UNIVERSIDAD MICHOACANA
DE SAN NICOLÁS DE HIDALGO

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

CARACTERIZACIÓN BIOQUÍMICO-MOLECULAR DE LA ENZIMA PECTIN LIASA 2 (*Clpnl2)* DE *Colletotrichum lindemuthianum*

TESIS

QUE PRESENTA

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

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Morelia, Michoacán Marzo de 2012

El presente trabajo se realizó en el Centro Multidisciplinario de Estudios en Biotecnología de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Michoacana de San Nicolas de Hidalgo, bajo la asesoría del Dr. Horacio Cano Camacho y la co-asesoría de la Dra. María Guadalupe Zavala Páramo.

Este trabajo recibió apoyo de FOMIX CONACyT-Gobierno del estado de Michoacán (Proyecto 2009-05 Clave 116208), beca otorgada por CONACyT (Registro de becario No. 165268) para la realización de estudios de posgrado y por la Coordinación de la Investigación Científica de la UMSNH (Clave 14.8).

AGRADECIMIENTOS

A mis padres: Francisco Lara Klahr y Alicia Márquez Melgoza, gracias por todo el amor, apoyo e inspiración que me han dado siempre.

A mis hermanas: Elizabeth y Mónica, gracias por estar conmigo siempre apoyarme y hacer los días mas divertidos.

A Ivan, gracias mi negro por tu amor y apoyo incondicional.

A mis asesores: Horacio Cano Camacho y Guadalupe Zavala Páramo, gracias por sus consejos y su apoyo durante todos estos años.

A el Dr. Everardo Lopez Romero, gracias por su paciencia, sus valiosas observaciones y por el tiempo que dedico en las revisiones de este trabajo.

Al Dr. Gerardo Vázquez Marrufo y al Dr. Rodolfo López Gómez, miembros de mi comité tutorial por sus comentarios y sugerencias siempre valiosas.

A el Dr. Alberto Ken Oyama y la Dra. Patricia Ponce, por amabilidad y comentarios a la tesis.

A Nancy y Luis, Ulices, Lupita y todos mis compañeros del laboratorio, gracias por su apoyo y por su compañía.

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RESUMEN

En este trabajo se reporta la caracterización del gen *Clpnl2* que codifica la pectin liasa 2 (CLPNL2) del hongo patógeno de frijol *Colletotrichum lindemuthianum*. El análisis se realizó utilizando la secuencia genómica de *Clpnl2* [GenBank: JN034038] y la secuencia del ADNc de *Clpnl2* [GenBank: JN034039] obtenida mediante amplificación por RT-PCR. La región codificante del gen *Clpnl2* consiste de 1,428 pb interrumpida por cuatro intrones con tamaños entre 60 a 87 pb. Se identificaron varias posibles secuencias regulatorias en las regiones no-codificantes 5´ y 3´ de *Clpnl2*. El ADNc de *Clpnl2* contiene un ORF de 1,140 nucleótidos que codifican un supuesta proteína de 379 aa con una señal de secreción N-terminal de 19 aa, un tamaño molecular de 37.4 KDa, pI de 9.1, y un sitio potencial de N-glicosilación en posición 110. De acuerdo a la estructura tridimensional de CLPNL2 predicha por modelaje por homología, la estructura terciaria de CLPNL2 coincide con la topología típica de las pectin liasas (PNLs) en β-hélice paralela.

Se realizó un análisis filogenético de la secuencia deducida de amino ácidos de CLPN2 y las secuencias reportadas de PNLs en las bases de datos. El análisis muestra la separación temprana de dos grupos: uno representado por PNLs bacterianas y otro compuesto por PNLs de hongos y oomicetos. Los árboles inferidos también muestran la formación de un grupo monofilético compuesto principalmente por secuencias de hongos saprófitos/oportunistas. Sin embargo, las secuencias de hongos y oomicetos fitopatógenos no forman un grupo monofilético. La PNL 2 de *C. lindemuthianum* se encontró agrupada con las secuencias de *C. gloesporioides*. Adicionalmente, se realizó el modelaje por homología de las secuencias utilizadas en el análisis filogenético y se compararon sus estructuras (incluyendo el sitio de unión a carbohidrato) utilizando tres métodos de comparación. Los resultados de éste análisis concuerdan con los obtenidos del análisis filogenético.

Se analizó la expresión de *Clpnl2* en una raza patógena (1472) y una no patógena (0) de *C. lindemuthianum* crecidas en diferentes fuentes de carbono. Los resultados indican diferencias en los tiempos y niveles de expresión entre las razas. De manera adicional, se expresó el ADNc de *Clpnl2* en la cepa X-33 de *Pichia pastoris*, bajo el control del promotor de la alcohol oxidasa. Como resultado de la transformación con la construcción, se obtuvieron 16 cepas de *P. pastoris* (X-33/*Clpnl2* 1-16); la cepa X-33/*Clpnl2*-2 se utilizó en el resto de los

análisis por su resistencia a zeocina (2000 μg/ml). De acuerdo al análisis de expresión por RT-PCR, *Clpnl2* se expresa en la cepa X-33/*Clpnl2*-2 desde el inicio de la inducción con 0.5% de metanol y ésta se mantiene durante todo el periodo experimental. La pectin liasa recombinante se secreta de manera eficiente en el medio a partir de las 48 h de inducción con 0.5% de metanol (22.9% de la proteína total), con un peso molecular estimado de 44.4 kD, similar al calculado por SDS-PAGE para la proteína nativa en *C. lindemuthianum*. La actividad total de la PNL recombinante detectada en el extracto inducido durante 48 h fue de 5090.91 nmol de UUE/min, 1.5 veces más que la actividad total detectada en el extracto crudo de *C. lindemuthianum* inducido con pectina esterificada al 92% (3369.6 nmol de EU/min).

Para purificar tanto la enzima recombinante como la nativa, se fraccionaron los extractos crudos de la cepa X-33/*Clpnl2*-2 de *P. pastoris* y de *C. lindemuthianum* con sulfato de amonio. La mayor parte de la actividad específica de PNL se detectó en la fracción 50-60% (28.7 nmol de EU/min/mg de proteína), con un incremento en la actividad específica de 6 veces en comparación con el extracto crudo (4.55 nmol de EU/min/mg de proteína).

El fraccionamiento salino del extracto crudo de *C. lindemuthianum* mostró dos picos de actividad específica de PNL en las fracciones 30-50% y 60-80% (146.44 y 91.53 UUE/min/mg de proteína, respectivamente). La fracción 60-80% del extracto de *C. lindemuthianum* se sometió a cromatografía en una columna de DEAE celulosa. El perfil de elución de la enzima mostró un pico correspondiente a 0.1–0.4 M de NaCl donde se concentró la mayoría de la actividad específica de PNL de *C. lindemuthianum*.

SUMARY

Here we report the isolation and sequence analysis of the *Clpnl2* gene, which encodes the pectin lyase 2 (CLPNL2) in the phytopathogenic fungus *C. lindemuthianum*. The analysis was performed using the genomic sequence of *Clpnl2* [GenBank: JN034038] and the *Clpnl2* cDNA sequence obtained by RT-PCR amplification. The coding region of the *Clpnl2* gene consisted of 1428 bp interrupted by four introns ranging in size from 60 to 87 bp. Several possible regulatory sequences were identified in the 5´and 3´ untranslated regions of *Clpnl2*. The *Clpnl2* cDNA contains an ORF of 1140 nucleotides that encodes a putative protein of 379 aa with a N-terminal secretion signal sequence of 19 aa. The molecular mass and pI calculated were 37.4 KDa and 9.1 respectively, and the one potential N-glycosylation site was located at position 110. The tertiary structure of Clpnl2 predicted by homology modeling coincided with the typical topology of the parallel β-helix of pectin lyases (PNLs).

A phylogenetic analysis of the deduced amino acids sequence of Clpnl2 and PNLs sequences reported in databases was performed. The analysis showed an early separation of pectin lyases into two groups, one represented by bacteria and another represented by fungi and oomycetes. The inferred tree also showed that the analyzed sequences of saprophytic/opportunistic fungi are clustered into a monophyletic group. However, phytopathogenic fungi and oomycetes were not clustered together. *C. lindemuthianum* clustered with the aa sequences of the fungal pathogen *C. gloeosporioides*. The tertiary structures corresponding to the aa sequences used in phylogenetic analyses were also predicted (including the carbohydrate-binding site) and compared their structures using three comparison methods. In agreement with the phylogenetic analyses, it was possible to distinguish the cluster formed mainly by sequences of fungi and oomycete pathogens, including Clpnl2 from the clusted formed by saprophytic/opportunistics fungi.

We analyzed the *Clpnl2* expression in pathogenic (1472) and non-pathogenic (0) races of *C. lindemuthianum* grown on different carbon sources. The results indicate differences in the timing and expression levels between the two fungal races.

Additionally, the *Clpnl2* cDNA was expressed in *Pichia pastoris* under the control of alcohol oxidase promoter. There were obtained 16 strains of *P. pastoris* (X-33/*Clpnl*2 1-16) as a result of the transformation with the construction; the X-33/*Clpnl2*-2 strain was used in the rest of the tests by its resistance to high concentrations of Zeocin (2000 μg/ml). According to the RT-PCR expression analysis, *Clpnl2* was expressed in X-33/*Clpnl2*-2 since the start of induction with 0.5% methanol, and it was maintained throughout the induction kinetics. The recombinant pectin lyase was efficiently secreted into the induction medium after 48 h (22.9% of the total protein), with an estimated molecular mass of 44.4 kDa, similar to that estimated in *C. lindemuthianum* by SDS-PAGE. Total activity of recombinant PNL detected in the extract induced by 48h was of 5090.91 nmol of UUE/min/mg, 1.5 times of the total activity detected from crude extract of *C. lindemuthianum* induced with 92% esterified pectin (3369.6 nmol de EU/min/mg protein).

To purify the PNL recombinant and native, the extracts of *P. pastoris* X-33/*Clpnl2*-2 and *C. lindemuthianum* were fractionated with ammonium sulfate. Most of the PNL specific activity in extract of *P. pastoris* X-33/*Clpnl2*-2 was detected in the 50-60% fraction (28.7 nmol de EU/min/mg protein) with an increase in the specific activity of 6 times (28.7 nmol de EU/min/mg protein) compared to the crude extract (4.55 nmol de EU/min/mg protein). Nevertheless, the specific activity in the 50-60% fracction of *P. pastoris* X-33/*Clpnl2*-2 only represented 52% of PNL activity in the extract of *C. lindemuthiamun* induced with esterified pectin.

There were detected two peaks of specific activity in fractions 30-50% and 60-80% of the extract of *C. lindemuthianum* (146.44 y 91.53 UUE/min/mg protein, respectively). The 60-80% fraction of the extract of *C. lindemuthianum*, was subjected to a DEAE cellulose column. The elution profile of the enzyme showed a peak corresponding to elution with 0.1 to 0.4 M NaCl where most of the PNL specific activity was detected.

INTRODUCCIÓN GENERAL

1.1 PARED CELULAR VEGETAL

La pared celular vegetal es la principal fuente de carbono de la biósfera y uno de los complejos macromoleculares más sofisticados que se conocen (Humphrey et al. 2007) y es considerada por algunos autores como un organelo activo que funciona como una interfase entre la planta y el medio (Walton 1994; Lagaert et al. 2009). 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, es responsable de la forma celular y fortaleza de la planta (Carpita y Gibeaut 1993; Humphrey et al. 2007; Sarkar et al. 2009). 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 pared celular está compuesta principalmente por carbohidratos en forma de fibras como la celulosa, la cual se encuentra incluida en una matriz amorfa de otros polisacáridos complejos (hemicelulosa y pectina), glicoproteínas, proteoglicanos, componentes de bajo peso molecular y iones (Willats et al. 2001), además de lignina. La pared celular primaria se compone de aproximadamente 10% de proteínas y 90% de polisacáridos (celulosa, hemicelulosa y pectina) (Fig 1). La composición de estos polisacáridos puede variar entre especies, tejidos y estado del desarrollo de la planta (McNeil et al. 1984; Lagaert et al. 2009). Sin embargo, en paredes celulares primarias tipicamente se encuentran valores aproximados a 30% de celulosa, 30% hemicelulosa y 35% pectina (Cosgrove 1997), y en paredes celulares secundarias con valores aproximados a 35% - 50% de celulosa, 20% - 50% de hemicelulosa y 7% - 10% de lignina (Vogel 2008; King et al. 2011).

Siendo la principal fuente de carbono de la biósfera y la principal barrera con la que cuentan las células vegetales contra los patógenos, la pared celular vegetal es blanco del ataque de un gran número de microorganismos que pretenden utilizar esta fuente de energía y acceder a las células vegetales. Esta es la razón de una larga batalla co-evolutiva entre los mecanismos de defensa de las plantas y las estrategias de los patógenos por evadirlos y

degradar los componentes de la pared celular (Walton 1994; Warren 1996; Cantu et al. 2008; Sarkar et al. 2009).

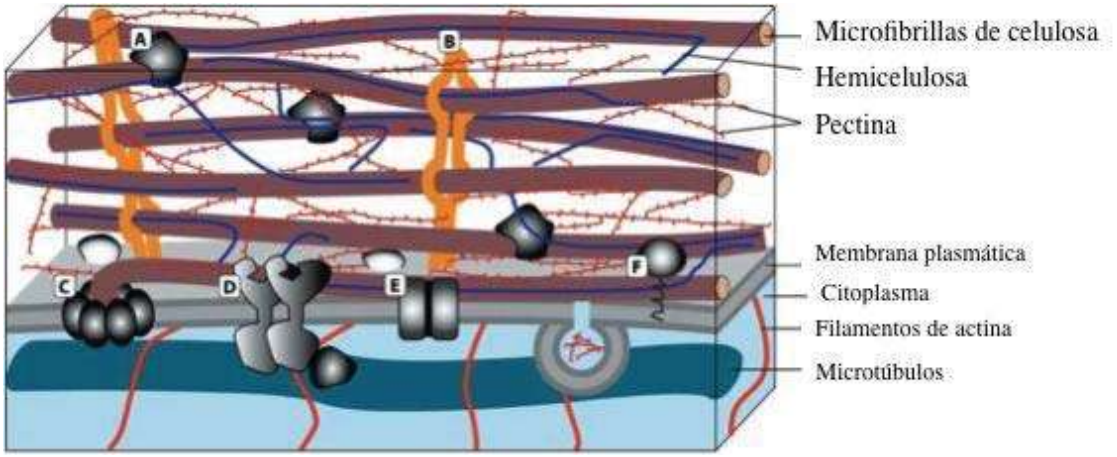

Figura 1. Modelo de la pared celular ilustrando los principales polisacáridos y componentes proteicos. (A) expansinas, (B) extensinas y (F) glicosilfosfatidilinositol (GPI) anclando proteínas glicosiladas y asociándolas con la red de polisacaridos. (C) complejo de la celulosa sintasa, (D) kinasas, (E) canáles de iones, y (F) proteínas GPI de anclaje (Humphrey 2007).

La producción de enzimas que degradan los polímeros de la pared celular es una de las principales estrategias de los patógenos para evadir la barrera que esta representa. La producción extracelular de estas depolimerasas se ha encontrado en microorganismos saprófitos y patógenos, principalmente entre bacterias, nemátodos y hongos (Walton 1994). La actividad de estas enzimas contribuye a que los patógenos accedan a la célula vegetal y proporciona nutrientes que son aprovechados durante el proceso de infección. Sin embargo, su actividad también genera señales (oligosacáridos productos de la degradación) que desencadenan respuestas de defensa de la planta (Sarkar et al. 2009).

La complejidad de la pared celular vegetal, en si misma constituye una de las principales dificultades a las que se enfrentan los patógenos. Algunos de los polisacáridos que la constituyen son relativamente fáciles de degradar y las enzimas necesarias para ello se encuentran ampliamente distribuidas. Sin embargo, otros polisacáridos son mucho más complejos, disminuyendo así el número de microorganismos que pueden degradarlos. Algunos otros componentes de la pared, como la lignina, son tan complejos que solo algunos microorganismos son capaces de enfrentarse a este polímero (Walton 1994; Sarkar et al. 2009).

La complejidad de los polisacáridos de la pared celular también es un factor que contribuye a su resistencia a la hidrólisis para la producción bioenergética y de bioproductos derivados de ella (Merino y Cherry 2007; King et al. 2011) y por lo tanto un reto para la biotecnología. El conocimiento de la composición de la pared celular vegetal, las interacciones entre los polisacáridos que la conforman y las enzimas que componen los sistemas de degradación, es entonces fundamental para su aprovechamiento biotecnológico.

Las enzimas que degradan pectina son capaces de macerar las paredes celulares por si mismas y generalmente son las primeras enzimas secretadas durante el proceso de infección. Se cree que su acción facilita el acceso a otros polímeros de la pared para ser degradados y han sido consideradas como factores de patogénesis y/o virulencia (Wattad et al. 1997; Soriano 2006). Por estos motivos constituyen uno de los principales focos de atención de muchas investigaciones durante la última década, tanto en estudios enfocados en la interacción planta/patógeno como por su potencial biotecnológico en diversas industrias.

1.2. PECTINA

La pectina es uno de los polisacáridos más complejos en la naturaleza y es uno de los principales componentes de la pared celular de las plantas (Fig. 1). Es el mayor constituyente de la lámina media y se encuentra en células en división activa y en áreas de contacto entre células con pared celular secundaria, incluyendo células del xilema y células fibrosas. La pectina comprende entre el 30% y el 35% de la pared celular de dicotiledóneas y monocotiledóneas no gramíneas (Cosgrove 1997; Willats et al. 2001; Cosgrove 2005; Mohnen 2008). El contenido de pectina en pared celular secundaria es reducido; sin embargo, la pectina tiene un papel importante en la estructura y función de las paredes celulares primaria y secundaria (Willats et al. 2001; Mohnen 2008).

