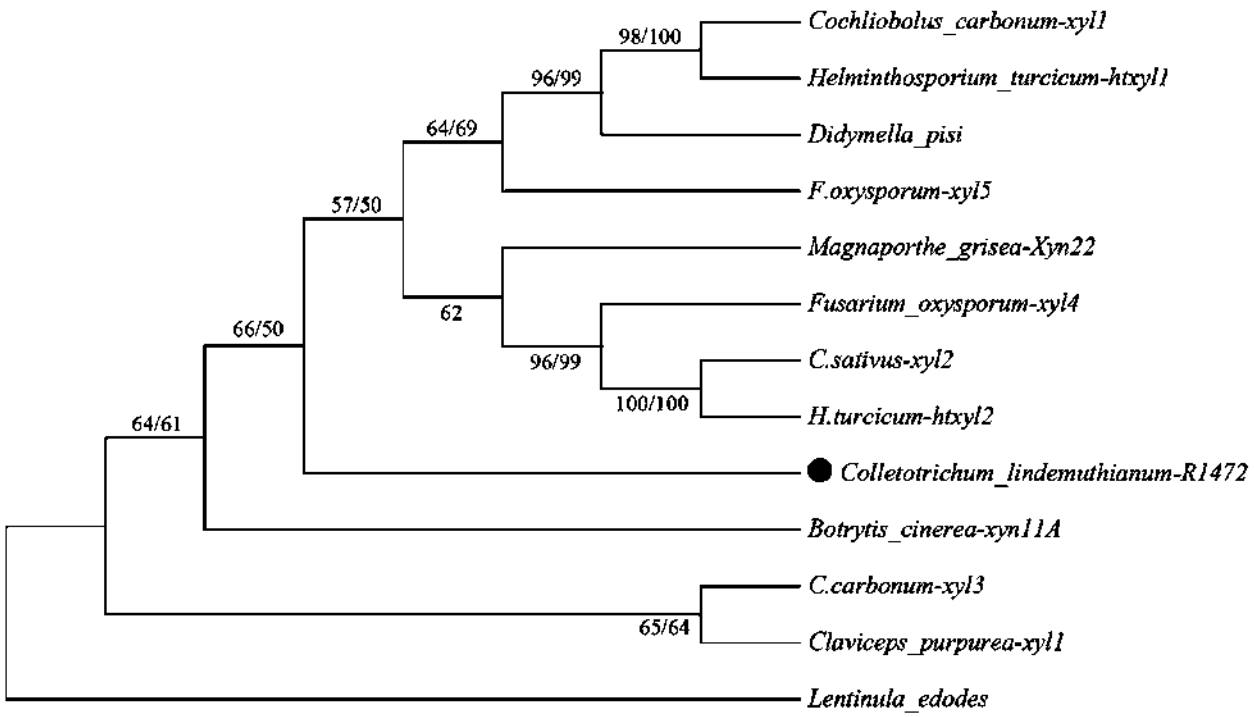
7d
9d
Time

**Figure 6**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Fusarium_oxysporum-xyl4
C.sativus-xyl2
H.turcicum-htxyl2
Colletotrichum_lindemuthianum-R1472
Botrytis_cinerea-xyn11A
C.carbonum-xyl3
Claviceps_purpurea-xyl1
Lentinula_edodes
98/100
96/99
64/69
57/50
62
96/99
100/100
66/50
64/61
65/64

## CAPÍTULO III

Artículo de investigación. Protein homology modeling, docking and phylogenetic analyses of an endo-1,4-β-xylanase GH11 of *Colletotrichum lindemuthianum* and similar endoxylanases in Colletotrichum species. Sometido a Journal of Molecular Modeling.

# Protein homology modeling, docking and phylogenetic analyses of an endo-1,4-β-xylanase GH11 of *Colletotrichum lindemuthianum* and similar endoxylanases in *Colletotrichum* species

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**Abstract**

Endo-1,4-β-xylanase (EC 3.2.1.8) is a crucial enzyme that randomly cleaves the β-1,4-glycosidic linkages of the xylan backbone, releasing xylooligomers of different lengths. The three-dimensional structure of the endo-β-1,4-xylanase gene (*xyl1*) from *Colletotrichum lindemuthianum* was modeled and docked with various xylan model compounds. Docking analyses revealed significantly higher stability of *C. lindemuthinum* XYL1 with the xylopentaose oligomer. Residues interacting with the model oligomers at the respective enzyme active sites were found to be in accord with their role in xylan degradation. Nevertheless, docking analyses of xylanases GH11 from *Colletotrichum* sp, revealed significative differences in structure, integration of the substrate in the active site and the glutamate residues of the catalytic site that carry out the substrate hydrolysis; of these proteins, 36%, 60%, and 4% integrate xylotetraose, xylopentaose, and xylohexaose in the active site, respectively. Since endoxylanases GH11 from *Colletotrichum* species interact much more efficiently with xylopentaose and xylotetraose, and xylanases GH11 from different fungi do not seem to have the same substrate binding subsites, we propose that they are enzymes with different affinity to xylooligosaccharides. In agreement with this idea, phylogenetic analyses of xylanases from *Colletotrichum* sp. shows four lineages suggesting diversifying selection. Most likely, the polydiversity or structural polymolecularity of xylan in plant cell walls processed by these organisms play a determinant role in this phenomenon.

**Keywords:** *Colletotrichum*; endo-1,4-β-xylanase; three-dimensional structure; molecular docking; phylogeny

## Introduction

Analysis of genomic sequences from plant saprophytic and pathogenic fungi has led to the identification of putative genes encoding for carbohydrate-active enzymes (CAZymes) involved in the degradation of plant cell walls. Comparison of these genes has contributed to our understanding of their lifestyle and helped to create infection models [1]. For example, biotrophic fungi tend to have fewer CAZymes than necrotrophic and hemibiotrophic fungi, saprophytic fungi have fewer CAZymes than plant pathogenic fungi,

and dicot pathogens often contain more pectinases than monocot pathogens [1]. For the *Colletotrichum* genus, there exist reports of sequenced genomes of some species where it is possible to identify gene sequences of CAZymes, but there no reports are dealing with their function and role in the different lifestyles or invasion/nutrition strategies of these organisms. The ascomycete *Colletotrichum* genus includes species with different lifestyles such as endophytes, saprophytes and a large number of plant pathogens with hemibiotrophic invasion strategy. The genus is also characterized by containing species of plant pathogenic fungi with a wide variety of host ranges and different levels of virulence, from saprophytes to very destructive pathogens that affect a large number of economically important crops [2-4]. The study of species in this group is therefore of great significance because of the economic impact caused by pathogenic species of the genus.

Endoxylanases are CAZymes produced by saprophytic and pathogenic fungi [5,6] and are responsible for the depolymerization of xylan in plant cell walls [7]. There are currently more than 100 families of glycoside hydrolases (GHs) in the CAZymes database [8](http://www.cazy.org/). Endoxylanases are distributed in families GH10 and GH11, which correspond to the F and G families, respectively [9]. The structural differences among these enzymes influence their physicochemical characteristics. The three-dimensional structure of the active center of xylanases belonging to family GH10 results in a reduced substrate specificity. Therefore, these xylanases are more active on xylooligosaccharides with a low degree of polymerization than endoxylanases of family GH11 [10-12]. The endo-β-1,4-xylanases (EC 3.2.1.8) belonging to family GH11 hydrolyze the β-1,4 bond of xylan generating xylooligosaccharides, which are further hydrolyzed by β-xylosidase to xylose units (EC 3.2.1.37) [13]. These endoxylanases fold into β jellyroll sheets that define their secondary structure [14]. To date, the three-dimensional structure and mechanisms of ligand binding of *Colletotrichum* sp. endoxylanases have not been described despite the availability of strategies designed to derive functional information from three-dimensional images of enzymes. Databases describing the structure of xylanolytic enzymes and their interaction with the ligand can facilitate the analysis of minor structural differences responsible for the specific recognition of a unique oligosaccharide sequence within a heterogeneous mixture [15]. Moreover, phylogenetic analysis of amino acid sequences derived from the endoxylanases of plant

pathogens can contribute to the understanding of the evolutionary process in relation to host types and different invasion/nutritional strategies (biotrophic, necrotrophic, or hemibiotrophic).

Previously, we isolate and characterize a *xyl*1 cDNA which encodes an extracellular endo-β-1,4-xylanase GH11 of *C. lindemuthianum* race 1472 (manuscript in press). A flexible docking study was performed to understand the mechanisms of ligand binding and the interaction between the ligand and the three-dimensional XYL1 model, with four xylooligosaccharides, in the range of xylotriose to xylohexaose. Moreover, to get an insight into the evolutionary process, we also present results of Clustal alignment of the deduced amino acid sequences, three-dimensional structure, mechanisms of ligand binding and phylogenetic analyses of the putative protein model generated by homology modeling of endo-β-1,4-xylanase GH11 from *C. lindemuthianum* and corresponding genes from genomes of *Colletotrichum* sp.

## Methods

### Sequence analysis

In the present work, *xyl1* cDNA encoding an endo-β-1,4-xylanase (XYL1) from *C. lindemuthianum*, race 1472, previously isolated and described, was used [Gen Bank Access: KM587707] (manuscript in press). Twenty-five homologous xylanase protein sequences from *Colletotrichum* species were selected from NCBI database [16] and from databases for the fungal plant cell wall-degrading enzymes [17] (Table 1).