Las funciones de la pectina en la pared celular vegetal son diversas e incluyen crecimiento y desarrollo, morfogénesis, defensa, adhesión celular, estructura y expansión celular, porosidad, hidratación de semillas, abscisión de hojas y desarrollo de frutos, entre otras (Willats et al. 2001; Mohnen 2008).

La pectina es un polisacárido rico en ácido galacturónico (GalA) que se presenta en forma de dominios estructurales unidos covalentemente: homogalacturonanos (HG), xylogalacturonanos (XGA), ramnogalacturonanos I (RG-I) y ramnogalacturonanos II (RG-II) (Willats et al. 2001; Mohnen 2008) (Fig. 2). Los HG son los componentes más abundantes de la pectina (comprenden aproximadamente el 65% de la pectina), estan formados por cerca de 100 residuos de GalA unidos por enlaces α-D-1,4 que pueden ser modificados por metil-esterificación en el carbono C-6, o por la presencia de grupos acetil en O-2 y O-3 (Mohnen 2008). Los XGA están formados por una base de HG, donde el 25-75% de las unidades de GalA están substituidas en C-3 con un residuo de xilosa y ocasionalmente por un segundo residuo de xilosa en C-4 (Coenen et al. 2007; Mohnen 2008).

Los RG-I comprenden el 20-35% de la pectina, se trata de un polímero más complejo que consiste en un soporte de unidades repetidas del disacárido -α-D-GalA-1, 2-α-L-Rha-1-4, donde el 20 y 80% de los residuos de ramnosa están sustituidos con cadenas laterales de oligómeros de arabinosa y galactosa de tamaño variable. Ocasionalmente, se presentan cadenas laterales de fucosa y ácido glucurónico (Willats et al. 2001; Mohnen 2008).

El RG-II es el dominio estructural más complejo de la pectina. Esta formado por una cadena principal de residuos de GalA unidos por enlaces -α-D-1,4 con candemas laterales de 12 diferentes azúcares (arabinosa, galactosa, xilosa, fucosa, ácido acerico, ácido glucurónico, apiosa, etc.) y forma dímeros a través de ésteres de borato (Mohnen 2008).

Los dominios estructurales de la pectina interactúan entre ellos mismos y con otras moléculas como calcio, borato, poliaminas y compuestos fenólicos que contribuye a las propiedades de la matriz de pectina. Los polímeros que conforman la pared celular también interactúan por medio de enlaces de hidrógeno con microfibrillas de celulosa, hemicelulosa, pectina y xiloglucanos o xilanos por medio de enlaces covalentes (Popper y Fry 2008).

1.3. DEGRADACIÓN ENZIMÁTICA DE LA PECTINA

Debido a que la pectina es una estructura muy compleja y heterogénea, su degradación requiere de la acción combinada de varias enzimas que pueden ser clasificadas de acuerdo a su modo de acción y al substrato que degradan. Estas enzimas incluyen poligalacturonasas (PG), que degradan el HG por la hidrólisis de los enlaces glicosídicos y se clasifican como endo-PG (PGA; E:C. 3.2.1.15) y exo-PG (XPG; E.C. 3.2.1.67) (de Vries y Visser 2001; Benen y Visser 2003). Las liasas como pectato liasas (PL; E:C: 4.2.2.9 y 4.2.2.2) y pectin liasas (PNL; E:C: 4.2.2.10) catalizan la degradación del poligalacturonato y la pectina esterificada, respectivamente, por medio de β-eliminación removiendo un protón y generando un enlace insaturado entre el carbono C-4 y C-5 del extremo no reducido de la pectina (Fig. 2A) (Herron et al. 2000; Jayani et al. 2005). Los residuos acetil, metil y feruloil de la pectina son removidos por las pectin metilesterasas (PME; E.C. 3.1.1.11) (Fig. 2A), acetilesterasas (AE; E.C. 3.1.1.6), ramnogalacturonan acetilesterasas (RGAE; E.C. 3.1.1.) y feruloil esterasas (FAE; E.C. 3.1.1.73). La actividad de estas enzimas es importante para la degradación completa de la pectina, ya que su actividad promueve la activación de otras enzimas; por ejemplo, la degradación eficiente de la pectina por las PGs y PLNs depende significativamente de la actividad de las PMEs (de Vries et al. 2000; de Vries y Visser 2001).

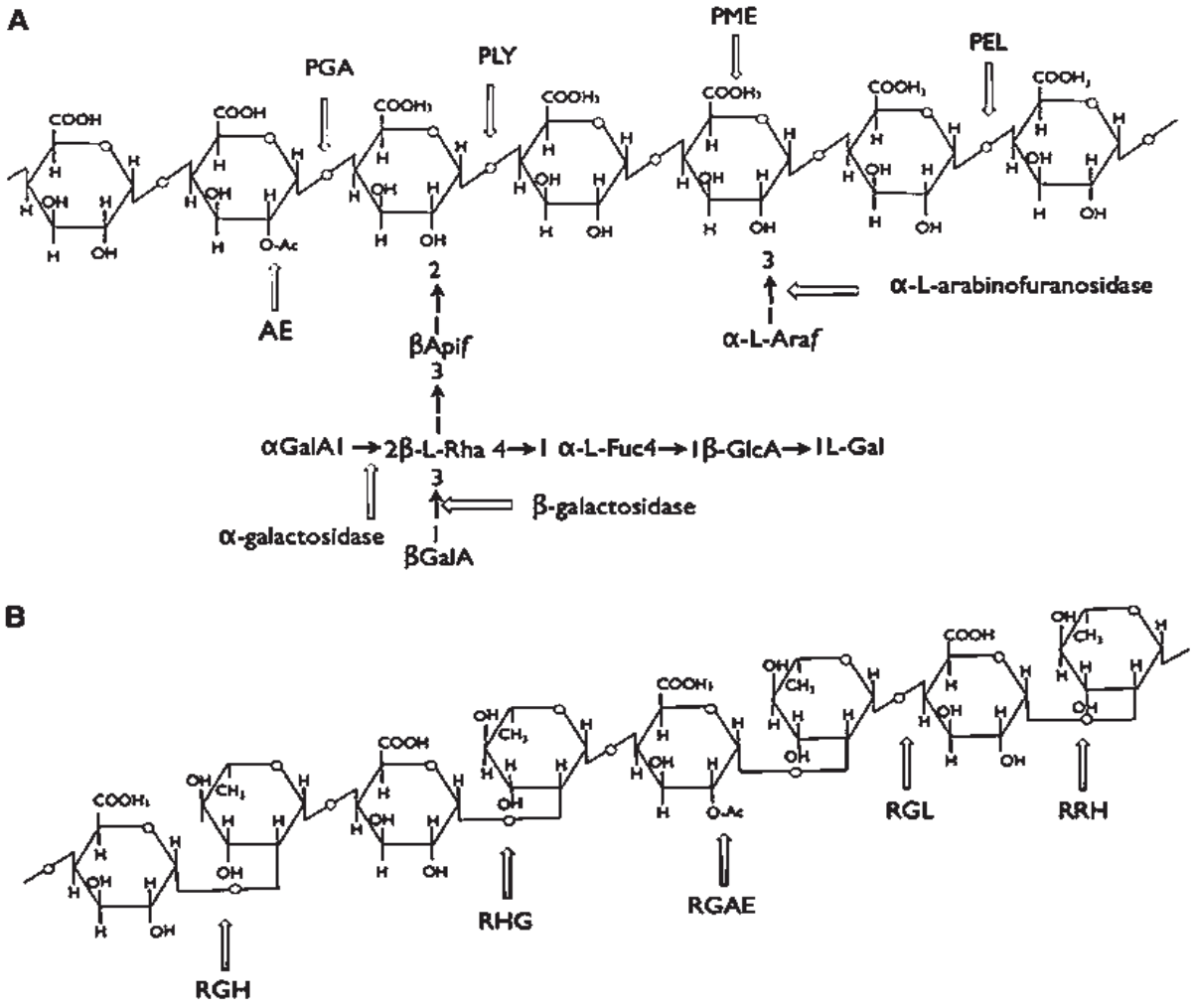

Figura 2. Estructura representativa de la pectina y sitio de acción de las enzimas involucradas en su degradación. A) enzimas pectinolíticas involucradas en la degradación de la cadena principal de HG y cadenas laterales de RG-II, B) depolimerasas de la cadena principal de RG-I. (PGA) endo-poligalacturonasa, (PL) pectin liasa (PLN) pectaton liasa, (PME) pectin metilesterasa, (AE) acetilesterasa, (RGH) ramnogalacturonan galacturonohidrolasa, (RHG) endoramnogalacturonan hidrolasas, (RGAE) ramnogalacturonan acetilesterasas, (RGL) ramnogalacturonan liasas, (RRH) ramnogalacturonan ramnohidrolasa (Lara-Márquez et al. 2011)

Las endoramnogalacturonan-hidrolasas (RHG; E.C. 3.2.1) degradan los enlaces -α-D-GalA-1, 2-α-L-Rha en el RG-I por hidrólisis, mientras que las ramnogalacturonan liasas (RGL; E.E. 4.2.2.) degradan los enlaces -α-L-Rha-1, 4-α-D por β-eliminación (Fig. 2B). La ramnogalacturonan ramnohidrolasa (RRH; E.C. 3.2.1.) y ramnogalacturonan galacturonohidrolasa (RGH; E.C. 3.2.1.) degradan oligosacáridos del extremo no-reducido por ataque exo (de Vries y Visser 2001; Voragen et al. 2008). Se han encontrado dos nuevas

pectinasas capaces de degradar XGA en *Aspergillus tubingensis*: una exogalacturonasa capaz de remover el disacárido Xyl-GalA y una endoxilogalacturonanhidrolasa que corta los enlaces 1,4-α-D-GalA en el XGA (Beldman et al. 1996; van der Vlugt-Bergmans et al. 2000).

Las cadenas laterales del RG-I y RG-II son degradadas por enzimas accesorias tales como endogalactanasas (E.C. 3.2.1.89), exogalactanasas (E.C. 3.2.1.145), α- y β-galactosidasas (E.C. 3.2.1.22 y 3.2.1.23) (Fig. 2A), α-L- arabinofuranosidasas (E.C. 3.2.1.55), endoarabinasas (E.C. 3.2.1.99) y exoarabinasas (E.C. 3.2.1.) (de Vries y Visser 2001; de Vries 2003).

1.3.1. Genes que codifican para enzimas pectinolíticas

No obstante que se han clonado muchos genes que codifican para enzimas pectinolíticas, estamos lejos de un conocimiento completo del sistema pectinolítico a nivel genétco (de Vries et al. 2005). Actualmente, se han secuenciado un gran número de genomas de hongos filamentosos, incluyendo especies de importancia biotecnológica y patogénica. El análisis de los genomas de *A. nidulans* y *A. niger* ha demostrado el potencial de estos estudios. De Vries et al. (2005) reportan que más de dos tercios de los ORFs potencialmente involucrados en la degradación de polisacáridos de la pared celular encontrados en el genoma de *A. nidulans* codifican para enzimas nuevas. También identificaron ORFs que aparentemente codifican para enzimas putativas que degradan oligosacáridos intracelulares y otros con homología a trasportadores de oligosacáridos. El análisis del genoma de *A. niger* reveló 21 genes que codifican para pectinasas de la familia 28 de las glicosil hidrolasas (Martens-Uzunova et al. 2006), 39 genes que codifican para enzimas involucradas en la depolimerización de la cadena principal de pectina y genes que codifican para enzimas con actividades en las cadenas laterales de la pectina (Martens-Uzunova y Schaap 2009).

Las secuencias genómicas de los hongos filamentosos y el uso de herramientas bioinformáticas han permitido un progreso rápido en el estudio de los genes que codifican para enzimas pectinolíticas, lo que ha mostrado la complejidad del sistema de degradación de la pectina y lo mucho que falta por conocer (Vries et al. 2005; Martens-Uzunova et al. 2006).

Se han aislado varios genes que codifican para pectin liasas, principalmente de hongos saprófito/oportunistas y bacterias (Tabla 1).

Tabla 1. Genes que codifican para pectin liasas

Microorganismo	Numero de Acceso	Referencia
Aspergillus niger	GenBank: CAD34589, GenBank: AAW03313, GenBank: CAA39305, GenBank: CAA01023, GenBank: ACE00421, GenBank: AAA32701	(Gysler et al. 1990; Kusters-van Someren et al. 1991; Kusters-van Someren et al. 1992; de Vries et al. 2002; Martens-Uzunova y Schaap 2009)
Aspergillus nidulans	GenBank: ABF50854	(Bauer et al. 2006)
Aspergillus oryzae	GenBank: BAB82468, GenBank: BAB82467	(Kitamoto et al. 2001a; Kitamoto et al. 2001b)
Aspergillus fumigatus	GenBank: EDP48344, GenBank: EAL91566, GenBank: EAL91586, GenBank: EAL87726	(Nierman et al. 2005; Fedorova et al. 2008)
Aspergillus terreus	GenBank: EAU31855, GenBank: EAU37973	
Aspergillus clavatus	GenBank: EAW12911	(Fedorova et al. 2008)
Emericella nidulans	GenBank: EAA64674	(Bauer et al. 2006)
Colletotrichum gloeosporioides	GenBank: AAA21817, GenBank: AAD43565, GenBank: AAF22244	(Templeton et al. 1994; Wei et al. 2002)
Penicillium occitanis	GenBank: ABH03046	(Trigui-Lahiani y Gargouri 2007)
Penicillium. Griseoroseum	GenBank: AF502280	(Bazzolli et al. 2006)
Neosartorya fischeri	GenBank: EAW17753, GenBank: EAW23742	(Fedorova et al. 2008)
Pyrenophora tritici-repentis	GenBank: XP_001934252, GenBank: XP_001930850	
Ustilago maydis	GenBank: EAK86184	(Kämper et al. 2006)
Verticillium albo-atrum	GenBank: XP_003001443	
Phytophthora infestans	GenBank: XP_002909420, GenBank: XP_002903922	
Bacillus subtilis	GenBank: BAA12119, GenBank: AAB84422	(Sakamoto et al. 1996; Kunst et al. 1997)
Pectobacterium atrosepticum	GenBank: CAG74408	(Bell et al. 2004)
Pectobacterium carotovorum	GenBank: AAA24856	(Chatterjee et al. 1991)

1.3.2. Regulación de genes que codifican para pectin liasas

Los genes que codifican para pectin liasas son regulados a nivel transcripcional por las condiciones ambientales, así como por el pH del medio y las fuentes de carbono como la pectina. También sufren represión catabólica; sin embargo, en algunos casos se ha reportado su expresión constitutiva (Guo et al. 1995; de Vries y Visser 2001; Wei et al. 2002; de Vries 2003).

El sistema fúngico mejor estudiado es el de *Aspergillus* sp., en el que se han localizado algunos elementos regulatorios en las regiones promotoras de estos genes. Estos elementos incluyen sitios de unión a represores catabólicos como CreA, Hap2-3-4 y sitios de unión a activadores transcripcionales, o factores que modulan la expresión (Kitamoto et al. 2001b; Aro et al. 2005).

Las vías de regulación que controlan la expresión de genes que codifican para pectinasas se encuentran mejor documentadas en bacterias fitopatógenas, especialmente en *Erwinia* sp.; sin embargo, es poco lo que se conoce sobre su regulación en hongos fitopatógenos (Aro et al. 2005).

Como se mencionó anteriormente, la composición de la pectina y de la pared celular vegetal es compleja, por lo que los microorganismos que requieren degradarla necesitan poseer un sistema de degradación eficiente y bien coordinado para enfrentarse de manera exitosa a esta complejidad. De acuerdo a esto, se ha sugerido la existencia de un sistema multifuncional de degradación de la pectina (Martens-Uzunova y Schaap 2009). de Vries (2003) propone que ya que *Aspergillus* es incapaz de importar polisacáridos grandes, los verdaderos inductores del sistema de regulación son los pequeños oligosacáridos o monosacáridos generados como producto de la degradación de la pectina. Existen evidencias que señalan la expresión de un grupo de genes pectinolíticos en respuesta a ácido galacturónico, donde los productos generados por estas reacciones enzimáticas como L-arabinosa, L-ramnosa, ácido ferúlico y D-xylosa activan a otros genes del sistema de degradación (Parenicová et al. 2000; de Vries et al. 2002; Martens-Uzunova et al. 2006). Martens-Uzunova y Schaap (2009) analizaron la expresión de 46 genes de pectinasas en tres transcriptomas de *A. niger* por microhileras de ADN y de acuerdo a sus resultados proponen

un modelo en cascada para la degradación de la pectina donde interactúan enzimas que degradan las cadenas principales de este polímero con enzimas accesorias de manera sirnegística resultando en la liberación de azúcares monoméricos que a su vez inducen la actividad de otras enzimas del sistema.

Las pectinasas son utilizadas en diversas aplicaciones biotecnológicas para modificar o degradar la pectina y por lo tanto es de gran interés la producción de enzimas pectinolíticas. Las poligalacturonasas y pectato liasas son las enzimas mas utilizadas en estas aplicaciones. Sin embargo, de acuerdo a los antecedentes mencionados anteriormente para degradar completamente a la pectina es necesario conocer todas las enzimas involucradas en el sistema de degradación y la regulación de los genes que las codifican.

1.3.3. Propiedades bioquímicas de las pectin liasas

Las pectin liasas catalizan la degradación de la pectina por β-eliminación; el mecanismo de β-eliminación comienza con la remoción del protón del carbono C-5 en el anillo del azúcar del ácido urónico o éster, posteriormente se estabiliza el anión resultante por la deslocalización de carga en el C-6 y el corte lítico del enlace entre el O-4:C-4, facilitando la donación del protón, para dar un ácido hexenurónico en el extremo no reducido de la cadena (Yip et al. 2004; Yip y Withers 2006; Lombard et al. 2010). El reconocimiento de polisacáridos en las pectin y pectato liasas a menudo depende de interacciones con cationes divalentes (Ca^{2+}), en el caso de las pectato liasas o con cadenas laterales de amino ácidos cargados positivamente (Arg), o con grupos de ácido urónico en el sustrato en el caso de las pectin liasas (Herron et al. 2000; Jayani et al. 2005; Lombard et al. 2010).