### Modeling of protein homology

To determine the possible functional and structural domains of *C. lindemuthianum* XYL1, the online services Pfam and ProDom (v2006.1) were used. A computer analysis of protein secondary structure using the online service Jpred was performed [18]. The tertiary structure of the deduced amino acid sequence (mature protein) of XYL1 and endo-β-1,4-xylanases of *Colletotrichum* species (Table 1) were predicted by a homology modeling approach using the Swiss-Model Server [19], and the Protein Data Bank (PDB; http://www.rcsb.org/pdb/) as template sources [20].

**Quality and energy minimization prediction**

The prediction quality and structural parameters of the modeled structures were estimated with the SPDBV v. 4.01 program [21]. The energy minimization of the model was made through Anolea [22] and GROMOS96 [23], from the SPDBV program. SPDBV v. 4.01 and Jmol [24](http://www.jmol.org/) were employed to visualize molecular structures. The stereochemical quality of the protein structure was analyzed by PROCHEK program [25] and SPDBV v. 4.01. Superposition of three-dimensional structures was performed using the PDBeFold online service [26,27] and the STRAP program [28].

**Preparation of xylooligosaccharide model compounds**

The ligands xylotriose, xylotetraose, xylopentaose, and xylohexaose were built and optimized with MarvinSketch software ver.14.10. and the resulting molecules served as starting structures for the docking experiments.

**Molecular docking**

CLC Drug Discovery Workbench program, version 2.4.1 (CLC Inc A, Denmark)[29] was used for docking simulation of xylooligosaccharides to the active site of the XYL1 structure. For docking score analysis we used the $PLANTS_{PLP}$ score method of Drug Discovery Workbench program [30].

Basic settings such as ligand and protein preparation were performed. The docking assistant software used default algorithms with 200 numbers of runs and basic parameters. The center of the grid was assigned between the catalytic residues Glu97 and Glu187.

Xylooligosaccharides were docked into the active site recognized in the macromolecule cavity. Docking studies were designed to predict the binding affinities based on the hydrogen interactions and docking score. The best-qualified models were selected, and their binding residues were displayed using the CLC Drug Discovery visualization tool [29].

**Phylogenetic analyses**

Phylogenetic analyses were made on the deduced amino acid sequence of *C. lindemuthianum* XYL1, race 1472, and 25 putative endo-β-1,4-xylanase genes from

genomes of *Colletotrichum* species and one sequence from the basidiomycete *Lentinula edodes* as an outgroup (Table 1). Deduced amino acid sequences were aligned with Clustal X software [31] using the default parameters. Previous to phylogenetic analyses, we eliminate the signal peptide sequences and N- and C-terminal extensions. Phylogenetic analysis was made under Bayesian and maximum parsimony criteria with MrBayes Vs. 3.1.2 [32] and Mega6 software [33]. The amino WAG evolution model with gamma correction was utilized for Bayesian analysis.

A total of 10,000 trees were obtained using the settings ngen = 100 000 and sample freq = 100 for the Bayesian criteria. Previous to evaluating the support for the found topologies, the convergence of the overall chains (4) when the log likelihood values reached the stationary distribution was checked. The first 1000 trees were 'burn-in' and discarded, and a 50% majority rule consensus tree of the remaining trees was generated. In the analysis of maximum parsimony, the most parsimonious trees were assessed by the heuristic search option (Nearest-Neighbor-Interchange-NNI) with random sequence addition (five random replicates). A WAG+G substitution model was used, and 1000 bootstrap replicates were performed.

## Results and discussion

### Protein homology modeling of *C. lindemuthianum* XYL1

The data obtained by homology modeling of XYL1 from *C. lindemuthianum* were consistent with those of enzymes within the endoxylanase family. Analysis of energy minimization revealed values of -8055.405 KJ/mol. The quality of the generated models was assessed by plotting the dihedrals Φ and Ψ onto Ramachandran plots (SPDBV v. 4.01) [21]. The results were drawn from a PROCHECK program, which validates the model with 83.1% of total residues confined to the core region, 16.2% in additional allowed region, remaining no residues in generously allowed region and 0.7% residues in the disallowed region in XYL1 (Fig. 1A). The sequence showed 66.84% of identity and 79% of similarity with an endoxylanase II (XYLII) from *Trichoderma reesei* Chain A (PDB: 4hk9)[34]. This template is the crystallized structure of the enzyme-xylotriose substrate complex. The three-dimensional model structure of XYL1 from *C. lindemuthianum* race 1472 was also

compared against protein database (PDB) using a DaliLiteV3.1 server [35], which illustrates RMSD 0.3, an identity of 67% and a Z-score value of 38.3 with template 4hk9-1.A.

The three-dimensional model structure of XYL1 from *C. lindemuthianum,* race 1472, exhibits the typical structure of glycosylhydrolases belonging to family GH11, which have a folding β-jellyroll as an additional element of its secondary structure. The β-jelly roll structure resembles the shape of a partially closed right hand. The two β-sheets constitute the ‘‘fingers’’, whereas the twisted part of β-sheet B and the α-helix forms the ‘‘palm’’ of the hand (Fig. 1B). The active site with the two catalytic glutamates is confined to the concave site of the palm. The loop between β-strands β10 and β11 makes the ‘‘thumb’’. The ‘‘cord’’ part of a long irregular loop connects the fingers with the base of the thumb and partially closes the active site on one side (Fig. 1C) [36,37].

These results suggest that the β-sheets of the *C. lindemuthianum* XYL1 are twisted around a long deep cleft, which is well suited to serve as a specific site to accommodate the substrate. The substrate is aligned with several tyrosine residues and other aromatic amino acids, which are specifically placed to stabilize four to six xylose residues, and the α-helix is adjusted to the rear side of the β-sheets (Fig. 1B). The active center of the endoxylanase consists of a slit of variable depth and width that involves two highly conserved glutamate residues at positions 96 and 187 (Fig. 1D). These residues are required in the hydrolysis of xylan glycosidic bond, with one acting as an acid catalyst/base and the other as a nucleophilic residue [38].

The protein is primarily stabilized by hydrophobic interactions between the β-sheets and also by several hydrogen bonds and electrostatic interactions between the highly conserved amino acids [39,40]. These characteristics are consistent with the mode of action of endoxylanases and the model of *C. lindemuthianum* XYL1 presented here.

The thumb region, with its characteristic half-folded shape, is very well conserved among xylanases GH11. Its tip has the consensus sequence PSIXG, where X can be almost any residue [41] as in XYL1 (Pro136, Ser137, Ile138) (Fig. 1D).

Finally, to know the motifs conserved between *T. reesei* XYLII Chain A (PDB: 4hk9) and *C. lindemuthianum* XYL1, their three-dimensional structures were superimposed. The merging results revealed the β-jellyroll folding with the presence of the same β-sheets, α-

helix and loops, including the two glutamates in the active center and residues critical for the interaction with the substrate (Pro, Ser, Ile) (Fig. 2A, B, C, and D). Further, alignment of the two amino acid sequences showed that identity and similarity are related with conserved or similar residues in motifs that generate 14 β-sheets and one α-helix. We observed different residues particularly in the N- and C-terminals where *C. lindemuthianum* XYL1 contains some more residues than the *T. reesei* protein; others not conserved residues are dispersed in the sequence of loops (Fig. 2E).

**Molecular docking of *C. lindemuthianum* XYL1**

Molecular docking of XYL1 from *C. lindemuthianum* with xylooligosaccharides was carried out using CLC Drug Discovery Workbench. Results of this approach revealed that binding efficiency of xylooligosaccharides increased with the size of the model compounds; however, the degree of binding efficiency of xylohexaose compound was lower than the others (Table 2).

The active-site geometry of xylanases GH11 is highly conserved, and it is recognized in five to six subsites, which are divided considering the reducing and non-reducing end of the xylooligosaccharide on the two catalytic residues (Glu96, Glu187) in the protein [37]. In this site the substrate interacts with the amino acid residues that make up the subsites and it has been defined as -3, -2, -1, +1, +2, +3, on the direction of the reducing end / non-reducing end of the xylooligosaccharide respectively [42,34].

Previous experiments showed that xylanase TRXII (GH11) from *T. reesei* preferably bind longer chain xylooligosaccharides [43] and the specificity constant for enzymatic hydrolysis of xylohexaose by *Neocallimastix patriciarum* (NpXyn11A ) xylanase was almost 12 times higher than the xylopentaose hydrolysis [44]. However, differences have been observed in the binding site of two xylanases of *T. reesei*, namely, XYNI has only three subsites whereas XYNII contains five [37]. In *Aspergillus kawachii*, xynC probably contains five subsites while *Streptomyces* sp. *38* XYL1 exhibits six subsites [45]. Docking analysis on a xylanase GH11 (Tx-Xyl) from *Thermobacillus xylanilyticus* revealed that the existence of a subsite -3 is uncertain since the measurement of subsite-binding energies showed that subsite -3 displays the lowest energy [46,47,44].