La actividad de las pectin liasas es altamente dependiente de la distribución de los metil ésteres sobre la cadena del homogalacturonano. Las pectin liasas exhiben un pH óptimo entre 6.0-8.5 (Tabla 2) y, a diferencia de las pectato liasas, su actividad es independiente de iones Ca^{2+}; se cree, sin embargo, que el residuo Arg^{236} juega un papel similar al del Ca^{2+} (Herron et al. 2000; Jayani et al. 2005).

Tabla 2. Propiedades bioquímicas de las pectin liasas

Especie	Enzima	KDa	pH_{opt}	T_{opt} (ºC)	pI	Referencia
Aspergillus japonicus		32	6.0	55	7.7	(Ishii y Yokotsuka 1975)
Aspergillus niger	PL I	37.5			3.6	
Aspergillus niger	PL II	37.5			3.7	
Aspergillus niger	PL B	40	8.5-9.0		5.9	(Kusters-van Someren et al. 1991)
Aspergillus oryzae	PL		4.0-8.5	50-55		(Ueda et al. 1982)
Aspergillus oryzae	PEL 2	44	6.0	50		(Kitamoto et al. 2001a)
Aspergillus oryzae	PEL 1	39				(Kitamoto et al. 2001b)
Aspergillus flavus		38	8.0	50		(Yadav et al. 2008)
Aspergillus terricola		35	8.0	50		(Yadav et al. 2009)
Aspergillus sp.			8.5-8.8	40-45		(Delgado et al. 1992)
Colletotrichum gloeosporioides	PNL A				9.4	(Templeton et al. 1994)
Curvularia inaequalis			50	45		(Afifi et al. 2002)
Penicillium expansum	PEL 1	40			9.4	(Cardoso et al. 2007a)
Penicillium griseoroseum	PLG 1	40				(Bazzolli et al. 2006)
Penicillium italicum	PNL	22	6-7	35-40		(Alaña et al. 1990)
Penicillium occitanis	PNL1	37.5				(Trigui-Lahiani y Gargouri 2007)
Penicillium oxalicum	PNL		8.0	40		(Yadav y Shastri 2005)
Pythium splendens		23	8.0	50	8.0	(Chen et al. 1998)

La complejidad de la pared celular se puede comparar con la cantidad de enzimas producidas por los microorganismos que degradan los polisacáridos que la componen. Las enzimas que degradan los enlaces glicosídicos se encuentran clasificadas en múltiples familias, determinadas de acuerdo a la similitud estructural y de secuencia de sus módulos catalíticos y de unión a carbohidrato (Henrissat 1991; Henrissat 1993; Henrissat et al. 1995; Lombard et al. 2010). La base de datos de enzimas activas sobre carbohidratos (CAZy) incluye a las enzimas que rompen los enlaces glicosídicos de forma no-hidrolítica, como las pectin liasas dentro de la familia 1 de las polisacárido liasas la cual comprende, además de pectin y pectato liasas, proteínas del polen/estilo de plantas. Los miembros de esta familia

muestran una amplia variedad de estructuras, desde β-hélices a barriles α/α. Sin embargo, las pectato liasas y en particular las pectin liasas se caracterizan por presentar una estructura conservada en β-hélice (Fig. 3). Esta topología esta formada por hebras β paralelas que se pliegan formando un anillo largo hacia la derecha (Herron y Jurnak 2003; Lombard et al. 2010).

La glicosilación es la principal modificación post- tranduccional que sufren las pectin y pectato liasas. En algunos casos, la falta de esta modificación o la hiperglicosilación tienen efectos en la actividad y/o la estabilidad de la enzima (Jeoh et al. 2008). Sin embargo, también se han reportado casos en los que no existen diferencias significativas entre la proteína nativa y la modificada (Benen et al. 2000; Nevalainen et al. 2005).

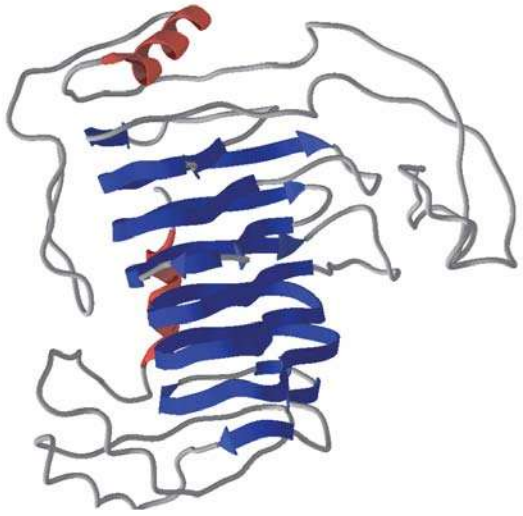

Figura 3. Estructura tridimensional de la pectin liasa B de *A. niger* [PDB: 1QCX].

Hasta la fecha, unicamente se ha determinado la estructura tridimensional de cinco miembros de la superfamilia de las pectato liasas. Estas incluyen la pectato liasa C (Yoder et al. 1993) y la pectato liasa E (Lietzke et al. 1994) de *Erwinia chrysanthemi*. Una pectato liasa de *Bacillus subtilis* (Pickersgill et al. 1998), pectin liasa A (Mayans et al. 1997) y la pectin liasa B (Vitali et al. 1998) de *A. niger*. A pesar de que las pectin y pectato liasas exhiben una arquitectura estructural similar, así como un mecanismo catalítico relacionado, estas divergen significativamente en su estrategia de unión al sustrato (Mayans et al. 1997; Herron et al. 2000).

A pesar de los avances obtenidos gracias a las estrategias actuales, capaces de obtener información funcional a partir de imágenes tridimensionales, aún faltan muchos aspectos por conocer como por ejemplo la capacidad de reconocimiento de secuencias únicas de oligosacáridos en una mezcla heterogénea (Mayans et al. 1997).

1.4. POTENCIAL BIOTECNOLÓGICO DE LAS PECTIN LIASAS

Como ya lo mencionamos, la pared celular de las plantas es la mayor fuente de biomasa en la naturaleza y en la actualidad la misma es utilizada en una amplia variedad de procesos, si bien gran parte de ella es desaprovechada. En la mayoría de los procesos biotecnológicos se utilizan métodos mecánicos o químicos y muchos de ellos son poco eficientes, muy complejos, agresivos con el medio ambiente y tienen costos elevados. El primer paso en el procesamiento industrial de biomasa frecuentemente involucra la degradación parcial de la fracción polimérica. Por ello, el uso de enzimas capaces de degradar los componentes de la pared celular como celulasas, hemicelulasas y pectinasas, ha sido extensamente estudiado debido a su potencial biotecnológico y por las ventajas que ofrecen.

Comparado con los procesos industriales aplicados comúnmente, el uso de enzimas tiene características atractivas, tales como una mayor estabilidad y alta actividad, son biocatalizadores muy específicos y reducen los productos indeseables (Hoondal et al. 2002; MacCabe et al. 2002).

Las pectin liasas son las enzimas más importantes involucradas en la depolimerización de la pectina ya que son las únicas capaces de cortar los enlaces glicosídicos internos de la pectina altamente metilada, como es el caso de la pectina de la fruta, sin la acción de otras enzimas (Alaña et al. 1989). Por estas razones, las pectin liasas tienen diversas aplicaciones en la industria de los alimentos, en la preparación de jugos, café, vinos, aceites, en la alimentación animal y el tratamiento de agua, entre otras (Kashyap et al. 2001; Hoondal et al. 2002). La demanda de éstas enzimas esta creciendo rápidamente, así como los esfuerzos por mejorar su producción é implementar su uso en los diversos procesos industriales.

Se han desarrollado diversas estrategias para la obtención de enzimas con interés biotecnológico, con el fin de estudiar sus características bioquímicas y para su uso en diversos procesos industriales. Entre estas estrategias se encuentran:

- Producción de extractos utilizando fermentación sumergida (SmF) o fermentación en estado sólido (Diaz-Godinez et al. 2001; Martin et al. 2004).
- Sobre-expresión de enzimas introduciendo copias múltiples de los genes que las codifican en los receptores adecuados (Kitamoto et al. 2001a; Kitamoto et al. 2001b; Cardoso et al. 2007b).
- Utilización de tratamientos mutagénicos y selección de cepas sobre-productoras de enzimas de interés (Antier et al. 1993; Hadj-Taieb et al. 2002; de Vries 2003).
- Fusión de protoplástos para generar cepas hipersecretoras de enzimas con propósitos industriales (Bussink et al. 1992; El-Bondkly 2006; Solís et al. 2009).
- Sistemas de expresión heterólogos (Benen et al. 2000; Nevalainen et al. 2005; Yoon et al. 2010) entre otros.

Una de las técnicas más utilizadas para la producción de enzimas de interés biotecnológico es la expresión heteróloga de los genes que las codifican. *Escherichia coli* se ha utilizado en diversos casos como sistema de expresión heterólogo. Sin embargo, la falta de modificaciones post-traduccionales y la deficiente secreción de proteínas son el principal problema de este sistema (Yoon et al. 2010). No obstante lo anterior, existen ejemplos de expresión de genes que codifican pectinasas de hongos en donde la falta de glicosilación no tiene efectos sobre la actividad (Zhang et al. 2007; Zhao et al. 2007).

Los hongos filamentosos y levaduras tienen una alta capacidad de producción y secreción de proteínas, por lo que son ampliamente utilizados como sistemas de expresión heteróloga. Sin embargo, las modificaciones post-transcripcionales como la glicosilación, corte proteolítico y la formación de enlaces disulfuro, puede diferir entre los hongos filamentosos y levaduras y aún entre hongos como *Aspergillus* y *Trichoderma* (Benen et al. 2000; Nevalainen et al. 2005; Jeoh et al. 2008).

1.5. MODELOS DE OBTENCIÓN DE GENES QUE CODIFICAN ENZIMAS PECTINOLÍTICAS

Los hongos filamentosos poseen características que los hacen buenos modelos para aplicaciones industriales (de Vries 2003). Entre estas características destacan su capacidad de fermentación, la producción de grandes cantidades de enzimas extracelulares, la facilidad con la que pueden ser cultivados y los bajos costos de producción en grandes biorreactores.

1.5.1. *Colletotrichum lindemuthianum*

C. lindemuthianum, es un hongo ascomiceto causante de la antracnosis, una de las enfermedades más severas y comunes del frijol (*Phaseolus vulgaris)*. Es un patógeno hemibiotrófico intracelular que se caracteriza por presentar un proceso de infección en dos fases. La fase biotrófica o benigna comienza una vez que el hongo ha penetrado las células epidérmicas del huésped de un cultivar susceptible. Durante esta fase, *C. lindemuthianum* desarrolla una vesícula de infección y se extiende a través del tejido del huésped mediante hifas primarias sin penetrar las membranas celulares del huésped. Una vez que gran parte de la planta ha sido colonizada, se desarrolla la fase necrotrófica de la infección, durante la cual se desarrollan hifas necrotróficas por el tejido del huésped, y es durante esta fase que se observa la aparición de las manchas típicas de la antracnósis (O'Connell y Bailey 1988). La producción de un gran número de enzimas que degradan los componentes de la pared celular del huésped esta estrechamente relacionada con ésta última fase (Wijesundera et al. 1989; Dodds et al. 2009).

En el género *Colletotrichum* se ha reportado la producción extracelular de diferentes enzimas: cutinasa (Chen et al. 2006), glucoamilasa (Krause et al. 1991), lacasa (Guetsky et al. 2005), polifenol oxidasa (Singh 1968), celulasa, celobiohidrolasa, β-glucosidasa (Acosta-Rodríguez et al. 2005), xilanasa, poligalacturonasas (Albersheim y Anderson 1971) y β-xilosidasa (Piñon-Escobedo 2005) y varias pectin liasas (Templeton et al. 1994; Wei et al. 2002; Hernández-Silva et al. 2007; Ramos et al. 2010).

Al parecer, las enzimas pectinolíticas secretadas por el hongo tales como poligalacturonasas, pectin y pectato liasas, juegan un papel fundamental en el paso de la fase biotrófica a la necrotrófica y por lo tanto se cree que deben ser consideradas como factores de

patogénesis y/o de virulencia. Sin embargo, a pesar de que su papel como factores de virulencia se ha comprobado en algunos casos de bacterias fitopatógenas, su papel en la patogénesis aún esta en discusión (Wattad et al. 1997; Soriano 2006).

C. lindemuthianum es el primer patógeno de plantas en el cual se han reconocido razas fisiológicas de las que se tiene evidencia desde 1918 (Skipp et al. 1995). Estas razas fisiológicas son morfológicamente idénticas pero difieren en su capacidad para infectar a variedades distintas de un mismo huésped. Hernández-Silva et al. (2007) reportaron diferencias significativas entre una raza patogénica (1472) y una no patogénica (0) de *C. lindemuthianum* en cuanto a su crecimiento en diferentes fuentes de carbono y nitrógeno y en los niveles de actividad extracelular de pectin liasa en las distintas fuentes de carbono en donde es posible observar un retraso en los niveles máximos de actividad en la raza no patogénica de *C. lindemuthianum*.

La mayoría de la información sobre genes que codifican para enzimas pectinolíticas y su regulación proviene de hongos saprófitos como *A. niger* y *A. nidulans* (de Vries 2003). Los hongos fitopatógenos representan una buena fuente alternativa poco explorada de genes pectinolíticos (Tabla 1) y en general para la obtención de genes que codifican para enzimas que degradan la pared celular vegetal (Mendgen et al. 1996). En un análisis de perfiles hidrolíticos de hongos saprófitos y patógenos utilizando diversos sustratos, King et al., (2011) reportaron que los hongos patógenos son más activos que los no patógenos en seis de los ocho sustratos analizados, y que entre más relacionado esté el sustrato con el huésped natural de los hongos patógenos utilizados, mayor es la eficiencia de degradación de los mismos.

Por lo mencionado anteriormente, es importante estudiar microorganismos patógenos como una fuente alternativa de genes que codifican para enzimas pectinolíticas y en general para enzimas que degradan componentes de la pared celular con características catalíticas distintas a las enzimas utilizadas actualmente y posiblemente más específicas que pueden ser utilizadas en aplicaciones biotecnológicas. En *C. lindemuthianum* se ha detectado la producción extracelular de enzimas de diferentes sistemas de degradación de la pared celular vegetal por lo que es un buen candidato para la obtención de los genes que las codifican. Por otra parte, *C. lindemuthianum* es un buen modelo de estudio de los procesos de interacción

planta/patógeno, de la inducción de mecanismos de resistencia, así como de las bases bioquímicas y moleculares de la especificidad del patógeno y del huésped.

HIPÓTESIS GENERAL

El sistema pectinolítico del hongo *C. lindemuthianum* está compuesto por enzimas que degradan las cadenas principales de la pectina y de enzimas accesorias que actúan de manera coordinada para la degradación de la pectina de la pared celular de su huésped.

HIPÓTESIS ESPECÍFICA

El sistema pectinolítico de *C. lindemuthianum* contiene al menos una actividad de pectin liasa con potencial de ser utilizada en aplicaciones biotecnológicas.

OBJETIVO GENERAL

Caracterizar bioquímica y molecular el sistema pectinolítico de *C. lindemuthianum*

OBJETIVOS ESPECÍFICOS

- Realizar la caracterización estructural del gen (*Clpnl 2*) que codifica la pectin liasa 2 de *C. lindemuthianum*
- Establecer un sistema de expresión heterólogo del ADNc correspondiente a la pectin liasa 2 de *C. lindemuthianum*
- Realizar la caracterización bioquímica de la enzima recombinante CLPNL 2 purificada a partir de su expresión heteróloga.

CAPITULO I

ARTÍCULO I

Se reporta la clonación y caracterización del gen que codifica la pectin liasa 2 de *Colletotrichum lindemuthianum* (*Clpnl 2*), y un análisis filogenético/ estructural de los genes que codifican pectin liasas de microorganismos fitopatógenos y saprófitos/oportunistas. Artículo publicado en la revista internacional indizada BMC Microbiology.

BMC Microbiology

RESEARCH ARTICLE Open Access

Cloning and characterization of a pectin lyase gene from *Colletotrichum lindemuthianum* and comparative phylogenetic/structural analyses with genes from phytopathogenic and saprophytic/opportunistic microorganisms

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Abstract

Background: Microorganisms produce cell-wall-degrading enzymes as part of their strategies for plant invasion/nutrition. Among these, pectin lyases (PNLs) catalyze the depolymerization of esterified pectin by a β-elimination mechanism. PNLs are grouped together with pectate lyases (PL) in Family 1 of the polysaccharide lyases, as they share a conserved structure in a parallel β-helix. The best-characterized fungal pectin lyases are obtained from saprophytic/opportunistic fungi in the genera *Aspergillus* and *Penicillium* and from some pathogens such as *Colletotrichum gloeosporioides*.

The organism used in the present study, *Colletotrichum lindemuthianum*, is a phytopathogenic fungus that can be subdivided into different physiological races with different capacities to infect its host, *Phaseolus vulgaris*. These include the non-pathogenic and pathogenic strains known as races 0 and 1472, respectively.

Results: Here we report the isolation and sequence analysis of the *Clpnl2* gene, which encodes the pectin lyase 2 of *C. lindemuthianum*, and its expression in pathogenic and non-pathogenic races of *C. lindemuthianum* grown on different carbon sources. In addition, we performed a phylogenetic analysis of the deduced amino acid sequence of Clpnl2 based on reported sequences of PNLs from other sources and compared the three-dimensional structure of Clpnl2, as predicted by homology modeling, with those of other organisms. Both analyses revealed an early separation of bacterial pectin lyases from those found in fungi and oomycetes. Furthermore, two groups could be distinguished among the enzymes from fungi and oomycetes: one comprising enzymes from mostly saprophytic/opportunistic fungi and the other formed mainly by enzymes from pathogenic fungi and oomycetes. Clpnl2 was found in the latter group and was grouped together with the pectin lyase from *C. gloeosporioides*.