Our results of *C. lindemuthianum* XYL1 are consistent with some previous reports in

terms that a substrate such as xylopentaose has higher energy levels than the other xylooligosaccharides (Fig. 3). This suggest that the XYL1 interacts on five subsites in the active site -3, -2, -1, +1, +2 (Fig. 3C). According to the XYL1-xylopentaose model, there is a clear interaction of the subsite -3 with Ser137, a highly conserved amino acid residue that participates in the mechanism of enzyme catalysis by accommodating the substrate and lock its position [48,46,41]. Ser26, Tyr87, and Tyr181 residues form hydrogen bonds with the sugar unit in the subsite -2. Glu96 (one of the catalytic residues), Arg132, and Pro136 form hydrogen bonds with the sugar unit in the subsite -1. Asn55, Arg132, and Glu187 (one of the catalytic residues) form hydrogen bonds with the sugar moiety in the subsite +1. Tyr83 and Tyr106 forms a hydrogen bond with the sugar unit in subsite +2 (Fig. 4).

The results of our study suggest that the active subsites of *C. lindemuthianum* XYL1, and perhaps some xylanases GH11 have five possible binding sugar from subsites -3 through +2. The docking shows the presence of numerous intermolecular interactions among the five subunits of xylose residues (Fig. 4). In Table 3, potential residues in the individual subsites of the modeled *C. lindemuthianum* XYL1 are compared to other xylanases GH11 whose binding sites have been studied in a limited number of fungi. All residues at subsite -3 of XYL1 were not similar. Accordingly, at subsite -2 prevails the Trp and Tyr residues, yet it is not a clear trend; at subsite -1 there are more variable characteristics, a diversity of amino acids is observed and the presence of Pro, Arg and Glu is retained. On the other hand, subsites +1 and +2 were not similar to the substrate binding subsites of other xylanases GH11 (Table 2). Thus, based on the present study, it is concluded that sugars as xylopentaose and xylotetraose can be interacting much more efficiently with the *C. lindemuthianum* XYL1 than xylohexaose and that xylanases GH11 from different fungi would not have the same substrate binding subsites.

**Phylogenetic analyses**

To elucidate the relationship among endo-β-1,4-xylanases from *Colletotrichum* sp., XYL1 and other 25 nucleotide sequences identified in five species with genomes available in databases, were analyzed along with the corresponding sequence of the basidiomycete *L. edodes* as an outgroup. Comparison of deduced amino acid sequences from putative proteins for these 25 genes revealed an identity of 33 to 99% suggesting a diversification

process that gave rise to proteins that shared identity mainly in the sequence and the structure of the catalytic site, with some differences in entire three-dimensional structures. Clustal alignment identified the location of amino acids in xylanases expected to have a catalytic role (Fig. 5) [41,49]. The tertiary structures were predicted to determine structural domains, (Fig. 6). The phylogenetic analysis revealed a clustering into four clades, where the enzyme from *C. lindemuthianum* race 1472 was grouped with the xylanase designated as xyl1 of *C. orbiculare* (group I) (Fig. 6), which is the nearest species according to a phylogenetic analysis of *Colletotrichum* sp. [50,51]. Despite the diversity showed by the amino acid sequences (Fig. 5), superimposed three-dimensional structures of each protein lineage exhibited the typical β jellyroll fold structure found in members of Family GH11; however, some differences among them are observed (Fig. 6). The phylogenetic analysis also revealed an early separation of xylanases into group IV, which may represent ancestral versions of the enzyme. In an evolutionary progression, it is conceivable that new versions of the enzyme gave rise to members of group III followed by the widely diversified group II with xyl3 of *C. orbiculare* as basal. The more recent modification of these enzymes into group I would have preserved the presently known three-dimensional structure, size and characteristics of members of family GH11 (Fig. 6).

With more than one gene coding for putative endo-β-1,4-xylanases, our observations in the *Colletotrichum* sp. support the notion of intraspecific genetic diversification in lineages, suggesting a diversifying selection [52]. Similar results were obtained after a phylogenetic analysis of the nucleotide sequences of the *htxyl1* and *htxyl2* xylanase genes from the corn pathogen *Helminthosporium turcicum.* These genes showed a differential expression related to the substrate type (xylan and/or xylose) or stages of infection of maize, suggesting a role in saprophytic or pathogenic lifestyles [53,49]. In this context, the differential expression of cutinases, cellulases, hemicellulases and pectinases related to different stages of the life cycle, namely, biotrophic, necrotrophic and saprophytic, has been reported in the hemibiotrophic pathogen *Zymoseptoria tritici* [52]. In some of these genes, diversifying selection has also been detected, which is possibly related to the adaptation to the host and/or the life cycle of the fungus [52].

It has been observed that pathogens of monocot and dicot plants are better adapted to degrade the cell walls of the corresponding plants, a phenomenon that reflects host preference

[54]. An analysis of *Rhizoctonia cerealis, Fusarium cuhnorum,* and *Pseudocercosporella herpotrichoides* revealed a similar enzymatic profile for pectinases, xylanases, and arabinases; however, the pathogens of dicot plants exhibited a lower pectinase profile [55]. In our analysis, each group or lineage of endo-β-1,4-xylanases included pathogens of monocot and dicot plants, suggesting that evolution not is necessarily influenced by the host preference.

**Docking analyses of *Colletotrichum* sp. xylanases**

The biochemical properties of xylanases GH11 may depend on very discrete structural differences [14]. Thus, to know if the differences observed between superimposed three-dimensional structures of xylanases from *Colletotrichum* species could be related to differences in the molecular binding of xylooligosaccharides, docking analyses were carried out with the proteins of this genus (Table 1). Results showed that 36%, 60% and 4% of these proteins integrate xylotetraose, xylopentaose, and xylohexaose, respectively (Table 4).

Interestingly, although the three-dimensional structures analyzed maintain the same typical topology of xylanases GH11 (Fig. 6), docking analyses showed significant differences in structure, integration of the substrate into the active site, and in the glutamate residues of the catalytic site involved in substrate hydrolysis. For instance, in the *C. fioriniae* PJ7-*xyl2* and *C. fioriniae* PJ7-*xyl3* proteins model, the glutamate orientation (near the N-terminal) is different; this difference is visible in other xylanases from *Colletotrichum* sp. (Fig. 7A), which generates changes in the direction and characteristics of the substratum binding. Moreover, a difference was observed in the location of both glutamates inside of active site in xylanase of C. *gloeosporioides* Nara-*xyl5* (Fig. 7B). *C. gloeosporioides* Nara-*xyl5* shows the Glu110 location outside of the β-sheet and in a different region respect to other proteins model. We observed that this change generates a wider opening in the active site, allowing the entrance of a greater polymer (Table 4; *C. gloeosporioides* Nara-*xyl5*). Considering the protein lineages observed in the phylogenetic analyses, we propose that these could be proteins that represent ancestral versions (*C. fioriniae* PJ7-*xyl2,* into group V), or products of a diversifying evolution (*C. gloeosporioides* Nara-*xyl5* and *C. fioriniae* PJ7-*xyl3*, into the group III) (Fig. 6).

The polydiversity or structural polymolecularity of xylan in plant cell walls processed by

these organisms could play a determinant role in the evolution of these enzymes. The polydiversity or structural polymolecularity of xylan relies on the nature and content of mono-substituents (neutral or uronic) and short-chain oligosaccharides bound to the main chain of β-1,4 xylopyranosyl linkages, such as glucomannan, galactomannan, and arabinogalactan, which can connect to the ends of pectic polysaccharides and cellulose fibers [40,56]. The side chains do not affect the geometry of the glycosidic linkage, but they do determine the solubility, physical conformation and reactivity of the xylan molecule with other hemicellulose components, thus greatly affecting the mode of enzymatic breakdown [57,58].

## Conclusions

Docking analyses revealed that compounds such as xylopentaose and xylotetraose interact much more efficiently with xylanases from *Colletotrichum* sp. than xylohexaose. The xylanases of family GH11 from different fungi would not have the same substrate binding subsites, suggesting a different affinity to xylooligosaccharides. The phylogenetic analyses of amino acid sequences of xylanases of *Colletotrichum* sp. showed four lineages suggesting diversifying selection and studies of three-dimensional structure and docking analyses showed significant differences in structure, integration of the substrate in the active site, and in the glutamate residues of the catalytic site required for substrate hydrolysis. Most likely, the polydiversity or structural polymolecularity of xylan in plant cell walls processed by these organisms play a determinant role in this phenomenon. Finally, we consider that the information presented here is of great importance in studies of the biotechnological potential of these enzymes, as their efficiency should depend on the complexity of the vegetal material they process and also from the source organism of enzymes and/or genes.

## Acknowledgements

The authors thank the financial support provided by Secretaría de Educación Pública-Consejo Nacional de Ciencia y Tecnología (SEP-CONACyT) (Project 2012-01-182755 to MGZP) and CONACyT for scholarships granted to UCS and MGVR.