Conclusions: The *Clpnl2* gene of *C. lindemuthianum* shares the characteristic elements of genes coding for pectin lyases. A time-course analysis revealed significant differences between the two fungal races in terms of the expression of *Clpnl2* encoding for pectin lyase 2. According to the results, pectin lyases from bacteria and fungi separated early during evolution. Likewise, the enzymes from fungi and oomycetes diverged in accordance with their differing lifestyles. It is possible that the diversity and nature of the assimilatory carbon substrates processed by these organisms played a determinant role in this phenomenon.

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Background

Pectin is one of the major components of the primary cell wall of plants and is also found in dividing cells and in the areas of contact between cells that have a secondary cell wall, including xylem and the fibrous cells of woody tissue. Pectin comprises approximately 35% of the primary cell wall of dicots and non-graminaceous monocots. Although its content in secondary walls is greatly reduced, it is believed that pectin plays an important role in the structure and function of both primary and secondary cell walls. The functions of pectin in cell walls are diverse and include plant growth and development, morphogenesis, defense, cell adhesion, cell wall structure, cellular expansion, porosity, ion binding, hydration of seeds, leaf abscission and fruit development, among others [1,2]. In general, pectin is considered to be a group of polysaccharides that are rich in galacturonic acid (GalA) and present in the form of covalently linked structural domains: homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) [1,2]. The main enzymes involved in the degradation of the HG backbone of pectin are polygalacturonases (PGA, E.C. 3.2.1.15 and XPG, E.C. 3.2.1.67), pectate lyases (PL, E. C. 4.2.2.9 and 4.2.2.2) and pectin lyases (PNL, E.C. 4.2.2.10) [3].

Pectin lyases (PNLs) catalyze the degradation of pectin through β-elimination; they remove a proton and generate an unsaturated bond between the C-4 and C-5 carbons of the non-reducing end of pectin, which is a neutral form of pectate in which the uronic acid moiety of galacturonic residues has been methyl-esterified. The activity of PNLs is highly dependent on the distribution of the methyl esters over the homogalacturonan backbone. PNLs exhibit pH optima in the range of 6.0-8.5 and, unlike PLs, their activity is independent of Ca^{2+} ions; it is believed, however, that the residue Arg^{236} plays a role similar to that of Ca^{+2} [4,5]. Pectinase gene expression is regulated at the transcriptional level by the pH of the medium and by carbon sources, as it is induced by pectin and pectic components and repressed by glucose [6-8].

PNLs are grouped into Family 1 of the polysaccharide lyases [9] and into the pectate lyase superfamily that, in addition to pectin lyases and pectate lyases, also includes plant pollen/style proteins. The three-dimensional structures of five members of the pectate lyase superfamily have been determined. These include *Erwinia chrysanthemi* pectate lyase C (PELC) [10] and pectate lyase E (PELE) [11], *Bacillus subtilis* pectate lyase [12] and *Aspergillus niger* pectin lyase A (PLA) [13] and pectin lyase B (PLB) [14]. These enzymes fold into a parallel β-helix, which is a topology in which parallel β-strands are wound into a large right-handed coil [15]. Although PLs and PNLs exhibit a similar structural architecture and related catalysis mechanisms, they nonetheless diverge significantly in their carbohydrate binding strategy [4,13]. Currently, strategies are available for developing functional information from three-dimensional images of enzymes. The growing number of databases on the structure of pectinolytic enzymes has facilitated the analysis of minor structural differences that are responsible for the specific recognition of a unique oligosaccharide sequence in a heterogeneous mixture [4].

Most of the available information about fungal PNLs and their corresponding encoding genes has been obtained from saprophytic/opportunistic fungi such as *Aspergillus niger* [16-19], *A. orizae* [20,21], *A. fumigatus* [22], *Penicillium griseoroseum* [23], *P. occitanis* [24] and to a lesser extent from the phytopathogenic fungi *Glomerella cingulata* [25] and *C. gloeosporioides* [26].

The ascomycete *C. lindemuthianum* is an economically important phytopathogen, and along with its host *Phaseolus vulgaris*, it provides a convenient model to study the physiological and molecular bases of plant-pathogen interactions [27]. It is an intracellular hemibiotrophic pathogen with physiological races that invade the plant in an interaction consistent to the gene-for-gene model [28], and monogenic dominant resistance in common bean cultivars leads to the appearance of localized necrotic spots typical of the hypersensitive response (HR) [29]. After penetration of a host epidermal cell in a susceptible cultivar, the pathogenic races of *C. lindemuthianum* develop an infection vesicle and extend into adjacent cells by producing large primary hyphae, which invaginate without penetrating the host cell membrane and thus persist as a biotrophic interaction. Once a large area of the plant tissue has been colonized, necrotrophic hyphae develop [29], and this step closely correlates with the production of a number of host cell-wall-degrading enzymes that are characteristic of phytopathogenic fungi [30-32]. Up to know, race 0 is the only strain of *C. lindemuthianum* unable to infect *P. vulgaris*, which contrasts with 1472, one of the most virulent races isolated in México [33]. This difference makes the two races an excellent model to investigate the role of pectinolytic enzymes in virulence of *C. lindemuthianum*. Previous results from this laboratory revealed significant differences between pathogenic (1472) and non-pathogenic (0) races of *C. lindemuthianum* in terms of growth and production of extracellular PNL activity on different carbon and nitrogen sources in liquid culture. Accordingly, race 1472 grew faster in media containing glucose or polygalacturonic acid, and on 92%-esterified pectin, it produced levels of PNL activity that were approximately 2-fold higher than those produced by race 0. In contrast, cell walls isolated from *P. vulgaris* hypocotyls and, to a lesser degree, from

cellulose sustained the growth of both races but induced PNL only in the pathogenic race [34].

Here we report the isolation and sequence analysis of the *Clpnl2* gene, which encodes pectin lyase 2 of *C. lindemuthianum*, and its expression in pathogenic and non-pathogenic races of *C. lindemuthianum* in response to cultivation on different carbon sources. To determine the relationship among the three-dimensional structures of PNLs and the lifestyle of PNL-producing microorganisms, we performed a phylogenetic analysis using protein sequences and deduced amino acid sequences reported for PNLs. A comparative analysis of the three-dimensional structure of the Clpnl2 protein predicted by homology modeling, covering the main body of the protein and the carbohydrate binding site, and the three-dimensional structures of the PNLs used in the phylogenetic analysis was also performed.

Methods

Strain and culture conditions

C. lindemuthianum races 0 (non-pathogenic) and 1472 (pathogenic) were kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, México) and maintained on potato dextrose agar (PDA, Difco) at 20°C. For DNA extraction, mycelia from *C. lindemuthianum* race 1472 grown on potato dextrose (PD) for 9 days at 20°C with continuous shaking (150 rpm), was recovered by filtration through Whatman paper No. 1 and stored at -85°C. For induction, 1.6 mg (about 5 cm^2) of mycelia from races 0 and 1472 were inoculated in 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 250 ml-Erlenmeyer flasks containing 50 ml of modified Mathur's medium (10 mM $MgSO_4.7H_2O$, 20 mM KH_2PO_4, 36 mM L-glutamic acid, distilled water up to 1 L; final pH, 5.5) [35] supplemented with either 2.5% glucose, 92%-esterified pectin or cell walls from *P. vulgaris*. Flasks were shaken (150 rpm) at 20°C and after different periods of growth, mycelia was collected by filtration, washed with water and stored at -85°C until use.

Preparation of plant cell walls

Seedlings of *P. vulgaris* cv. Flor de Mayo were grown for 7 days, and cell walls were extracted and purified from hypocotyls as described elsewhere [36].

DNA and RNA isolation

Genomic DNA was isolated from *C. lindemuthianum* mycelia that had been grown for 9 days in PD medium according to standard protocols [37]. Total RNA was purified from mycelia using TRIzol reagent (Invitrogen). RNA samples were treated with DNAse I according to manufacturer's instructions (Invitrogen) to eliminate DNA. The quality and concentration of total RNA were verified using the RNA 6000 Nano LabChip kit (2100 Agilent Bioanalyzer).

Isolation of the homologous DNA *Clpnl2* probe from *C. lindemuthianum*

Genomic DNA from race 1472 was amplified by PCR using the upstream primer pnlD (5'-CAGTACGTCTG GGGTGGTGA-3') and downstream primer pnlR (5'-AAGTAGTTGTTGACGACGTGG-3', which are homologous to sequences between 595 and 614 nt and 891 and 911 nt, respectively, of exon 3 of the *Clpnl2* gene from *C. gloeosporioides* [GenBank: AAD43565]. The PCR incubation mixture was heated at 95°C for 5 min in a thermocycler (Eppendorf Master Cycler Gradient, Brinkmann, Westbury, NY), followed by denaturation for 1 min at 95°C, annealing for 2 min at 48°C and extension for 2 min at 72°C. PCR was then performed for 35 cycles, followed by a final extension for 10 min at 72°C. A PCR product of 383 bp corresponding to *pnl2* gene (*clpnl2* fragment) was ligated into the pCR 2.1 vector and introduced into *E. coli* TOP 10 strain from the TOPO TA Cloning kit (Invitrogen).

Genomic DNA library construction and screening

Partial *Sau*3AI digestion of genomic DNA from race 1472 was used to construct a genomic library in Lambda DASH II/*Bam*HI according to manufacturer's instructions (Stratagene). Screening was performed using 15×10^4 UFP with three rounds of hybridization filters and the homologous *Clpnl2* fragment, which was ^{32}P-radiolabeled using the Radprime DNA Labeling System Life Technologies Kit (Tech-Line).

Molecular cloning of the *Clpnl2* full-length cDNA and expression analyses

The cDNA was amplified by RT-PCR as specified by the manufacturer. SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to prepare cDNA from total RNA. PCR was performed using the upstream primer Pnl67 (5'-ATGAAGTCTACCATCTTCTCCG-3') and downstream primer Pnl1569 (5'-TTAGATCTTGC-GAAACCGGC-3') designed from the DNA *Clpnl2* genomic sequence of *C. lindemuthianum*. The PCR incubation mixture was heated at 94°C for 5 min in a thermocycler (Eppendorf Master Cycler Gradient, Brinkmann, Westbury, NY), followed by 30 cycles of denaturation for 20 sec at 94°C, annealing for 30 sec at 54°C, extension for 1.5 min at 72°C and then by a final extension for 7 min at 72°C. A PCR product of 1,140 bp obtained from total RNA of race 1472 induced with pectin for 4 h and corresponding to the *Clpnl2* gene, was ligated into the pCR 2.1 vector (Invitrogen) and three clones were selected and sequenced. The 5' end of cDNA was amplified by 5'RACE as specified by the manufacturer (5'RACE System for

Rapid Amplification of cDNA Ends, Invitrogen), with total RNA from race 1472 induced for 4 h with 92%-esterified pectin, using the specific reverse primers Pnl1249 (5'-GTA GTT GTT GAC GAC GTG GAC G-3') and Pnl975 (5'-CGA TGT GCT GGC GGC CG-3'). The amplification products were cloned and five clones were selected and sequenced. For expression analysis, total cDNA (1140 pb) was amplified with specific primers Pnl67 and Pnl1569 in the same conditions described above using total RNA of mycelia from both races induced with 92%-esterified pectin or cell walls from *P. vulgaris* for 2, 4, 6, 8, 10 and 12 h. For expression analysis, cDNA obtained from cells grown under different conditions was also amplified by PCR using oligonucleotides prepared from ribosomal 18S RNA as a control (5'- TTAGCATGGAATAATRRAATAGGA-3'and 5'-ATTGCAATGCYCTATCCCCA-3) [38]. The PCR incubation mixture was heated at 94°C for 3 min, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 56°C, extension for 1 min at 72°C and then a final extension for 10 min at 72°C. The cDNA of *Clpnl2* and the amplified RT-PCR products were analyzed in a Bioanalyzer using the system for quantification and molecular size Agilent DNA 7500 (2100 Agilent Bioanalyzer).

Southern blot hybridization

Genomic DNA of mycelia from race 1472 was digested with selected restriction endonucleases. Digestion products were size-fractionated on a 0.8% agarose gel, transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotec, England), hybridized and detected with a ^{32}P-radiolabeled *Clpnl2* probe. Hybridizations were carried out at 60°C in 2X SSC containing 0.5% blocking agent (Roche) and 0.1% SDS. After hybridization, the blot was washed at 60°C for 15 min with 2X SSC containing 1% SDS and then at 60°C for 15 min with 0.2X SSC containing 0.1% SDS.

Sequencing and DNA analysis

The sequences of both strands of DNA of race 1472 and cDNA of both races were determined by the dideoxy-chain termination method using the ABI Prism Dye Cycle Sequencing Ready Reaction Kit in an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences were analyzed using the DNAsis (Hitachi) and 4Peaks v 1.7.2 software (http://mekentosj.com). *In silico* analyses of putative transcription factor binding sites were performed using the AliBaba2.1 software [39] and the Transfac 7.0 database [40]; the regulatory sequences reported for genes of fungal lytic enzymes were also compared. The N-terminal secretion signal sequence was identified with the SignalP 3.0 web server [41]. The protein molecular mass, pI and *N*-glycosylation sites were calculated on an ExPASy Proteomics Server [42].

Phylogenetic analyses

Phylogenetic analyses were performed on the *Clpnl2* deduced amino acid sequence and the deduced amino acid sequences of 34 pectin lyases that were previously reported (Table 1). Protein sequences were aligned with Clustal × software [43] using default parameters. Prior to phylogenetic analyses, signal peptide sequences and N-terminal and C-terminal extensions were excluded. Phylogenetic analyses were performed under Bayesian, maximum parsimony and neighbor-joining criteria, using the programs MrBayes Vs. 3.1.2 [44], PAUP*v 4b10 [45] and Mega 4 [46]. We used the amino BLOSUM G2 evolution model with gamma correction for Bayesian analysis. In total, 10,000 trees were obtained based on the settings ngen = 1000 000 and sample freq = 100 for Bayesian criteria. Prior to estimating the support of the topologies that were found, we checked the convergence of overall chains (4) when the log likelihood values reached the stationary distribution. The first 2500 trees were 'burn-in' and discarded, and a 50% majority rule consensus tree of the remaining trees was generated. For maximum parsimony analyses, the most parsimonious trees were estimated using the heuristic search option (TBR branch swapping, saving only a single tree in each case) with random sequence addition (five random replicates). Support was evaluated by bootstrap analysis using the full heuristic search option with 1000 replicates. For the neighbor-joining method [47], a JTT matrix was used, and 1000 bootstrap replicates were performed. We used the *A. thaliana* pectate lyase [GenBank: CAB41092] as an outgroup for pectin lyase analyses.

Protein homology modeling

The tertiary structure of the deduced amino acid sequence of *Clpnl2* was predicted by homology modeling using the Swiss-Model Server [48] using Pel B from *A. niger* (PDB: 1qcxA) as template [14]. The prediction of three-dimensional structures of the deduced amino acid sequences used in the phylogenetic analysis was performed in a similar manner. The structural parameters and prediction quality of the modeled structures were evaluated using the program SPDBV v. 4.01 [49]. The energy minimization of the model was performed by GROMOS96 [50], which was provided by the SPDBV program. MMV 2010.2.0.0 (Molegro ApS) and SPDBV v. 4.01 were used for visualization of molecular structures.

Multiple comparisons of protein structures

The comparison of protein structures was performed using the Voronoi contact method [51] with the ProCKSI-Server [52]. Calculations were performed using default parameters, and the resultant similarity matrixes (Voronoi-contacts) were standardized and used as the input for

Table 1 Nucleotide and protein sequences of reported pectin lyases used for phylogenetic analyses

Microorganism	Access number
Aspergillus niger	GenBank: CAD34589, GenBank: AAW03313, GenBank: CAA39305, GenBank: CAA01023, GenBank: ACE00421, GenBank: AAA32701
Aspergillus nidulans	GenBank: ABF50854
Aspergillus oryzae	GenBank: BAB82468, GenBank: BAB82467
Aspergillus fumigatus	Swiss-Prot: BOYCL3, Swiss-Prot: Q4WV10, GenBank: EAL91586, Swiss-Prot: Q4W156
Aspergillus terreus	GenBank: EAU31855, GenBank: EAU37973
Aspergillus clavatus	GenBank: EAW12911
Emericella nidulans	Swiss-Prot: Q5BA61
Colletotrichum gloeosporioides	GenBank: AAA21817, GenBank: AAD43565, GenBank: AAF22244
Penicillium occitanis	GenBank: ABH03046
Penicillium griseoroseum	GenBank: AF502280
Neosartorya fischeri	GenBank: EAW17753, Swiss-Prot: A1CYC2
Pyrenophora tritici-repentis	GenBank: XP_001934252, GenBank: XP_001930850
Ustilago maydis	GenBank: EAK86184
Verticillium albo-atrum	GenBank: XP_003001443
Phytophthora infestans	GenBank: XP_002909420, GenBank: XP_002903922
Bacillus subtilis	GenBank: BAA12119, GenBank: AAB84422
Pectobacterium atrosepticum	GenBank: CAG74408
Pectobacterium carotovorum	GenBank: AAA24856

clustering of the protein set using the un-weighted pair group method for the arithmetic mean (UPGMA) [53].

Results and discussion

Isolation and sequence analysis of the *Clpnl2* gene

Nine positive clones were isolated from the screening of a *C. lindemuthianum* genomic library using the ^{32}P-radiolabeled fragment of *Clpnl2*. Southern blot analysis of the clones allowed the identification of a 4.0-kb fragment that hybridized with the PCR probe. The 4.0-kb fragment was subcloned, and 2,159 bp containing the *Clpnl2* gene was sequenced [GenBank: JN034038]. The full-length *Clpnl2* cDNA of races 1472 [GenBank: JN034039] and 0 [GenBank: JN653459] obtained by RT-PCR from total RNA of mycelium induced for 4 h with 92%-esterified pectin, was sequenced using specific primers designed from the *Clpnl2* gene sequence.