**Conflict of interest:** The authors declare that they have no conflict of interest.

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**Figure captions**

**Fig. 1** Ramachandran plot of modeled *C. lindemuthianum* XYL1 obtained by PROCHECK

validation package (A), three-dimensional structure of homology-modeled structure of *C. lindemuthianum* XYL1 (B), topology diagram (C) and the close-up view of the active site of XYL1 model structure (D)

**Fig. 2** Superimposition of three-dimensional modeled structures of *Trichoderma reesei* XYLII Chain A (PDB: 4hk9) complexed with xylotriose (green) and *C. lindemuthianum* XIL1 (blue) (A, B, C and D). Alignment of the two sequences where conserved motifs (blue and red) that generate β-sheets (E) and one α-helix (H) are indicated (E). The three-dimensional superimposition was generated using the STRAP program with 190 overlapping residues

**Fig. 3** Molecular docking models of *C. lindemuthianum* XYL1 with xylotriose (A), xylotetraose (B), xylopentaose (C) and xylohexaose (D). The cleft of the complex denotes some of the polar contacts occurring between ligand (represented as sticks) and receptor (gray)

**Fig. 4** Schematic diagram of the interactions of *C. lindemuthianum* XYL1 with three xylopentaoses. The ligand is represented as sticks and the amino acids as lines. The residues predicted to participate in substrate binding through hydrogen binding are labeled as -3, -2, -1, +1, +2, +3, on the direction of the reducing end/non-reducing end

**Fig. 5** Alignment of amino acid sequences of endoxylanases used in phylogenetic analyses. Identical and conserved catalytic residues are marked with an asterisk (*). Dots indicate the change of one amino acid for another of the same group

**Fig. 6** Phylogenetic analyses and protein homology model of endoxylanases from *Colletotrichum* species. The tree was constructed using the ML and Bayesian methods and includes only the conserved region of the catalytic domain (190 aa) of the genes utilized in the analysis. The general topology obtained is represented by the Bayesian 50% majority rule consensus tree, in which the Bayesian posterior probabilities and ML bootstrap support are indicated on the branches. The numbers at the nodes indicate bootstrap values based on 1,000

bootstrap replications. The models represent the superimposed three-dimensional structure of *C. lindemuthianum* XIL1 and the proteins of each clade or lineage identified on the tree

**Fig. 7** Protein homology model of endoxylanases from *Colletotrichum* species. Superimposition of three-dimensional modeled structures of *C. fioriniae PJ7-xyl2* and *C. fioriniae* PJ7-xyl3 (A). Superimposition of three-dimensional modeled structures of *C. gloeosporioides* Nara-xyl5 (green) and *C. lindemuthianum* XYL1 (red), showing a difference in the location of the glutamate (B)

**Table 1** Nucleotide sequences of endoxylanases

| Fungi | GenBank Access | PDB Template | In this study |
|---|---|---|---|
| *Colletotrichum fioriniae* PJ7 | XP_007592808.1 | 1pvx.1.A | *C. fioriniae* PJ7-*xyl1* |
| | XP_007597263.1 | 1yna.1.A | *C. fioriniae* PJ7-*xyl2* |
| | XP_007592531.1 | 1xyn.1.A | *C. fioriniae* PJ7-*xyl3* |
| | XP_007596900.1 | 1xnk.1.A | *C. fioriniae* PJ7-*xyl4* |
| *Colletotrichum gloeosporioides* Cg-14 | EQB49472.1 | 3akq.1.A | *C. gloeosporioides* Cg-*xyl1* |
| | EQB58440.1 | 1h1a.1.A | *C. gloeosporioides* Cg-*xyl2* |
| | EQB47911.1 | 4hk9.1.A | *C. gloeosporioides* Cg-*xyl3* |
| | EQB44117.1 | 1xyn.1.A | *C. gloeosporioides* Cg-*xyl4* |
| | EQB43516.1 | 2vuj.1.A | *C. gloeosporioides* Cg-*xyl5* |
| *Colletotrichum gloeosporioides* Nara gc5 | XP_007287342.1 | 2jic.1.A | *C. gloeosporioides* Nara-*xyl1* |
| | XP_007277672.1 | 1h1a.1.A | *C. gloeosporioides* Nara-*xyl2* |
| | XP_007287286.1 | 1yna.1.A | *C. gloeosporioides* Nara-*xyl3* |
| | XP_007273422.1 | 1xyn.1.A | *C. gloeosporioides* Nara-*xyl4* |
| | XP_007280278.1 | 1te1.1.B | *C. gloeosporioides* Nara-*xyl5* |
| *Colletotrichum graminicola* M1.001 | EFQ27362.1 | 1h1a.1.A | *C. graminicola* M1-*xyl1* |
| | EFQ30380.1 | 3zse.1.A | *C. graminicola* M1-*xyl2* |
| | EFQ27243.1 | 1yna.1.A | *C. graminicola* M1-*xyl3* |
| | EFQ30204.1 | 1xyo.1.A | *C. graminicola* M1-*xyl4* |
| | EFQ34818.1 | 1xyn.1.A | *C. graminicola* M1-*xyl5* |
| | EFQ27481.1 | 1xyn.1.A | *C. graminicola* M1-*xyl6* |
| *Colletotrichum higginsianum* | CCF32229.1 | 1h1a.2.A | *C. higginsianum-xyl1* |
| | CCF40067.1 | 1yna.1.A | *C. higginsianum-xyl3* |
| *Colletotrichum orbiculare* MAFF 240422 | ENH76821.1 | 1xnk.1.A | *C.orbiculare-xyl1* |
| | ENH79971.1 | 1pvx.1.A | *C.orbiculare-xyl2* |
| | ENH89093.1 | 1xnk.1.A | *C.orbiculare-xyl3* |
| *Pyrenophora tritici-repentis* Pt-1C-BFP | XP_001939205.1 | ---------- | *Pyrenophora tritici-repentis-xylI* |
| *Lentinula edodes* | AAL04152.1 | ---------- | *Lentinula edodes* |

**Table 2** Docking of *C. lindemuthianum* XYL1 with xylooligosaccharides model compounds

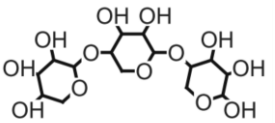
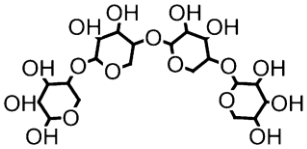
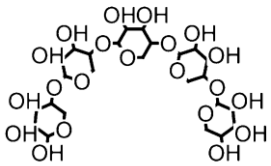
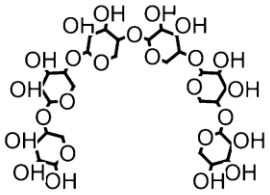
| Compound | Weight | Score | Hydrogen bond score | RMSD (A°) |
|---|---|---|---|---|
|  **Xylotriose** | 414.16 | -53.34 | -24.23 | 28.72 |
|  **Xylotetraose** | 546.48 | -52.31 | -25.81 | 28.77 |
|  **Xylopentaose** | 678.59 | -59.48 | -33.67 | 29.33 |
|  **Xylohexaose** | 810.71 | -40.62 | -30.19 | 23.50 |

**Table 3** Comparison of the amino acid residues in each subsite of modeled XYL1 of *C. lindemuthianum* and other fungal GH11 xylanases

| Organism | Complex-structure | Amino acid residues in individual subsite | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | -3 | -2 | -1 | +1 | +2 | +3 | |
| *C. lindemuthianum* 1472 (*xyl1*) | Xylopentaose | $S^{136}$ | $Ser^{26}$, $Y^{87}$ $Y^{181}$ | $R^{132}$, $P^{136}$, $E^{96}$ | $N^{55}$, $R^{132}$ $E^{187}$ | $Y^{83}$ $Y^{106}$ | - | In this study |
| *C. cinereus* | Xylohexaose | $Y^{87}$ | $W^{112}$, $Q^{127}$ | $R^{113}$, $Y^{79}$ | $W^{9}$, $Y^{68}$, $P^{117}$ | $Y^{168}$ | $S^{7}$, $Y^{167}$ | [59] |
| *T. reesei*-(XynII) | Xylohexaose | - | $Y^{77}$, $Y^{171}$ | $R^{122}$, $P^{126}$, $E^{86}$ | $Y^{73}$, $Y^{88}$, $O^{136}$, $E^{177}$ | $N^{71}$ | $N^{71}$ | [34] |
| *T. lanuginosus* (XynA) | Xylopentaose | - | $W^{18}$, $Y^{172}$ | $Y^{77}$, $E^{86}$, $R^{122}$, $P^{126}$, $Q^{136}$ | $N^{44}$ | - | - | [60] |
| *Penicillium griseofulvum* (*Pg*XynA) | Xylohexaose | $N^{5,}$ $R^{7,}$ $W^{18}$ | $R^{7,}$ $W^{18}$, $Y^{76}$, $Y^{171}$ | $S^{44}$, $P^{125}$, $E^{177}$ | $S^{44}$, $Y^{72}$ | $G^{98}$ | $Y^{95}$ | [47] |
| *A. kawachii* (XynC) | Xylohexaose | - | $Q^{8}$, $Y^{172}$ | $Y^{10}$, $P^{119}$ | $Y^{81}$, $R^{115}$ | - | - | [45] |
| *N. patriciarum* (XynCDBF*V*) | Xylobiose-Xylotriose | $W^{32}$ | $W^{32}$, $W^{125}$, $E^{30}$, $R^{61}$, $Y^{100}$ | $D^{57}$, $E^{202}$, $R^{148}$, $Q^{161}$, $P^{151}$, $E^{109}$ | $D^{57}$, $Y^{94}$, $Y^{111}$, $R^{148}$, $Q^{161}$, $E^{202}$ | $R1^{48}$ | $G^{93}$ | [49] |
| *H. jecorina* (XynII) | Xylopentaose | - | $W^{18}$, $Y^{77}$ | $E^{86}$, $Y^{88}$ | $E^{177}$ | $Y^{179}$ | $Y^{96}$ | [61,37] |

**Table 4** Docking of xylanases from *Colletotrichum sp.* with xylooligosaccharides model compounds

| Fungi (This study) | Xylotetraose Score | Xylopentaose Score | Xylohexaose Score |
|---|---|---|---|
| *C. fioriniae* PJ7-*xyl1* | **52.39** | 50 | 40.50 |
| *C. fioriniae* PJ7-*xyl2* | **52.47** | 48.57 | 43.02 |
| *C. fioriniae* PJ7-*xyl3* | 51.39 | **57.02** | 1.52 |
| *C. fioriniae* PJ7-*xyl4* | 52.84 | **68.76** | 44.53 |
| *C. gloeosporioides* Cg-*xyl1* | 31.71 | **75.47** | 27.80 |
| *C. gloeosporioides* Cg-*xyl2* | 45.78 | **61.26** | 48.49 |
| *C. gloeosporioides* Cg-*xyl3* | 41.99 | **82.79** | 43.42 |
| *C. gloeosporioides* Cg-*xyl4* | 54.52 | **61.65** | 13.32 |
| *C. gloeosporioides* Cg-*xyl5* | 50.05 | 55.52 | **58.64** |
| *C. gloeosporioides* Nara-*xyl1* | 55.07 | **58.49** | 32.83 |
| *C. gloeosporioides* Nara-*xyl2* | 38.83 | **62.58** | 46.49 |
| *C. gloeosporioides* Nara-*xyl3* | **55.34** | 53.28 | 30.84 |
| *C. gloeosporioides* Nara-*xyl4* | 48.95 | **63.25** | 24.65 |
| *C. gloeosporioides* Nara-*xyl5* | **58.91** | 49.59 | 30.72 |
| *C. graminicola* M1-*xyl1* | 37.43 | **62.63** | 52.28 |
| *C. graminicola* M1-*xyl2* | 25.39 | **55.58** | 40.58 |
| *C. graminicola* M1-*xyl3* | **57.59** | 49.74 | 10.72 |
| *C. graminicola* M1-*xyl4* | **54.52** | 48.63 | 32.77 |
| *C. graminicola* M1-*xyl5* | **59.26** | 44.52 | ------ |
| *C. graminicola* M1-*xyl6* | 52.18 | **53.68** | 38.17 |

| | | | |
|---|---|---|---|
| *C. higginsianum-xyl1* | **53.55** | **50.45** | 42.40 |
| *C. higginsianum-xyl3* | **55.94** | 53.37 | 48.50 |
| *C.orbiculare-xyl1* | 51.93 | **75.65** | 20.55 |
| *C.orbiculare-xyl2* | 52.25 | **54.94** | 34.88 |
| *C.orbiculare-xyl3* | 51.66 | **61.31** | 26.68 |

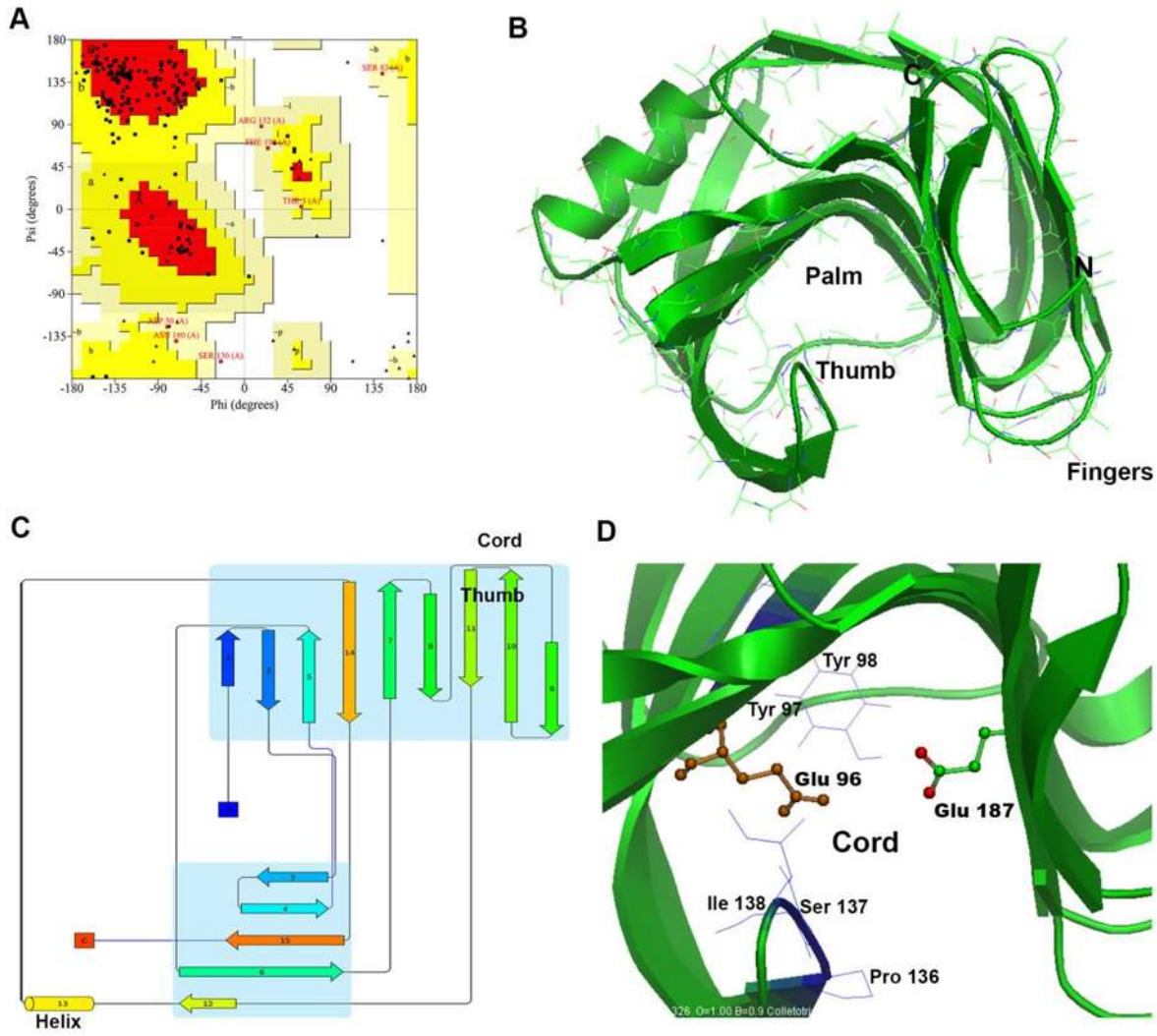



**Fig. 1** Ramachandran plot of modeled *C. lindemuthianum* XYL1 obtained by PROCHECK validation package (A), three-dimensional structure of homology-modeled structure of *C. lindemuthianum* XYL1 (B), topology diagram (C) and the close-up view of the active site of XYL1 model structure (D)