The analysis of the cDNA sequences showed no differences between the two races. The coding region of the *Clpnl2* gene consisted of 1428 bp interrupted by four introns ranging in size from 60 to 87 bp (Figure 1). According to the 5'RACE analysis, a putative transcription starting point was localized [19], and the context of the start codon ATG matched with the Kozak sequence for filamentous fungi [54]. Two possible regulatory sequences were identified in the 5' untranslated region of *Clpnl2*: a putative regulatory sequence for binding to RAP1, which is a transcriptional factor that participates in the activation of transcription and the silencing of genes in yeast cells, located at position +54 [55] and a possible binding sequence for the transcription factor AbaA at position +69. AbaA binding sites have been observed in several genes that participate in the control of cell development in organisms such as *A. nidulans* and the dimorphic fungus *P. marneffei*, where AbaA has been related to morphogenesis and dimorphism, respectively [56,57]. These putative regulatory elements were localized downstream the transcription site which is an uncommon finding. Multiple binding sites to AbaA have been reported in *cis* regulatory regions and some downstream the transcription starting site in *A. nidulans* genes. No attempts were made in this study to determine the function of these elements. Due to the size of the promoter region of *Clpnl2*, it was not possible to locate more elements commonly found in genes encoding for pectinolytic enzymes. The 5' and 3' untranslated regions (5'UTR and 3'UTR) were 129 and 563 bp, respectively. Two consensus sequences (AATAAA and TTTCACTGC) found in the terminal regions of eukaryotic mRNAs [58], and two of the three consensus sequences for yeast 3'-terminal regions (TAGT and YIT) [59] were detected in the *Clpnl2* 3'UTR.

The *Clpnl2* cDNA contains an ORF of 1140 nucleotides that encodes a putative protein of 379 aa with a N-terminal secretion signal sequence of 19 amino acids, according to the SignalP 3.0 web server [41]. A protein of molecular mass 37.4 kDa and a pI of 9.1 was calculated, and one potential *N*-glycosylation site was located at position 110 (ExPASy Proteomics Server) [42]. These

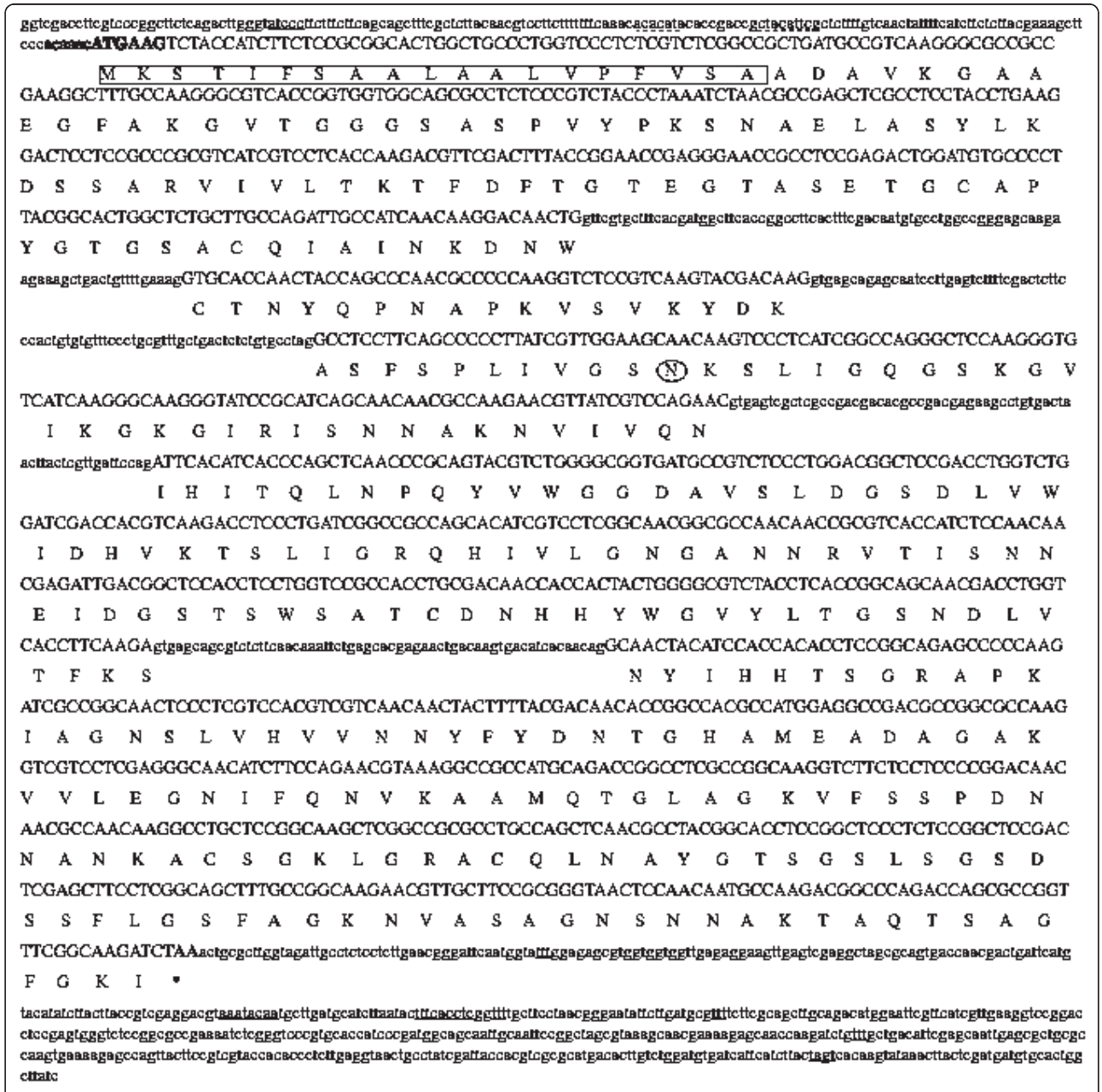

Figure 1 Nucleotide and deduced amino acid sequence of the *Clpnl2* gene. Intron and exon sequences are in lowercase and uppercase, respectively. The signal peptide sequence is boxed. The possible binding sequences of RAP1 and AbaA are underlined with a dotted line. The putative transcription start point is underlined, and the putative Kozak sequence is shaded. The sequences of the 3'-terminal region are underlined. An asterisk (*) marks the translation stop codon. The potential N-glycosylation site is circled. This sequence has been deposited in the GenBank nucleotide sequence database under accession number JN034038.

results are consistent with those reported for the amino acid sequences of *Pnl2* and *pnlA* of *C. gloeosporioides* [25,26]. Despite *N*-glycosylation is common in pectinolytic enzymes and has been reported in several fungal pectin lyases al similar positions, little is known about the function of this posttranslational modification. Although it is believed that it affect enzyme stability and activity [60,61].

Southern blot analysis

The genomic organization of the *Clpnl2* gene was investigated by Southern blot analysis. Total DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hind* III, *Xho*I, *Eco*RI/*Bam*HI and *Hind* III/*Xho*I. The digested DNA was fractionated on a 0.8% agarose gel and hybridized to the ^{32}P-radiolabeled *Clpnl2* probe. As depicted in Figure 2, commonly a single hybridization

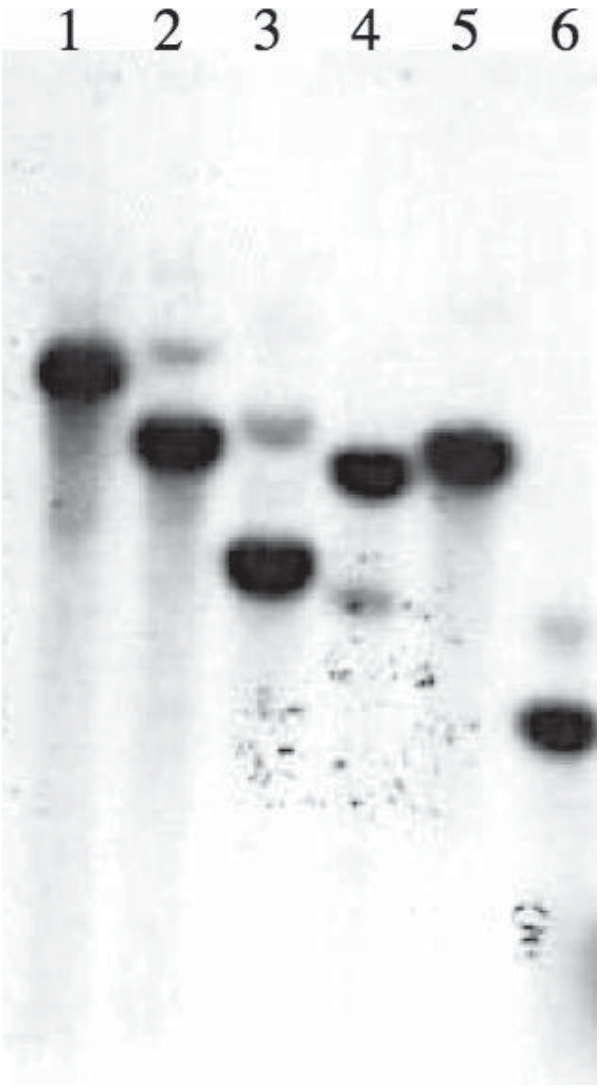

Figure 2 Southern blot analysis of total DNA from *C. lindemuthianum*. Total DNA was digested with BamHI (1), EcoRI (2), HindIII (3), XhoI (4), EcoRI/BamHI (5), or HindIII/XhoI (6), analyzed on a 0.8% agarose gel, transferred to nylon membrane and hybridized with a ^{32}P-radiolabeled *Clpnl2* fragment.

product was detected. In addition, a very faint signal probably resulting from hybridization with another gene of low similarity was observed. These results suggest that the *C. lindemuthianum* genome contains a single copy of the *Clpnl2* gene, as does *C. gloeosporioides* [26].

Protein homology modeling

The tertiary structure of *Clpnl2* predicted by homology modeling coincided with the typical topology of the parallel β-helix of PNLs (Figure 3). After energy minimization, the energy value was -17418.428 kJ/mol, and the quality of the model generated was assessed by plating dihedrals Φ and Ψ onto Ramachandran plots (SPDBV v. 4.01) [49]. The results are in agreement with the requirements for preferred and allowed regions, except for 3 non-glycine residues (0.8%).

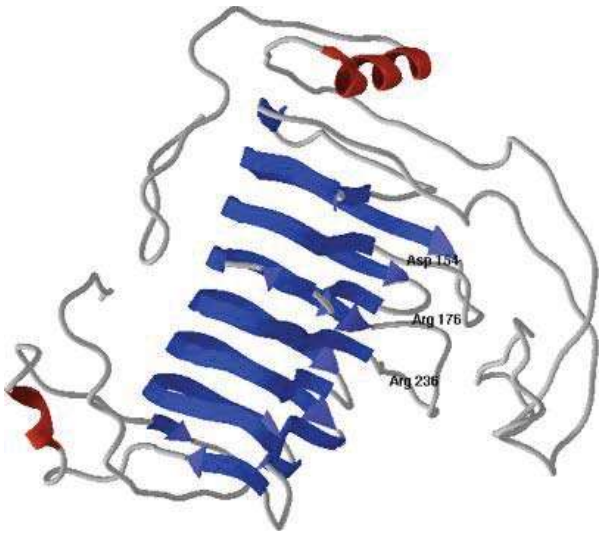

Figure 3 Three-dimensional structure of *Clpnl2* from *C. lindemuthianum* showing highly conserved residues involved in catalysis.

Phylogenetic analyses

To elucidate the relationship of *Clpnl2* from *C. lindemuthianum* with bacterial, oomycete and fungal pectin lyases, sequences reported in databases were analyzed. Protein or deduced amino acid PNL sequences from 14 fungal species including: basidiomycetes, ascomycetes and one oomycete species, three bacterial species, and a pectate lyase sequence from *A. thaliana* as an external group, were used to generate phylogenetic trees. Clustal alignment used for phylogenetic analysis (Figure 4) allowed to determine the location of amino acids expected to have a catalytic role in the PNLs [4,13]. Asp^{154} and Arg^{176} (numbered from *A. niger* PELA) are conserved in fungi and oomycetes, although Arg^{176} could not be located in *P. griseoroseum* [GenBank: AF502280], and Arg ^{236} is conserved in all analyzed sequences. Additionally, several conserved domains among the sequences of fungi and oomycetes were observed, and some of these were shared with bacterial amino acid sequences.

The trees inferred by the maximum parsimony (MP) and neighbor-joining (NJ) methods showed less resolution than those built by Bayesian analysis, as they had a number of unresolved branches. The general topology obtained is represented by the Bayesian 50% majority rule consensus tree, in which the Bayesian posterior probabilities, MP and NJ bootstrap support are indicated on the branches (Figure 5).

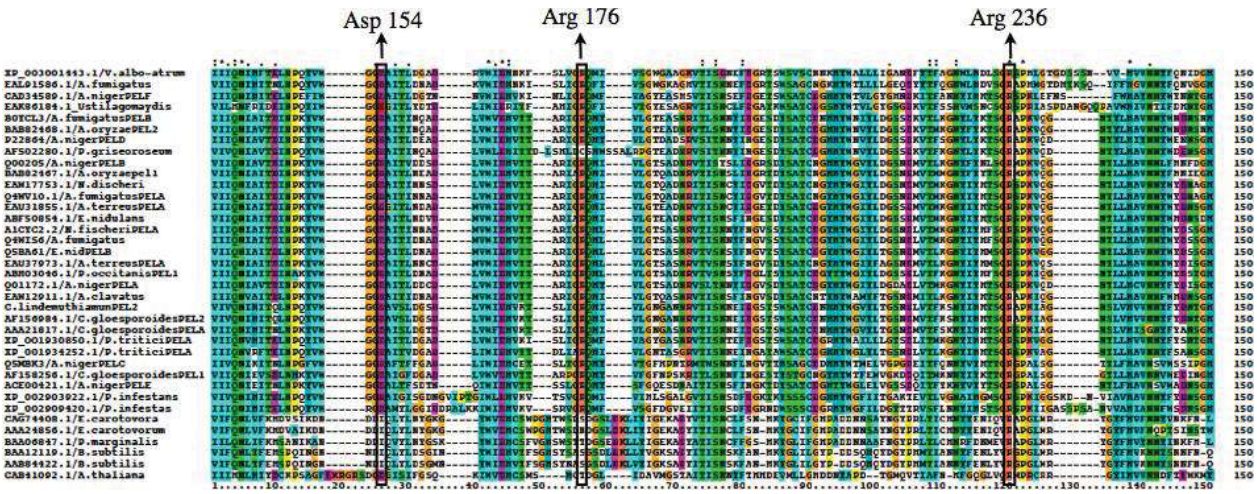

Figure 4 Alignment of the amino acid sequences of pectin lyases of bacteria, fungi and oomycetes used in phylogenetic analyses. Identical residues are marked with an asterisk (*). Dashes represent gaps introduced to preserve alignment. Conserved catalytic residues are indicated in boxes.

Bayesian analysis allowed the separation of pectin lyases into two groups: one representing bacteria with 100% posterior probability and 100% bootstrap support for MP and NJ analysis, and the other one representing fungi and oomycetes with 100% posterior probability and 98% bootstrap support for NJ. In the group formed by bacteria, sequences from *Pectobacterium atrosepticum*, *P. carotovorum* and *Bacillus subtilis* cluster together with 100% posterior probability. This early separation between amino acid sequences of bacteria and those of oomycetes and fungi can be explained in terms of the evolution of lytic enzymes in these microorganisms for different purposes. Bacteria and some anaerobic fungi produce multi-enzymatic complexes called cellulosomes, which are anchored to the cell surface, allow the microorganisms to bind to lignocellulose substrates and increase the breakdown efficiency of cellulose, hemicellulose and pectin [62,63]. In contrast, in the majority of fungi and oomycetes, cellulases, pectinases and hemicellulases are not integrated in cellulosome complexes, and the pectin degradation is regulated by a multifunctional control system in which the enzymes act in a synergistic manner and are induced by monosaccharides or small oligosaccharides that are generated as products of the same enzymatic reactions [64,65].

The inferred tree also showed that the analyzed sequences of saprophytic/opportunistic fungi are clustered into a monophyletic group with 98% posterior probability and 75% and 70% bootstrap support for MP and NJ analyses, respectively. However, phytopathogenic fungi and oomycetes were not clustered together. This may be the result of a reduced representation of sequences in the analysis arising from the few PNL sequences reported for members of these groups.

C. lindemuthianum is found clustered with the amino acid sequences of PnlA and Pnl2 of the fungal pathogen *C. gloeosporioides* with 100% posterior probability for Bayesian analysis as well as 96% and 99% bootstrap support for MP and NJ analysis, respectively.

Pectin and pectate lyases fold into a parallel β-helix, in which a high structural conservation occurs in regions distant from the active site and particularly in those that contribute to the parallel β-helix architecture. The binding cleft and surroundings constitute the most divergent part of the molecule, which allows variation in substrate specificity [13,15]. On this background, the results of the phylogenetic analyses and the fact that the classification of the pectin lyases is based both on amino acid sequence similarities as well as their structural features [9], we believe that a structural comparison would help to strengthen the phylogenetic analysis and to establish a relationship between the genes encoding PNLs with their three-dimensional structures involved in carbohydrate binding.

Multiple comparisons of protein structures

Once the tertiary structure of *Clpnl2* was predicted, the tertiary structures corresponding to the amino acid sequences used in phylogenetic analyses and covering the central body of the enzyme including the carbohydrate-binding site of these proteins were predicted and evaluated. The multiple comparisons of protein structures led to the formation of two clusters: one composed of the structures corresponding to the amino acid sequences of bacteria and another that was composed of fungal and oomycete structures (Figure 6). Furthermore, in agreement with the phylogenetic analyses, it was possible to

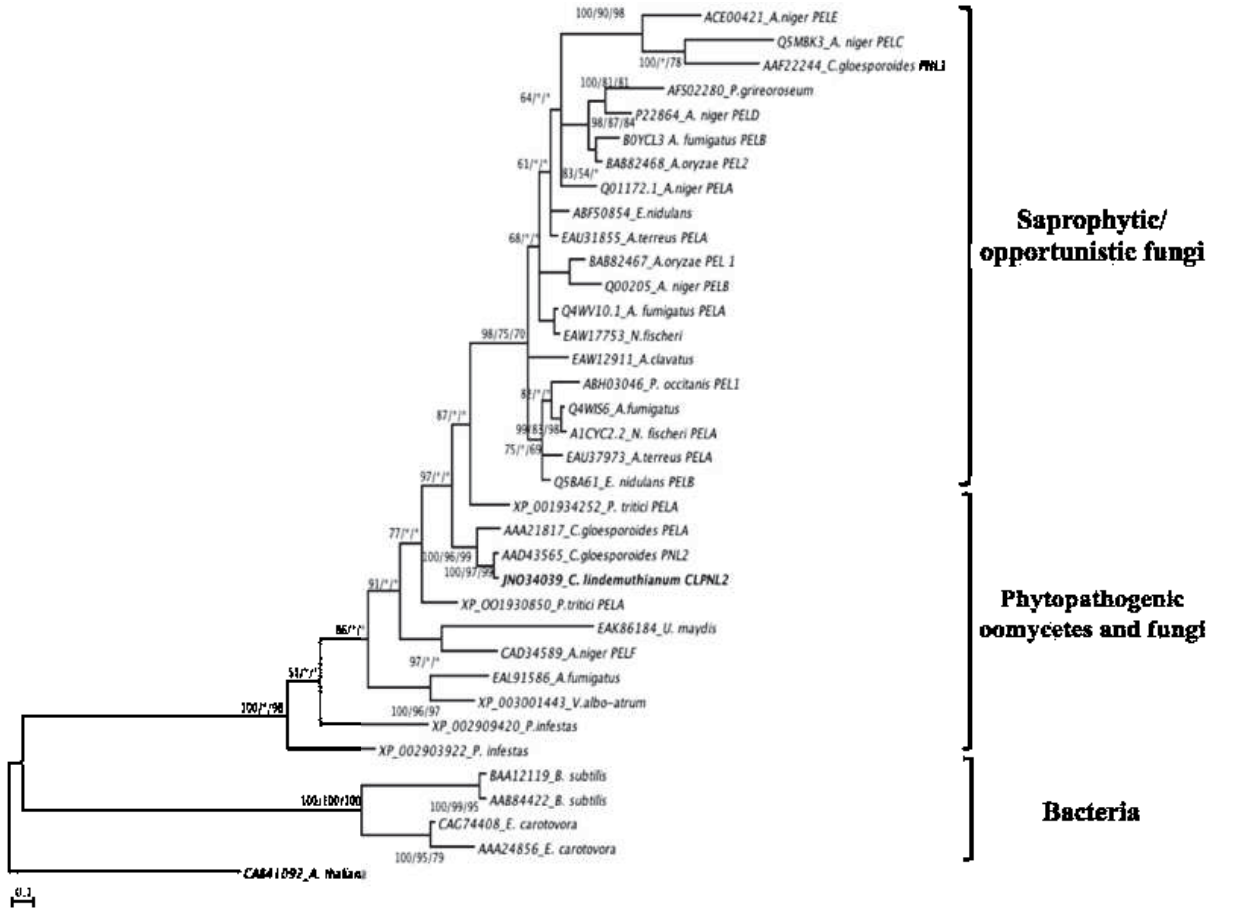

Figure 5 Phylogenetic tree of pectin lyases. The phylogeny shown is the Bayesian topology and branch lengths inferred using MrBayes vs. 3.1.2, with the Blosum 62 + G model. Numbers above the diagonal indicate posterior probability values from Bayesian analysis. Numbers below the diagonal indicate bootstrap percentage values from a bootstrap analysis inferred using the same alignment with PAUP*4.0 and Neighbor-J, respectively. *A. thaliana* pectate lyase was used as an outgroup. The asterisks represent branches that were not supported in 50% or more of the bootstraps. The scale bar represents the number of substitutions per site. The phylogenetic tree was edited using Dendroscope software [77].

distinguish the cluster formed mainly by sequences of fungi and oomycete pathogens, including Clpnl2, from the cluster formed by saprophytic/opportunistic fungi. Nevertheless, this analysis clustered the fungal sequences in two clearly defined groups: fungi and oomycete pathogens and saprophytic/opportunistic fungi. These results strongly support the notion that there is a close relationship between the tertiary structure of PNLs and the lifestyle of the microorganisms. The training of these groups was also observed for the elimination method FAST [66] and the hybrid heuristic URMS/RMS approach [67] using the ProCKSI-Server [52] (data not shown). Comparative modeling techniques and multiple comparisons of three-dimensional structures have been utilized for different purposes (e.g., searching for putative biological functions, drug design, protein-protein interaction studies). However, to our knowledge, this is the first study that uses a comparative analysis of protein structure in combination with a phylogenetic analysis to explore the evolution of lifestyle. We believe that a structural analysis can be an important tool for studying the evolution of microorganisms and their enzymes, since structural differences may reflect other important properties such as substrate specificity and others that can not be inferred from the analysis of amino acid sequences only.

Expression analysis of *Clpnl2*

Analysis of the *Clpnl2* transcript in cells grown with glucose as the carbon source showed similar low basal levels of expression in the 0 and 1472 races (Figure 7C). When grown on cell walls, levels of *Clpnl2* transcript in the pathogenic race, 1472, increased quickly after 2 h, reached a peak after 6 h, started to decrease and then again increased, giving a maximal value after 12 h of incubation (Figure 7B and 7C). Race 0 exhibited different expression kinetics: the amount of transcript peaked

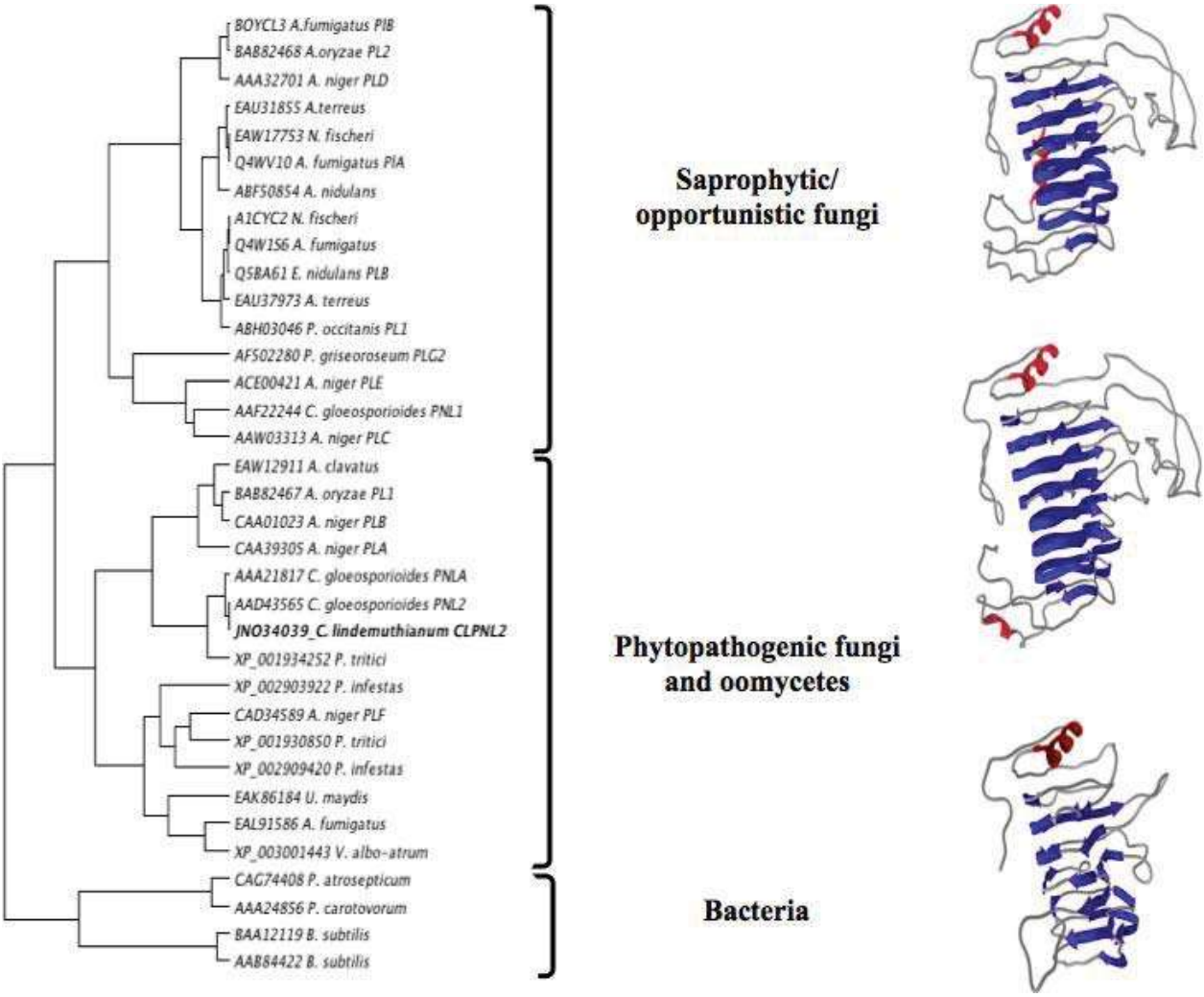

Figure 6 Clustering the three-dimensional structures of pectin lyases. The pectin lyase dataset was clustered by the un-weighted pair group method using the arithmetic mean (UPGMA) [53] with a similarity matrix obtained by the Voronoi contact method [51] using the ProCKSI-Server [52]. The tree image was generated using Dendroscope software [77]. A. Three-dimensional structure of PEL B from *A. niger* [PDB:1QCX]. B-C. Three-dimensional structures of the PNLs from *C. lindemuthianum* [GenBank: JN034039] and *P. carotovorum* [GenBank: AAA24856] respectively, predicted by homology modeling using the Swiss-Model Server [48].

after 6 h and then fell to undetectable levels after 10 h (Figure 7A and 7C). At all time points between 2 and 8 h, expression levels were lower than those observed in the pathogenic race. The transcript was expressed again after 12 h but at levels that reached only 23% of those observed in the pathogenic race.

The differences between the two races were much more noticeable when 92% esterified pectin was used as the sole carbon source. Transcript expression in the pathogenic race started to increase rapidly, reached the highest levels after 4-6 h and then started to decline, giving a still significant increase at the end of the experimental period (Figure 7B and 7C). The maximum transcript levels on this substrate were clearly higher than those observed on glucose. In contrast, the levels of the *Clpnl2* transcript in the non-pathogenic race remained undetectable after 8 h of incubation. Late expression occurred after 10-12 h of cultivation, reaching values comparable to those observed after 4 h of culture on cell walls (Figure 7A and 7C). These results agree with the differences found by Hernández et al. [34], who analyzed the extracellular activity of pectin lyase in both races of *C. lindemuthianum* under the same conditions employed in this study. When both races were grown with glucose, extracellular PNL activity was barely detected after 8 (race 1472) and 10 (race 0) days of incubation, as observed in this study. Plant cell walls from *P. vulgaris* induced a similarly low PNL activity in the

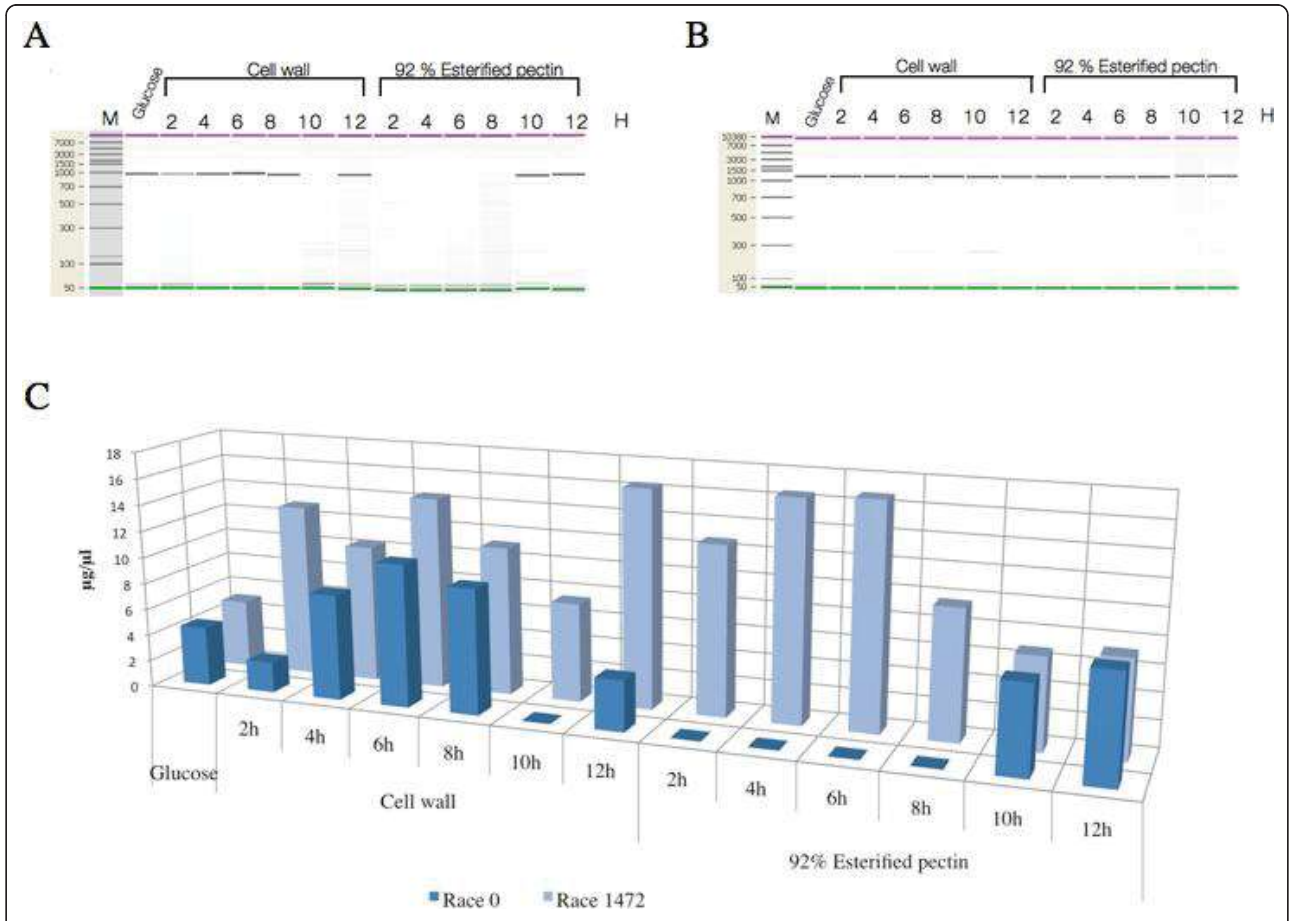

Figure 7 Analysis of the relative gene expression of *Clpnl2* in races 0 and 1472 of *C. lindemuthianum*. A-B. Gel-like images showing the expression of *Clpnl2* in races 0 and 1472, respectively, on the different carbon sources tested. C. Semi-quantitative data for the expression of *Clpnl2* in both races on the carbon sources. Total RNA was isolated from induced mycelia and amplified by RT-PCR with specific primers to yield the cDNA of *Clpnl2*. Amplification products were checked and quantified on a Bioanalyzer (2100 Agilent Bioanalyzer). The data were normalized using 18S rRNA as a control, and the results are expressed in µg/µl of amplified product.

two isolates after 7-8 days of incubation. When pectin esterified to 92% was used as the carbon source, the activity in the pathogenic race nearly doubled compared with the activity in the non-pathogenic race. Early transcription of genes encoding lytic enzymes and late detection of the corresponding activities is a well documented phenomenon in different fungi [8,30,65,68]. Apart from the presence of a regulatory system controlling gene expression, the production of active pectinase and probably other lyticases can be modulated by other mechanisms such as postranslational modification and protein transport [69]. These alternatives may help to explain the differences observed in this study.

The pectin lyase of the pathogenic race of *C. lindemuthianum* is able to degrade highly esterified pectin (92%), unlike that of the non-pathogenic race. Apparently, the differences between the pathogenic and non-pathogenic races of *C. lindemuthianum* occur as much at the expression level as at the level of enzymatic activity, and it is clear that the non-pathogenic and pathogenic races of *C. lindemuthianum* respond of different form to the carbon sources (except for glucose, where the mRNA of *Clpnl2* and the active enzyme is synthesized at basal levels).

It has been proposed that the basal level of enzymatic activity breaks down the substrate, generating degradation products that further induce enzymatic activity [64]. A similar behavior has been observed in our laboratory for other enzymes that degrade cell walls, such as cellulases and the xylanase and β-xylosidase of *C. lindemuthianum* (unpublished data).

Several studies have reported that the pectinolytic enzymes play an important role in pathogenesis [70,71]. These are the first enzymes that act during the infection of the plant, causing extensive degradation of the cell wall and the main symptoms of the disease [72]. However, in addition to enzyme production, the sequence in which the enzymes are produced, the speed of synthesis,

concentration and diffusion of enzyme are also fundamental aspects of the pathogenesis process [72]. 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 (0) and pathogenic (1472) races of *C. lindemuthianum* are related to the speed of activation of the lytic enzyme genes during the interaction with the host.

The number of pectin lyase sequences corresponding to different species of saprophytic/opportunistic fungi used in our analysis surpassed those of pathogenic oomycetes and fungi. This may be because more species of saprophytic/opportunistic have been studied and their degradation systems are better known. Alternatively, the enzymatic diversity may be the evolutionary effect of the heterogeneity of substrates that were encountered during interactions with an extended variety of hosts. For pectate lyases, it has been proposed that differences in the degree of pectin methylation can explain the existence of isozymes [4].