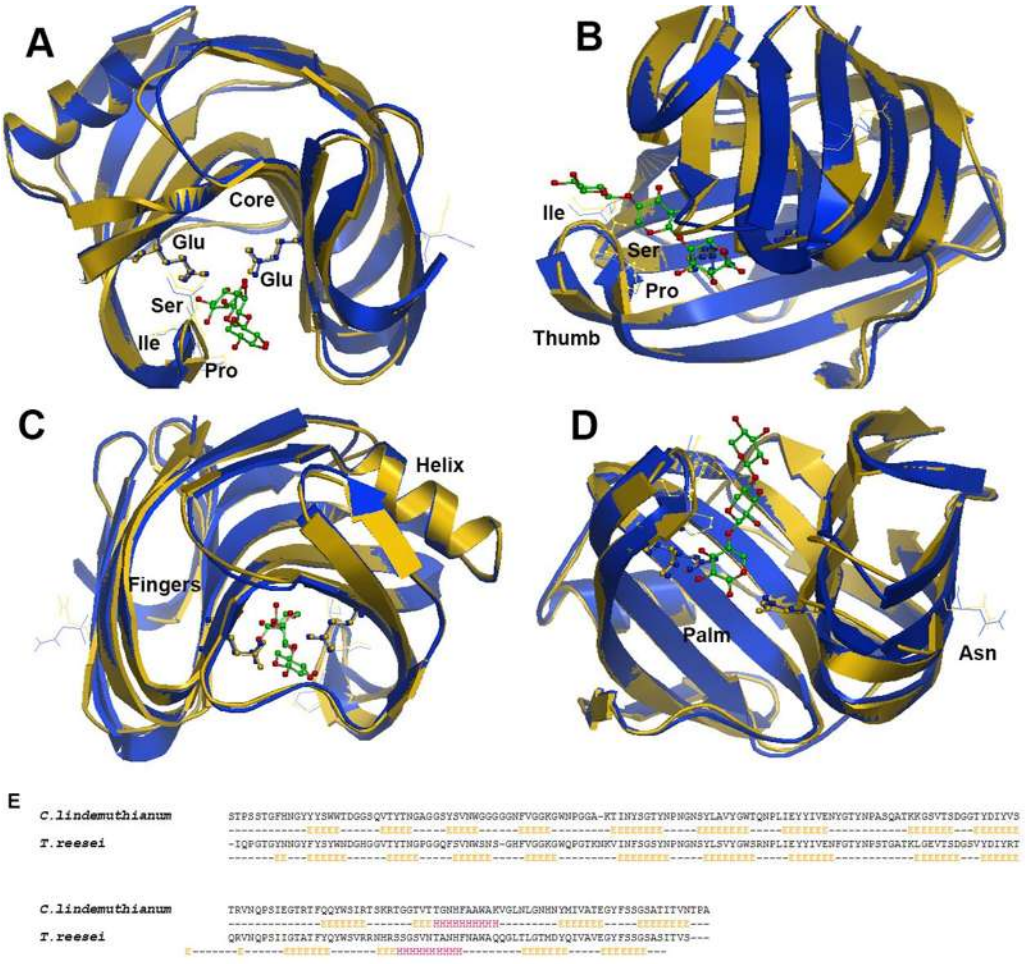



**Fig. 2** Superimposition of three-dimensional modeled structures of *Trichoderma reesei* XYLII Chain A (PDB: 4hk9) complexed with xylotriose (green) and *C. lindemuthianum* XIL1 (blue) (A, B, C and D). Alignment of the two sequences where conserved motifs (blue and red) that generate β-sheets (E) and one α-helix (H) are indicated (E). The three-dimensional superimposition was generated using the STRAP program with 190 overlapping residues

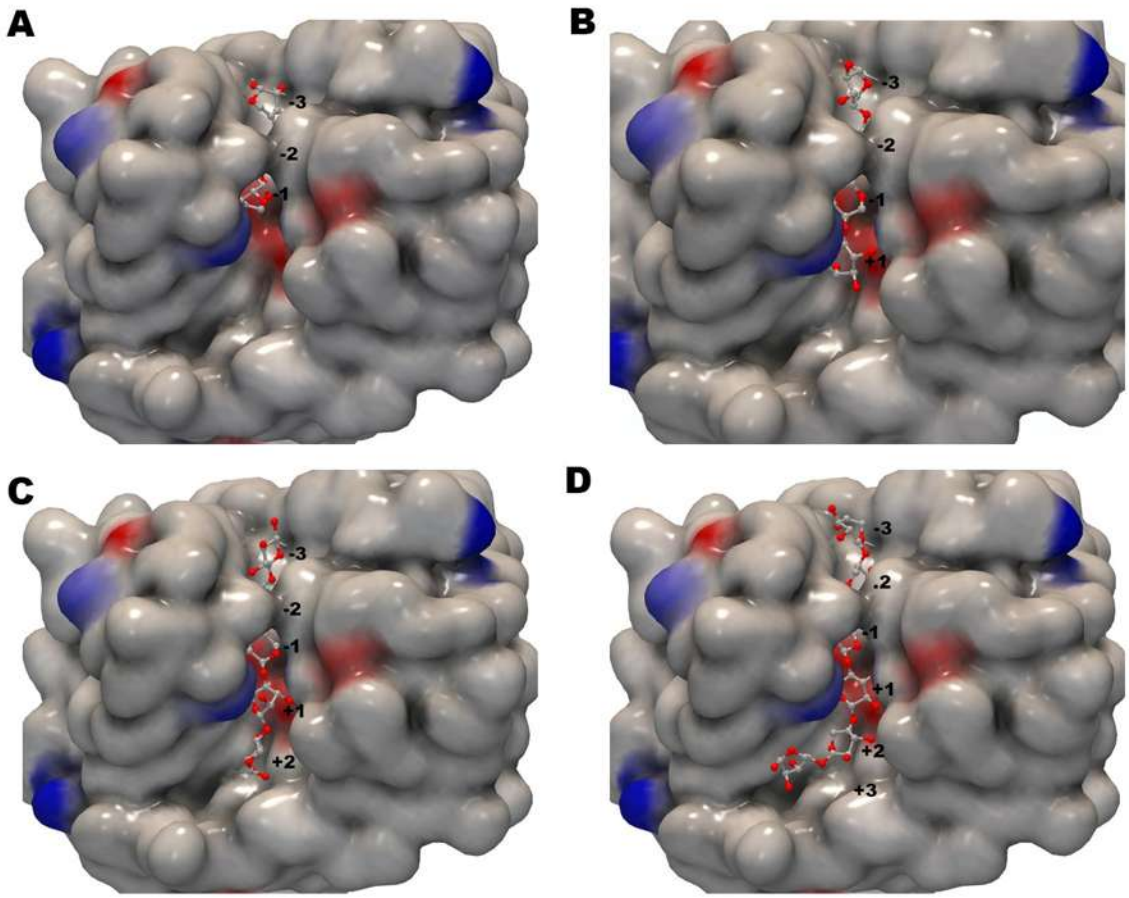



**Fig. 3** Molecular docking models of *C. lindemuthianum* XYL1 with xylotriose (A), xylotetraose (B), xylopentaose (C) and xylohexaose (D). The cleft of the complex denotes some of the polar contacts occurring between ligand (represented as sticks) and receptor (gray)

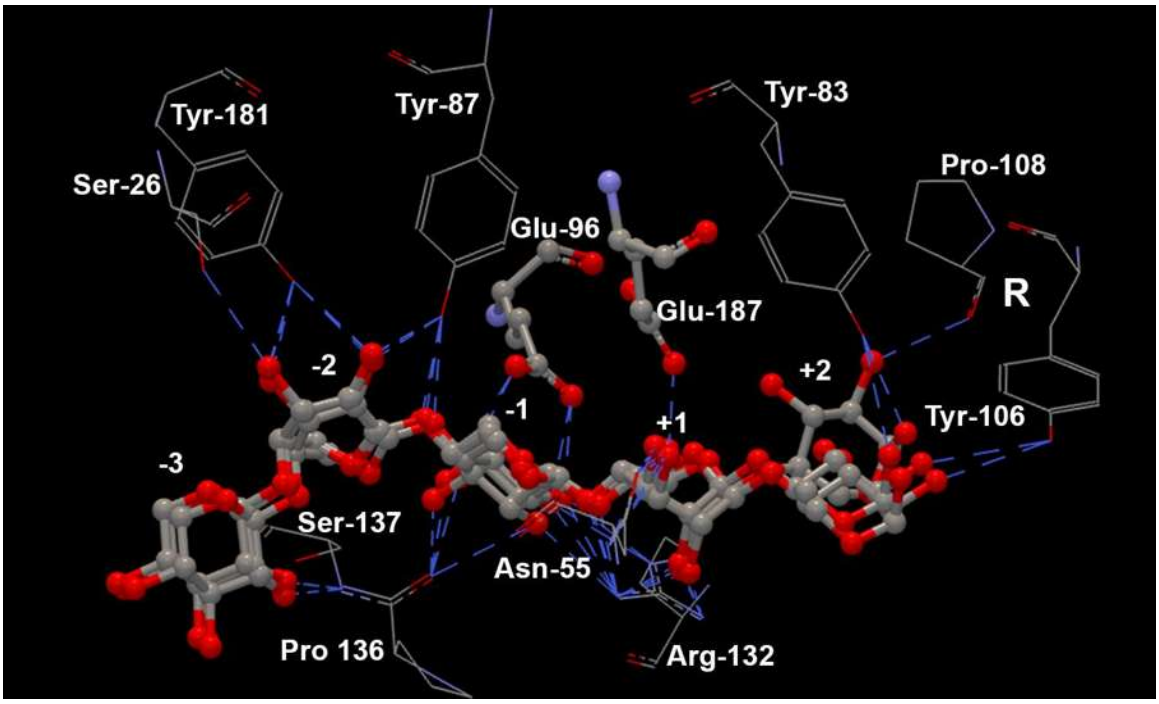



**Fig. 4** Schematic diagram of the interactions of *C. lindemuthianum* XYL1 with three xylopentaoses. The ligand is represented as sticks and the amino acids as lines. The residues predicted to participate in substrate binding through hydrogen binding are labeled as -3, -2, -1, +1, +2, +3, on the direction of the reducing end/non-reducing end

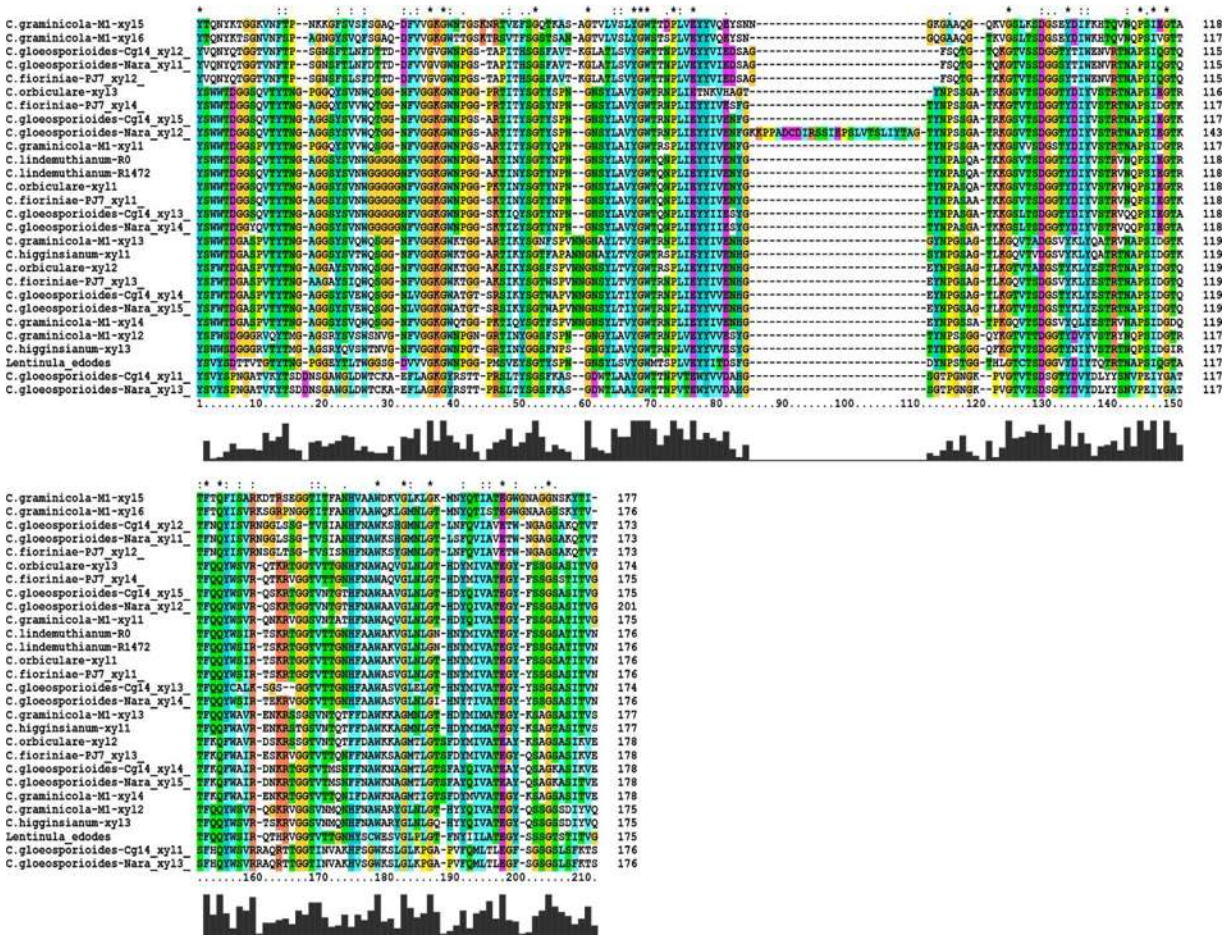


**Fig. 5** Alignment of amino acid sequences of endoxylanases used in phylogenetic analyses. Identical and conserved catalytic residues are marked with an asterisk (*). Dots indicate the change of one amino acid for another of the same group

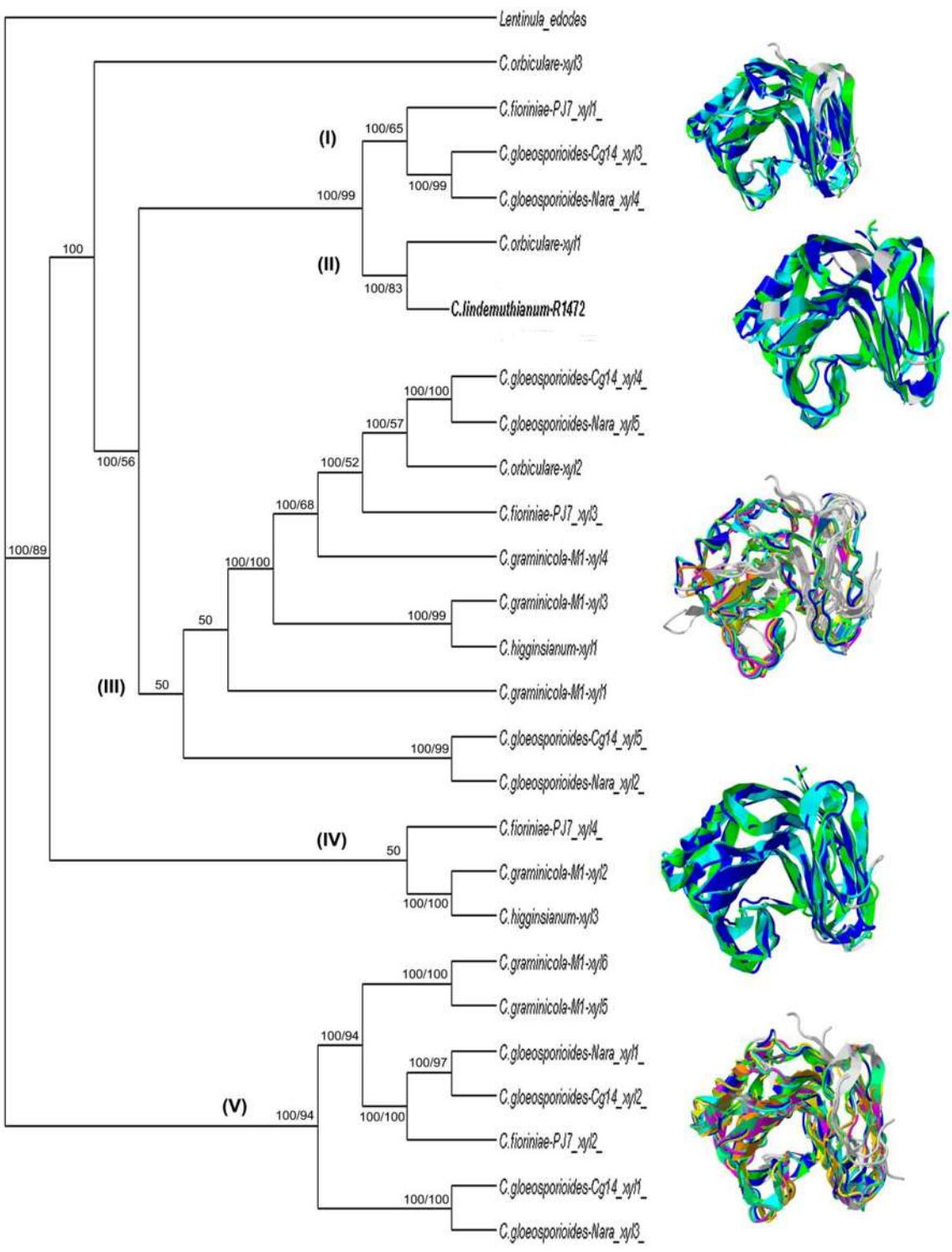



**Fig. 6** Phylogenetic analyses and protein homology model of endoxylanases from *Colletotrichum* species. The tree was constructed using the ML and Bayesian methods and

includes only the conserved region of the catalytic domain (190 aa) of the genes utilized in the analysis. The general topology obtained is represented by the Bayesian 50% majority rule consensus tree, in which the Bayesian posterior probabilities and ML bootstrap support are indicated on the branches. The numbers at the nodes indicate bootstrap values based on 1,000 bootstrap replications. The models represent the superimposed three-dimensional structure of *C. lindemuthianum* XIL1 and the proteins of each clade or lineage identified on the tree