Pathogenic fungi and those who have close relationships with their host have developmental strategies that allow them to avoid the plant defenses and penetrate cell walls through the use of lytic enzymes. Plants also rely on strategies that allow them to detect and to defend against the attack of pathogens by producing inhibitors of these enzymes [70,73,74]. It is therefore possible that the evolution of unique enzymes was induced in pathogenic fungi and that a greater variability of these enzymes was induced in those fungi with a saprophytic lifestyle, which would explain the presence of amino acid sequences and tertiary structures corresponding to enzymes of saprophytic/opportunistic fungi located between the sequences of pathogenic fungi and oomycetes in the phylogenetic analysis and comparison of structures.

There is evidence that supports a relationship between lytic enzyme production and the lifestyles of fungi and oomycetes. For instance, the genome of the oomycete *Hyaloperonospora arabidopsidis* has lost several of its hydrolytic enzymes compared with *Phytophthora* sp., which is likely its ancestor [75,76]. According to an analysis of the hydrolytic profiles of saprophytic/opportunistic and pathogenic fungi using diverse substrates, the species of phytopathogenic fungi are more active than the non-pathogenic fungi on six of eight tested substrates [74]. It has also been observed that pathogenic fungi of monocotyledonous plants are better adapted to degrade the cell walls of monocotyledonous plants, and pathogens of dicotyledonous plants are better able to degrade the cell walls of dicotyledonous plants, reflecting the host preference [74].

Conclusions

The *Clpnl2* gene, which was cloned from a genomic library of *C. lindemuthianum*, is a unique copy and contains the characteristic elements of a pectin lyase of Family 1 of polysaccharide lyases.

Phylogenetic analyses showed an early separation between the enzymes of bacteria and those of fungi and oomycetes as well as a tendency of the amino acid sequences of fungi and oomycetes to cluster together according to their lifestyle. These results were confirmed by multiple comparison analysis of structures. According to these results, we believe that is possible that the diversity and nature of the substrates processed by these microorganisms play a determining role in the evolution of their lifestyle. In addition, our results showed that both races of *C. lindemuthianum* express the *Clpnl2* gene, although some differences are observed in the timing and level of expression: the pathogenic race responds faster and at higher levels than the non-pathogenic race. This suggests that there are at least two levels of determination of the lifestyle of the microorganisms: one related to the evolution of the enzymes and one concerning the regulation of the expression of the enzymes. In our model, one race of *C. lindemuthianum* behaves as a hemibiotrophic pathogen and, according to its inability to infect bean, the other race behaves as a saprophyte. Although this study included the analysis of pectin lyase 2 only, we have observed this behavior with other enzymes of the complex involved in the degradation of the cell wall suggesting that it may be a general phenomenon. The differences at this level can be part of the general response of the fungi to host components. However future studies comparing the enzymatic complex of degradation of more fungi species with different lifestyles are needed to confirm this hypothesis.

Finally, we consider this type of information to be of great importance for the study of the biotechnological potential of these enzymes, as the efficiency of the enzymes could depend on the complexity of the vegetal material to be processed and the lifestyle of organism that is the source of enzymes and/or genes.

Acknowledgements

The authors thank the financial support provided by the FOMIX CONACYT-Gobierno del Estado de Michoacán (project 2009-05 Clave 116208 to HCC) and CONACYT (scholarship granted to ALM and UCS). We thank Gerardo Vázquez Marrufo by its comments to manuscript.

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Authors' contributions

ALM, MGZP and UCS carried out the experiments. ALM and NCC carried out data analysis. ALM, MGZP and HCC conceived and designed the study, guided data analysis, interpretation, and discussion, and wrote the

manuscript with comments from ELR and RLG. ELR participate in biochemical interpretation of data and RLG participate in genomic library construction. All authors read and approved the final manuscript.

Received: 3 June 2011 Accepted: 9 December 2011
Published: 9 December 2011

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doi:10.1186/1471-2180-11-260
Cite this article as: Lara-Márquez *et al.*: Cloning and characterization of a pectin lyase gene from *Colletotrichum lindemuthianum* and comparative phylogenetic/structural analyses with genes from phytopathogenic and saprophytic/opportunistic microorganisms. *BMC Microbiology* 2011 **11**:260.

CAPITULO II

ARTÍCULO II

En este artículo en preparación se reporta la expresión heteróloga del gen que codifica la pectin liasa 2 de *C. lindemuthianum* en la levadura metiltrófica *Pichia pastoris*, para someter en la revista internacional indizada Biotechnol Lett.

Expresión heteróloga de la pectin liasa 2 de *C. lindemuthianum* en *Pichia pastoris*

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Resumen

El (ADNc) del gen que codifica para la pectin liasa 2 de *Colletotrichum lindemuthianum* (*Clpnl2*) se expresó en *Pichia pastoris* bajo el control del promotor de la alcohol oxidasa (AOX). La pectin liasa (PNL) recombinante se secreta de manera eficiente en el medio a partir de las 48 h de inducción con 0.5% de metanol (22.9% de la proteína total), con un peso molecular estimado de 44.4 kDa, similar al calculado por SDS-PAGE para la proteína nativa. La actividad total de PNL recombinante detectada en el extracto inducido durante 48 h fue de 5090.91 nmol de UUE/min, 1.5 veces más que la actividad total detectada en el extracto crudo de *C. lindemuthianum* inducido con pectina esterificada al 92% (3369.6 nmol de EU/min).

Los extractos crudos de la cepa de *P. pastoris* X-33/*Clpnl2*-2 y de *C. lindemuthianum* se fraccionaron con sulfato de amonio. La actividad específica de la fracción 50-60% del medio extracelular de *P. pastoris* X-33/*Clpnl2*-2 se incrementó seis veces (28.7 nmol de EU/min/mg de proteína). En el caso de los extractos de *C. lindemuthianum* se detectaron dos posibles picos de actividad de PNL, sin embargo, en el zimograma del extracto de *C. lindemuthianum* se observa una sola banda de actividad. La fracción 60-80% del extracto de *C. lindemuthianum* se separó en una columna de DEAE celulosa donde se observó un pico de actividad enzimática específica que eluye con NaCl 0.1–0.4 M.

Palabras clave: Pectin liasa, Expresión heteróloga, *Pichia pastoris*, *C. lindemuthianum*

Introducción

La pectina es un polisacárido rico en ácido galacturónico (GalA) que se presenta en forma de dominios estructurales unidos covalentemente conocidos como homogalacturonanos (HG), xilogalacturonanos (XGA), ramnogalacturonanos I (RG-I) y ramnogalacturonanos II (RG-II) (Willats et al. 2001; Mohnen 2008). La pectina es el mayor constituyente de la lámina media, se encuentra en células en división activa y en áreas de contacto entre células con pared celular secundaria (Mohnen 2008). En frutas y vegetales, la pectina puede representar hasta el 30% de la biomasa vegetal (Hoondal et al. 2002). Su degradación requiere de la acción combinada de varias enzimas entre las cuales las poligalacturonasas (PG), pectato liasas (PL; E:C: 4.2.2.9 y 4.2.2.2) y pectin liasas (PNL; E:C: 4.2.2.10) son consideradas como las más importantes en la degradación de este polisacárido (de Vries and Visser 2001; Benen and Visser 2003).

Las pectin liasas catalizan la degradación de la pectina esterificada por el mecanismo de β-eliminación, removiendo un protón y generando un enlace insaturado entre el carbono C-4 y C-5 del extremo no reducido de la pectina (Herron et al. 2000; Jayani et al. 2005). Estas enzimas son las únicas capaces de cortar los enlaces glicosídicos internos de la pectina altamente metilada, como es el caso de la pectina de la fruta, sin la acción de otras enzimas (Alaña et al. 1989; Semenova et al. 2006). Por estas razones, las pectin liasas tienen diversas aplicaciones biotecnológicas, principalmente en la industria de los alimentos, en la preparación de jugos, vinos, café, aceites, en la alimentación animal y el tratamiento de agua, entre otras (Kashyap et al. 2001; Hoondal et al. 2002).

La principal estrategia que se ha utilizado para degradar y aprovechar pectina en procesos biotecnológicos es la utilización de preparaciones enzimáticas (Kashyap et al. 2001; Semenova et al. 2006). Sin embargo, el uso de estas preparaciones se ha cuestionado principalmente por la presencia de actividades minoritarias indeseables y su efecto en el producto final (Mantovani et al. 2005). Existe poca información sobre las características bioquímicas de las enzimas pectínolíticas, un conocimiento fundamental para su uso en la industria. La expresión heteróloga se ha utilizado para la producción de enzimas con el fin de estudiar sus características bioquímicas y para su producción con fines biotecnológicos, con buenos resultados en muchos de los casos (He et al. 2009; Wang et al. 2009; Huy et al 2011).

El ascomicete *C. lindemuthianum* es un hongo patógeno hemibiotrófico intracelular del frijol. Durante el proceso de infección, este hongo secreta una batería de enzimas que degradan los

componentes de la pared celular (Templeton et al. 1994; Acosta-Rodríguez et al. 2005) y entre éstas al menos una pectin liasa (Hernández-Silva et al. 2007; Lara-Márquez et al. 2011). Previamente reportamos el aislamiento y análisis de la secuencia del ADNc de *Clpnl2* que codifica para la pectin liasa 2 de *C. lindemuthianum* y su expresión en una raza patógena y una no-patógena del hongo crecidas en distintas fuentes de carbono. El ADNc de *Clpnl2* contiene un ORF de 1134 nucleótidos, codifica para una proteína putativa de 379 aa con una secuencia N-terminal de secreción de 19 aa, un peso molecular de 37.4 kDa, un pI de 9.1 y un sitio potencial de *N*-glicosilación en la posición 110 para la proteína madura (Lara-Márquez et al. 2011). En este trabajo se presentan los resultados de la expresión heteróloga del gen que codifica para la pectin liasa 2 de *C. lindemuthianum* en *Pichia pastoris* y los resultados de la purificación de la enzima nativa y recombinante.

Materiales y métodos

Cepas y vectores

Para la amplificación del ADNc de *Clpnl2* [GenBank: JN034039] se utilizó como fuente el plásmido pCR2.1-TOPO/*Clpnl2* (Lara-Márquez et al. 2011). Para la manipulación y mantenimiento de los plásmidos se utilizó *Escherichia coli* TOP10F (Invitrogen, USA) y para la expresión del ADNc de *Clpnl2* [GenBank: JN034039] se utilizó *P. pastoris* cepa X-33 y el vector pPICZαA (EasySelect™ Pichia Expression Kit, Invitrogen, USA). La raza 1472 *C. lindemuthianum* fue donada por la Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, México) y se mantuvo en medio de papa dextrosa agar (PDA, Difco) a 20 °C.

Aislamiento de ADN y ARN

El aislamiento de ADN de *P. pastoris* X-33 y X-33/*Clpnl2*-2 se realizó de acuerdo a la metodología estándar (Ausubel et al. 1999)**.** Se purificó ARN total de *P. pastoris* X-33 y X-33/*Clpnl2*-2 utilizando el protocolo del TRIzol (Invitrogen, USA). Las muestras de ARN se trataron con DNAsa I de acuerdo a las instrucciones del fabricante (Invitrogen, USA).

Construcción del vector de expresión heteróloga

El ADNc de *Clpnl2* codificante de la proteína madura se amplificó por PCR a partir del plásmido pCR2.1-TOPO/*Clpnl2* con el oligonucleótido directo pnl2-*Eco*RI/9 (5´-CGT AGA

ATT CGC TGA TGC CGT CAA-3´; se subraya la secuencia de restricción para *Eco*RI) y el oligonucleótido reverso pnl2-*Xba*I/1R (5´-CTA TTC TAG ATC AAG AGG AGA GGC AAT C -3´; se subraya la secuencia de restricción para *Xba*I). La mezcla de reacción se desnaturalizó a 95 ºC por 5 min en un termociclador (Techne, TC-412), seguido por 35 ciclos de 20 seg de desnaturalización a 95 ºC, 30 seg de alineamiento a 64 ºC y 1 min de extensión a 68 ºC. El fragmento amplificado se separó y se purificó a partir de gel (QIAquick gel Extration Kit, QUIAGEN, USA) y fue digerido con las endonucleasas *Eco*RI *y Xba*I (Invitrogene, USA) antes de clonarse en el vector de expresión pPICZαA, digerido previamente con las mismas enzimas. Con el plásmido recombinante resultante (pPICZαA-*Clpnl2*) se transformaron células de *E. coli* TOP10F y se seleccionaron las transformantes en agar Luria-Bertani conteniendo 25 μg/ml de zeocina (Invitrogene, USA). La clona seleccionada se revisó por análisis de restricción y secuenciación para verificar que el marco de lectura se encontrara en fase.

Transformación de *P. pastoris* y detección de las transformantes

Para la integración del gen en *P. pastoris*, se linearizaron 10 μg de plásmido pPICZαA-*Clpnl2* con la endonucleasa *Bst*XI (New England) y se transformaron por electroporación (*E. coli* Pulse™, Bio-Rad, USA) células competentes de *P. pastoris* X-33 (Ausubel et al. 1999). Las colonias transformantes se seleccionaron en placas de YPD conteniendo 100 μg/ml de zeocina. Con el fin de buscar colonias transformantes con copias múltiples de *Clpnl2*, se seleccionaron y se cultivaron 16 colonias en placas de YPD con 500, 1000 y 2000 μg/ml de zeocina. La colonia transformante con la mayor resistencia a zeocina fue seleccionada para la expresión de CLPN2 (X-33/*Clpnl2*-2). La integración del gen *Clpnl2* en el genoma de *P. pastoris* se confirmó por PCR con el oligonucleótido directo pnl2-*Eco*RI/9 (5´-CGT AGA ATT CGC TGA TGC CGT CAA-3´) y el oligonucleótido reverso pnl1590 (5´-TCA AGA GGA GAG GCA ATC TAC CA-3´). La mezcla de reacción se desnaturalizó a 95 ºC por 5 min en un termociclador (Techne, TC-412), seguido por 35 ciclos de 20 seg de desnaturalización a 95 ºC, 30 seg de alineamiento a 56 ºC y 1 min de extensión a 72 ºC. Los productos de amplificación se analizaron por electroforesis en geles de agarosa al 1% teñidos con 1 μ de SYBR Safe (Invitrogene, USA).

Ensayo de expresión de *Clpnl2* en *P. pastoris*

Para la inducción de la expresión de *Clpnl2* en *P. pastoris*, se creció la cepa transformante X-33/*Clpnl2*-2 en 20 ml de medio BMGY (1% de extracto de levadura, 2% de peptona, fosfato de potasio 100 mM, pH 6.0, 1.34% de YNB, 0.0004% de biotina y 1% glicerol) a 30 ºC en agitación (200 rpm) hasta una OD_{600} entre 5~6. Posteriormente, se centrifugó el cultivo y se resuspendió la pastilla de células en 100 ml de medio BMMY (medio BMGY suplementado con metanol al 0.5%). Se aseguró el suministro de oxígeno colocando el cultivo en un matraz de 2 L en agitación (250 rpm) a 30 ºC. Se agregó metanol absoluto cada 24 h a una concentración final de 0.5% para mantener la inducción. Se tomó una muestra de 1 ml cada 24 h, se centrifugó a 10,000 x g 5 min y se almacenó el sobrenadante y la pastilla a -80ºC.

Análisis de expresión

Para analizar la expresión de *Clpnl2* en la cepa transformante X-33/*Clpnl2*-2, se amplificó el ADNc utilizando el sistema de síntesis para RT-PCR (SuperScript III First-Strand Syntesis System for RT-PCR, Invitrogen, USA) a partir de ARN total purificado de células X-33/*Clpnl2*-2 inducidas con metanol al 0.5% por 24, 48, 72, y 96 h. Para las amplificaciones por PCR se utilizó el juego de oligonucleótidos específicos PliD (5´- CTG CGA CAA CCA CCA CTA CT -3´) y pnl1590 (5´-TCA AGA GGA GAG GCA ATC TAC CA-3´), diseñados para la amplificación de un fragmento de 556 pb. Las mezclas de reacción se desnaturalizaron por 2 min a 94 ºC, seguido por 30 ciclos de 1 min a 94 ºC, 1 min a 54 ºC , 1 min a 72 ºC, con una extensión final de 7 min a 72 ºC. Los productos de amplificación se analizaron por electroforesis en geles de agarosa al 1% teñidos con 1 μ de SYBR Safe (Invitrogene, USA).

Cultivo de *C. lindemuthianum*

Para la inducción enzimática se utilizaron matraces Erlenmeyer de 500 ml conteniendo 100 ml de medio modificado de Mathur ($MgSO_4.7H_2O$, 10 mM; KH_2PO_4, 20 mM y L-ácido glutámico, 36 mM, en un volumen final de 1 L; pH, 5.5) suplementado con 2.5% de glucosa (Sigma) o pectina esterificada al 92% (Sigma). Los matraces Erlenmeyer se inocularon con 1.6 mg (aproximadamente 5 cm^2) de micelio de *C. lindemuthianum* de una colonia de 9 días de crecimiento en PDA. Los cultivos se mantuvieron a 20 ºC con agitación continua (150 rpm) por 10 días, de acuerdo al tiempo de máxima actividad específica de PNL reportado por

Hernández-Silva (2007). Después de 10 días de crecimiento, los cultivos se centrifugaron a baja velocidad y el sobrenadante se mantuvo en hielo hasta que fue fraccionado con sulfato de amonio.