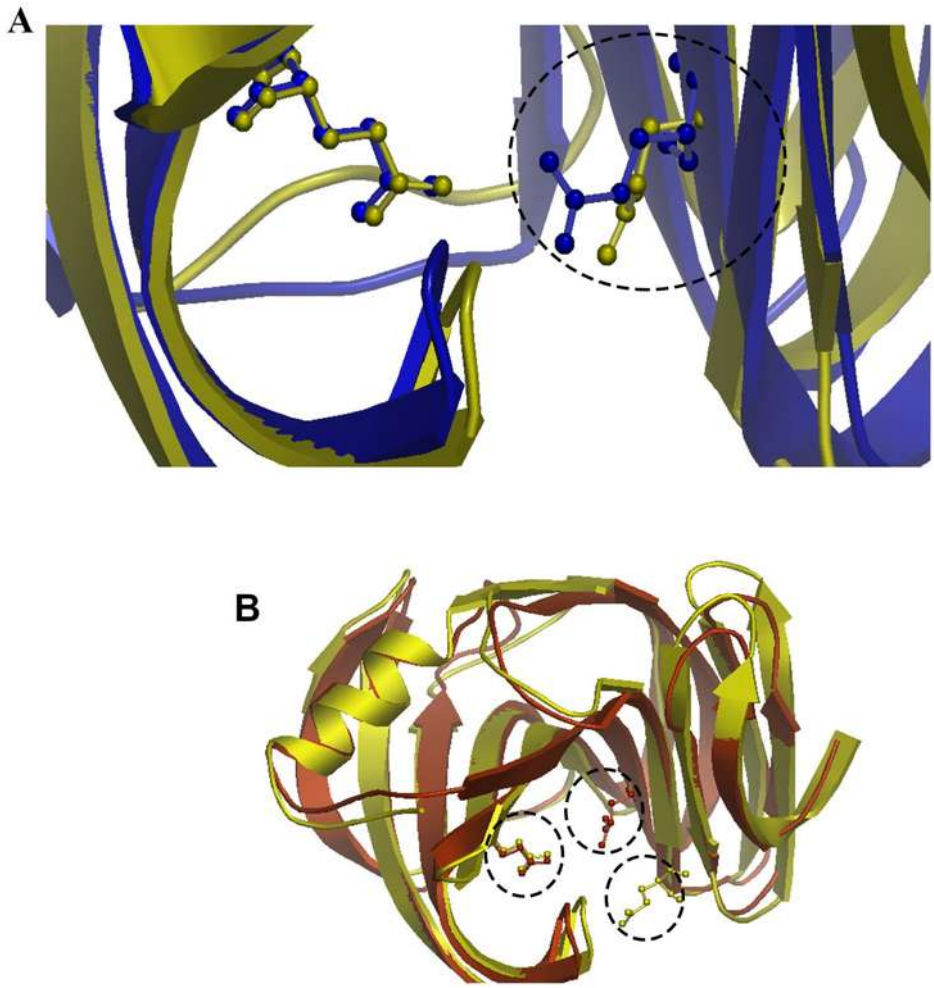



**Fig. 7** Protein homology model of endoxylanases from *Colletotrichum* species. Superimposition of three-dimensional modeled structures of *C. fioriniae PJ7-xyl2* and *C. fioriniae* PJ7-xyl3 (A). Superimposition of three-dimensional modeled structures of *C. gloeosporioides* Nara-xyl5 (green) and *C. lindemuthianum* XYL1 (red), showing a difference in the location of the glutamate (B)

## VII. DISCUSION GENERAL

Los carbohidratos son los productos predominantes de la fotosíntesis vegetal y son también los principales nutrientes de la mayoría de los microorganismos en los ambientes naturales. Las macromoléculas son comúnmente degradadas fuera de las células a unidades mono o diméricas por la acción de enzimas. Por su estructura compleja y heterogénea, la degradación de las paredes celulares vegetales por hongos requiere de un sistema enzimático complejo en el que las xilanasas actúan en conjunto con enzimas líticas como las celulasas, pectinasas, β-xilosidasas, etc, (Eichlerová et. al, 2000).

*C. lindemuthianum* es un agente patógeno natural del frijol que de manera similar a otros patógenos de plantas, utiliza una batería de enzimas capaces de degradar la pared celular vegetal de las células de su hospedero (Albersheim et. al., 1969). Al igual que otros hongos fitopatógenos se le puede considerar como una buena fuente alternativa y poco explorada de genes con capacidades hidrolíticas superiores a las enzimas de hongos saprófitos en sustratos relacionados a sus huéspedes.

De acuerdo a lo anterior, en este estudio se logró el aislamiento del ADNc correspondiente al transcrito completo del gen *Clxil1* que codifica para una xilanasa en ambas razas (R1472-R0) de *C. lindemuthianum*, las cuales fueron identificadas como miembros de la familia 11 de las glucosilhidrolasas. Adicionalmente se analizó la secuencia de aminoácidos de *CLXIL1* y las xilanasas reportadas para microorganismos patógenos, así como la comparación de sus estructuras tridimensionales. Se evaluó la expresión genética de *Clxil1* y se midio la expresión de esta enzima utilizando diversas fuentes de carbono en ambas razas. Se realizaron análisis filogenéticos de *Clxil1* en comparación con xilanasas de otros hongos fitopatógenos y con hongos del mismo género. Estos resultados se reforzaron con los obtenidos del análisis comparativo de las estructuras tridimensionales de las xilanasas. Mediante un estudió de docking o acoplamiento molecular se re-crearon *in silico* las condiciones de acoplamiento del sustrato natural de *CLXIL1* (raza 1472) y otras enzimas previamente modeladas, del género *Colletotrichum sp*. Este estudio mostró la preferencia y capacidad de cada una de las estructuras para asimilar el sustrato.

El análisis clustal de la secuencia deducida de aminoácidos mostró una identidad del 100% entre las dos secuencias de las xilanasas en ambas rasas y una similitud alta en comparación

con las secuencias que están reportadas en banco de datos NCBI. Sin embargo, existen pequeñas diferencias muy posiblemente originadas por la diversidad entre especies, ya que en algunos casos incluso dentro de una misma especie se han reportado hasta cuatro endoxilanasas (Apel-Birkhold y Walton 1996; Gómez et. al., 2002). Hasta el momento, no existe ningun reporte que presente dos razas patógenicas de la misma especie con la misma secuencia genética, que tenga niveles de expresión y de actividad significativamente diferentes y que genere, *in silico*, la misma estructura tridimensional.

Existe evidencia, de la presencia de más de una xilanasa en el genoma de hongos fitopatógenos como *Helminthosporium turcicum* (*htlyl1* y *htxl2)* y *Septosphaeria turcica (xil1* y *xil2).* Sin embargo, los análisis muestran una expresión diferencial en función del sustrato o la etapa de invasión en la que se presentan, por lo cual, se ha propuesto que la expresión diferencial es un factor importante en las fases saprófita y patógena del hongo (Degefu et.al, 2004).

Aunque en el estudio se presentan hongos que están relacionados fiogenéticamente y todos son patógenos de plantas, estos se adaptaron a diferentes hospederos, generando una amplia diversidad de xilanasas y una regulación diferencial, conservando una estructura ancestral que evolucionó debido a la estrategia de invación/nutrición generando una remodelación estructural, pero, conservando intacto el sitio catalítico y la manera de hidrolizar los sustratos. Este es un fenómeno que se ha observado en diferentes enzimas (celulasas, galactanasas, pectin liasas y β-xilosidasas) de *C. lindemuthianum* por lo que estos resultados aunados con aquellos derivados del análisis filogenético y de comparación de estructuras tridimensionales, sugieren que al menos existen dos niveles que parecen determinar el estilo de vida de los microorganismos, el primero, relacionado con la evolución de las enzimas y en segundo, con la regulación de la expresión de las mismas.

Los estudios filogenéticos de las xilanasas son muy pocos (Degefu et. al., 2004; Ellouze et. al., 2011) y solo existe un reporte que fusiona las relaciones filogenéticas con las estructuras tridimensionales en pectin liasas (Lara-Márquez et. al., 2011), por lo que este trabajo, sería el primer reporte de este tipo en xilanasas (GH11).

En este trabajo, se presenta, la predicción tridimensional de las xilanasas encontradas en el género de *Colletotrichum sp*., y el primer análisis de docking reportado para estas enzimas.

Cabe señalar, que existen diferencias estructurales importantes en el centro activo entre las

familias de las xilanasas. Por ejemplo, en las xilanasas de la familia GH10 el lugar de unión al sustrato es una hendidura poco profunda (*shallow groove*), mientras que en las de la familia GH 11 el lugar de unión al sustrato es una hendidura más profunda (*deep cleft*) (Roncero et. al., 2000; Collins et.al., 2005). Estas diferencias estructurales en la hendidura catalítica hacen que las xilanasas de la familia GH10 sean menos estrictas en cuanto al sustrato, mostrando una mayor versatilidad catalítica que las xilanasas de la familia GH11 (Biely *et al*., 1997).

Al parecer todas las estructuras de las xilanasas de la familia GH11 mantienen la misma conformación tridimensional (Paës et.al.,) y capacidad de intereacción con el sustrato, sin embargo, de acuerdo a nuestros análisis, las xilanasas GH11 de *Colletotrichum sp.* presentan diferencias de afinidad por sustrato; 36%, 60% and 4% integraron xylotetraosa, xylopentaosa y xylohexaosa en el sitio activo, respectivamente; esto podría estar relacionado con la capacidad de degradación de las diferentes xilanas. Las xilanasas de Colletotrichum sp. interactúan de manera mas eficiente con xilopentaosa y xilotetraosa que con xilohexaosa, contrastando con lo reportado (Jänis et. al., 2007).

Estos resultados no solo revelan diferencias significativas en la estructura, también demuestra las diferencias de integración del sustrato con el sitio activo y los aminoácidos que interactuan en estos procesos de degradación.

# VIII. PERSPECTIVAS

Para la complementación de estos trabajos seria importante identificar y caracterizar a todas las xilanasas que componen los sistemas de degradación de la pared celular vegetal en *C. lindemuthianum*, y estudiar la regulación de los genes que codifican dichas enzimas.

El estudio de los cambios evolutivos de estas enzimas, requiere de un análisis que permita determinar si han sufrido selección positiva. Identificar si los residuos de aminoácidos de las xilanasas de *Colletotrichum sp*. han estado bajo selección positiva de diversificación y determinar si estos sitios pueden estar contribuyendo a la especificidad hacia sus sustratos.

Estas enzimas presentan un gran potencial biotecnológico, por lo que es necesario establecer sistemas de expresión de Clxil1 y de los genes que codifican estas xilanasas, realizar la caracterización bioquímica de las mismas y estudiar el papel de la glicosilación en su actividad.

La remoción selectiva de los componentes de la pared celular requiere un complejo de enzimas con actividades específicas de diferentes xilanasas que puedan ser dirigidas, secuencialmnte, para degradar estos polímeros.

Finalmente, para el avance en el conocimiento sobre las xilanasas y otras enzimas degradadoras de pared celular vegetal, se requiere la secuencia los genomas y transcriptomas de diferentes razas o patotipos de *C. lindemuthianum*. Su comparación puede arrojar información básica de interés biotecnológico.

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