Purificación de las proteínas nativa y recombinante

Después de 48 h de inducción de la cepa X-33/*Clpnl2*-2 de *P. pastoris*, se recuperó el sobrenadante por centrifugación, se filtró utilizando papel Whatman No. 4 y se recuperaron aproximadamente 100 ml de sobrenadante. En el caso de *C. lindemuthianum*, después de 10 días de crecimiento se centrifugó el cultivo, se filtró utilizando papel Whatman No. 4 y se recuperaron 100 ml de filtrado. Los sobrenadantes de ambos sistemas se utilizaron como extractos crudos de la enzima para el fraccionamiento con sulfato de amonio. Con este propósito, se mantuvieron los extractos a 4 ºC en agitación mientras se les agregaba lentamente sulfato de amonio hasta alcanzar el 30% de saturación. Se dejo reposar por 1 h en hielo , se centrifugó a 10,000 g por 20 min y se almacenaron las pastillas a 4 ºC. El sobrenadante recuperado se saturó con sulfato de amonio al 50% y se incubó durante 1 h a 4 ºC, se recuperó la pastilla por centrifugación a 10,000 g por 20 min y se repitió el mismo procedimiento para obtener las fracciones correspondientes al 50-60% y 60-80% de saturación. Se disolvieron las pastillas en regulador de acetato de sodio 50 mM, pH 5.0, y se filtraron utilizando dos cambios de regulador de acetato de sodio 50 mM, pH 5.0, en cámaras de Amicon Ultra de 15 ml (Millipore) con membrana de corte de 10 kDa. Se recuperaron 200 µl de cada fracción, en las cuales se determinó la actividad enzimática y la concentración de proteína.

La fracción correspondiente al 60-80% de saturación, obtenida a partir del medio extracelular de *C. lindemuthianum* crecido por 10 días con 2.5 % de pectina esterificada al 92%, se aplicó a una columna (1.5 x 12 cm) de DEAE celulosa (Sigma, USA) equilibrada con regulador de Tris-HCl 10 mM, pH 8.0. La columna se lavó con 50 ml del mismo regulador y la proteína se eluyó con un gradiente de cloruro de sodio de 0.1 a 1 M en el mismo regulador. Las fracciones recuperadas (5 ml) se filtraron en cámaras de Amicon Ultra de 15 ml con un cambio de regulador de acetato de sodio 50 mM, pH 5.0, y cada fracción se concentró a un volumen final de 200 µl para analizar la actividad de PNL y la concentración de proteína.

Determinación de la actividad de PNL2

Para determinar la actividad de PNL se prepararon mezclas de reacción conteniendo 0.1% de pectina esterificada al 92% (Sigma), 100 µl de la muestra enzimática y regulador de acetato de sodio 50 mM, pH 5.0, en un volumen final de 1 ml. De forma paralela se prepararon mezclas control donde se omitió la enzima o el sustrato para hacer las correcciones correspondientes. Las mezclas de reacción se incubaron a 30 ºC por 10 min y se detuvieron colocándolas en agua hirviendo por 5 min. Una vez que las mezclas estuvieron a temperatura ambiente, se cuantificó por duplicado la cantidad de especies de esteres urónicos insaturados (UUE) en alícuotas de 0.4 ml del hidrolizado por el método del ácido thiobarbitúrico de Cooper y Wood (1975) modificado por Nedjman et al. (Nedjma 2001). La actividad se expresó como nmol de UUE liberados en 1 min, basada en el coeficiente de absorción molar de 5500 para la síntesis de derivados monoméricos de ácido galacturónico α, β-insaturados (Hashimoto et al. 1971). La actividad específica se expresó como nmoles de ésteres urónicos/min/mg de proteína.

Cuantificación de proteína

La concentración de proteína se determinó por el método de Lowry utilizando albúmina de suero bovino como estandar (Lowry et al. 1951). La pureza y el peso molecular de la proteína recombinante del medio extracelular de la cepa X-33/*Clpnl2*-2 inducida por 48 h, se estimó utilizando el kit de origen comercial Protein 80 Assay LabChip (2100 Agilent Bioanalyzer).

SDS-PAGE

Las muestras de proteína se analizaron por electroforesis en geles de poliacrilamida desnaturalizantes (SDS-PAGE) al 15% por el método de Laemmli (1970). Las muestras se hirvieron por 3 min antes de aplicar al gel. Las proteínas se visualizaron tiñendo los geles con Coomassie brillante azul R250 (Sigma). Se utilizó el estándar de tamaño molecular Kaleidoscope (Bio-Rad) conteniendo miosina (216 kD), β-galactosidasa (132 kD), albúmina (78 kD), anhidrasa carbónica (45.7 kD), inhibidor de tripsina de soya (32.5 kD), lisozima (18.4 kD) y aprotinina (7.6 kD).

Zimogramas

La proteína concentrada en cámaras de Amicon Ultra a partir de 15 ml de extracto crudo de *C. lindemuthianum* inducido por 10 días con 2.5% de pectina esterificada al 92%, se mezcló con regulador de SDS-PAGE. Después de la electroforesis en gel de poliacrilamida desnaturalizante al 12% (Laemmli 1970), se incubó el gel en Triton X-100 al 1% por 30 min a 4 ºC y 30 min en regulador de acetato 50 mM, pH 5.0, a 4 ºC. El gel se recubrió con un gel de poliacrilamida al 5% y 0.25% de pectina esterificada al 92% en regulador de acetato 50 mM, pH 5.0, y se incubó toda la noche a 30 ºC. El gel de poliacrilamida al 12% se tiño con Coomassie brillante azul R250 y el gel al 5% se tiño con Rojo de Rutenio (Sigma) al 0.05% por 20 min y se lavó en agua destilada hasta visualizar una banda clara correspondiente a la actividad de PNL.

Resultados

Detección y análisis de cepas transformantes de *P. pastoris*

En el sistema de expresión de *P. pastoris* utilizado en este trabajo, la transcripción de *Clpnl2* se induce por la presencia de metanol ya que la expresión se halla bajo el control del promotor de la alcohol oxidasa (AOX). La proteína recombinante es secretada al medio gracias a la fusión de un péptido de unión al factor α de *Saccharomyces cerevisiae* en el N-terminal del producto. A partir de la trasformación por recombinación homóloga de la cepa X-33 de *P. pastoris* con el plásmido pPICZαA-*Clpnl2,* se obtuvieron 16 cepas transformantes (X-33/*Clpnl2* 1-16). Las 16 cepas se sometieron a un gradiente de zeocina (500, 1000, y 2000 μg/ml) para seleccionar una transformante con copias múltiples de *Clpnl2* que potencialmente expresaran niveles altos de la proteína recombinante. De acuerdo a lo anterior, se seleccionó la cepa transformante X-33/*Clpnl2*-2 por su resistencia a 2000 μg/ml de zeocina. La integración de *Clpnl2* en el cromosoma de X-33/*Clpnl2*-2 de *P. pastoris* se confirmó por la presencia de un solo producto de amplificación correspondiente a *Clpnl2* en la cepa transformante y en el plásmido pCR2.1-TOPO/*Clpnl2* utilizado como control de amplificación (Fig, 1A). A pesar de que en la amplificación sobre ADN de la cepa X-33 de *P. pastoris* utilizada como control negativo se observa un producto de amplificación, éste es de un tamaño mayor al esperado que posiblemente corresponda a una amplificación inespecífica por el uso de un oligonuceótido con un sitio de reconocimiento para la endonucleasa *Eco*RI .

Expresión de la PNL recombinante en *P. pastoris*

Se realizó un ensayo de expresión a pequeña escala de la cepa X-33/*Clpnl2*-2 utilizando 0.5% de metanol para inducir la expresión de *Clpnl2* por 5 días. El análisis por RT-PCR confirmó que *Clpnl2* se transcribe desde las 24 h de inducción y se mantiene hasta 96 h (Fig. 1 B); sin embargo, una banda del tamaño esperado para la PNL recombinante se detectó hasta las 48 h de inducción en los extractos crudos fraccionados por SDS-PAGE y fue disminuyendo conforme avanzó el tiempo de inducción, aunque se mantuvo una baja concentración hasta las 120 h (Fig. 2A). De acuerdo a los análisis realizados en un Bioanalizador (2100 Agilent Bioanalyzer), la PNL recombinante secretada a las 48 h correspondió al 22.9% de la proteína total secretada por *P. pastoris*, y mostró un peso molecular de 44.4 kDa (Fig. 2B).

La actividad específica de la PNL producida por *P. pastoris* después de 48 h de inducción con 0.5% de metanol fue de 4.55 nmol de UUE/min/mg de proteína, mientras que no se observó la banda correspondiente a la PNL ni se detectó actividad específica en los extractos crudos de la cepa X-33/*Clpnl*2-2 de *P. pastoris* sin inducir ni en el extracto crudo de la cepa X-33 sin la construcción (Fig. 3A y B).

La actividad específica detectada en el extracto crudo de la cepa X-33*Clpnl*2-2 de *P. pastoris* inducida por 48 h correspondió al 8.24% de la actividad específica del extracto crudo de *C. lindemuthianum* inducido por 10 días con pectina esterificada (4.55 nmol de EU/min/mg de proteína vs 55.18 nmol de EU/min/mg de proteína, respectivamente) lo que corresponde a la actividad máxima con este sustrato de acuerdo a Hernández-Silva et al. (2007) (Fig. 4A). La actividad total del extracto de la cepa X-33*Clpnl*2-2 fue mayor que la detectada en *C. lindemuthianum* (5090.91 nmol de UUE/min vs 3369.6 nmol de EU/min, respectivamente) (Fig. 4B).

Purificación de las proteínas recombinante y nativa

Para purificar tanto la enzima recombinante como la nativa, los extractos inducidos de X-33/*Clpnl2*-2 y *C. lindemuthianum* se fraccionaron con sulfato de amonio. En el extracto de *C. lindemuthianum* se detectaron dos picos de actividad correspondientes a las fracciones 30-50% y 60-80% con una actividad específica de PNL de 91.53 y 146.44 UUE/min/mg de proteína, respectivamente (Fig. 5A). De acuerdo al análisis por SDS-PAGE de las fracciones (Fig. 5B),

en la fración 30-50% se observó una banda mayoritaria que de acuerdo al zimograma del extracto de *C. lindemuthianum* (Fig. 6) corresponde al tamaño en el que se detectó actividad de PNL. Sin embargo, a pesar de que no observó una banda correspondiente en la fracción 60-80%, es necesario repetir el ensayo para descartar un problema técnico (Fig. 5B). En el extracto de la cepa X-33/*Clpnl2*-2 de *P. pastoris*, la fracción 50-60% del fraccionamiento salino presentó la mayor parte de la actividad específica de PNL (28.7 nmol de EU/min/mg de proteína) aumentando al menos seis veces la actividad específica de PNL en comparación con el extracto crudo de X-33/*Clpnl2*-2 y se observó una banda mayoritaria correspondiente a la PNL recombinante en esta fracción (Fig. 7A y B).

Posteriormente, la fracción 60-80% del extracto de *C. lindemuthianum* se separó en una columna de DEAE celulosa. En la Fig. 8 se muestra el perfil de elución obtenido de la columna, donde se observa un pico de actividad enzimática específica que eluye con NaCl 0.1–0.4 M.

Discusión

Las pectinasas son enzimas ampliamente utilizadas en aplicaciones biotecnológicas y las pectin liasas en particular tienen un gran potencial en la industria de los alimentos (Kashyap et al. 2001; Hoondal et al. 2002; Semenova et al. 2006). A pesar de esto, son pocos los trabajos en los que se han utilizado sistemas de expresión heterólogos para producir estas enzimas (Templeton et al. 1994; Zhao et al. 2007; Qiang et al. 2009). En la industria, estas enzimas son utilizadas como componentes de mezclas enzimáticas; sin embargo, se tiene evidencia de la capacidad de las PNL para actuar sin la ayuda de otras enzimas en procesos como la purificación y/o clarificación de jugos, con resultados comparables al uso de mezclas enzimáticas y sin la presencia de actividades indeseables (Semenova et al. 2006).

En este trabajo se clonó el gen que codifica para la pectin liasa 2 de *C. lindemuthianum* en un sistema de expresión heterólogo de *P. pastoris* utilizando el promotor de la alcohol oxidasa para inducir la expresión de la PNL recombinante con metanol. De acuerdo a los resultados, el gen *Clpnl2* se transcribe y la PNL recombinante se sintetiza y es secretada al medio de cultivo, con un nivel máximo detectado por SDS-PAGE a las 48 h de inducción con metanol.

La actividad específica de la PNL detectada en el extracto crudo *P. pastoris* fue menor a la actividad específica del extracto de *C. lindemuthianum*. Sin embargo, cuando se consideraron

las actividades totales, la actividad del extracto de *P. pastoris* fue 1.5 veces mayor que la de *C. lindemuthianum*. De acuerdo a los resultados la PNL producida por *P. pastoris* correspondió al 22.9% de la proteína total producida en las condiciones de inducción utilizadas, lo que representa una ventaja en comparación con *C. lindemuthianum*. En general las levaduras tienen características que las hacen buenos sistemas heterólogos de producción de proteínas, tales como su capacidad de producción y secreción de proteínas, un rápido crecimiento (Damasceno et al. 2011) y en nuestro caso el bajo costo del inductor y la posibilidad de producir la PNL sin actividades relacionadas adicionales. A pesar de que la producción de la PNL en sistema de expresión realizado en este trabajo es buena, es posible incrementar la eficiencia de producción manipulando conducciones de crecimiento e inducción como pH, temperatura y concentración del inductor entre otras (Qureshi et al 2010).

De acuerdo al análisis *in silico* de la secuencia de amino ácidos de la pectin liasa 2 de *C. lindemuthianum,* la proteína madura tiene un peso molecular de 37.4 kDa y presenta un sitio probable de *N*-glicosilación en la Asp en posición 110 (Asn^{110}). Debido a que la proteína recombinante presentó un peso molecular de 44.4 kDa, un valor cercano al determinado por SDS-PAGE para la enzima nativa secretada por *C. lindemuthianum,* el incremento en el peso molecular sugiere un proceso de glicosilación. Sin embargo, el patrón de glicosilación realizado por *P. pastoris* en la proteína recombinante puede ser distinto al realizado por *C. lindemuthianum* a la proteína nativa.

Uno de los principales problemas de los sistemas de expresión heterólogos es la glicosilación de las proteínas recombinantes. Dado que esta modificación covalente puede influir en la termodinámica de la enzima y modular características cinéticas como la estabilidad, flexibilidad estructural y actividad (Nevalainen et al. 2005; Jeoh et al. 2008; Shental-Bechor and Levy 2009), es importante considerar que un cambio en el tipo ó estructura del oligosacárido unido a la Asp^{110} podría afectar al menos alguna de las propiedades antes mencionadas como se ha observado en otros casos (Benen et al. 2000; Tull et al. 2001) por lo sería importante analizar el papel de la glicosilación en la actividad de la PNL como parte de la caracterización de la enzima.

La purificación de la PNL de *C. lindemuthianum* por fraccionamiento salino, mostró dos picos de actividad en las fracciones 30-50% y 60-80%. Wijesundera et al. (1989), reportaron la presencia de dos isoformas de pectin liasa en cultivos de *C. lindemuthianum* con polipectato

de sodio y pared celular de frijol (*Phaseolus vulgaris*) como principales fuentes de carbono, con diferentes pI y tamaños moleculares. Con estos antecedentes, es posible que la actividad encontrada en las fracciones 30-50% y 60-80% correspondan a dos pectin liasas codificadas por dos genes distintos inducidos por la presencia de pectina esterificada en los medios de cultivo, uno de los cuales corresponde a la PNL2. La presencia de diferentes isoenzimas es un fenómeno que se ha observado comúnmente en genes que codifican pectinasas y en particular de pectin liasas (de Vries and Visser 2001; de Vries et al. 2002) por lo que no sería raro que por lo menos existieran dos genes que codifiquen pectin liasas como sucede en *C. gloeoesporoides* (Wei et al. 2002). Sin embargo, es posible que por la metodología utilizada se encontrara actividad de PNL dividida en varias fracciones pero que en realidad corresponda a una sola enzima. Como se pudo observar en el zimograma, son varias las proteínas expresadas bajo las condiciones de inducción utilizadas para *C. lindemuthianum*, pero una sola banda de actividad que coincide en tamaño con la proteína observada en la fracción 30-50%. Lamentablemente por las condiciones de la fracción 60-80 no es posible descartar la presencia de la banda correspondiente en esta fracción.

Se decidió continuar con la purificación de la enzima nativa utilizando la fracción 60-80% por ser la que presenta una actividad mayor, por lo que se hizo un segundo paso de purificación en una columna de DEAE celulosa, en donde se encontró que la máxima actividad específica de PNL se recuperó en las fracciones eluidas con 0.1M a 0.4 M de NaCl.

Comparadas con otras enzimas que degradan los componentes de la pared celular, es poca la información bioquímica que se tiene de las pectin liasas, lo cual contrasta con su enorme interés biotecnológico, por lo que la clonación de genes de PNL y la caracterización de las enzimas que codifican, es fundamental para impulsar y mejorar su uso biotecnológico. Se conoce muy poco sobre el papel de la glicosilación en las enzimas pectinolíticas, tanto a nivel de los azúcares que se unen como de su papel en la actividad y/o estabilidad de las mismas, y esto es válido para enzimas nativas y recombinantes. El estudio comparativo de la glicosilación de una enzima nativa y de su contraparte recombinante, podría darnos información valiosa para elegir sistemas de expresión heterólogos adecuados y eventualmente aumentar la eficiencia de estas enzimas en los procesos biotecnológicos.

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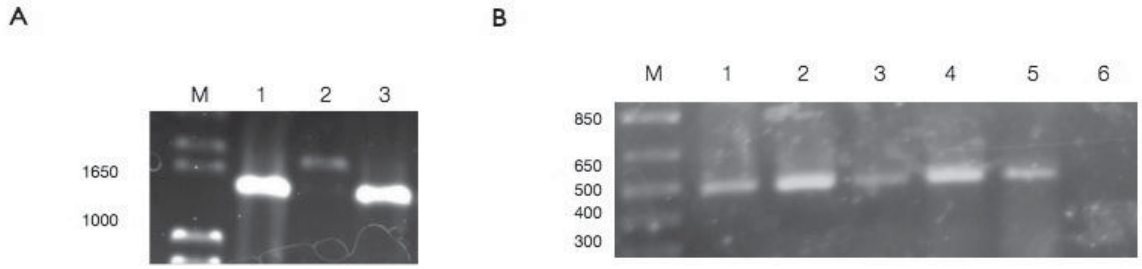

Figura 1. A) Amplificación del gen *Clpnl2* en la transformante X-33/*Clpnl2*-2 de *P. pastoris*. Carril M, marcador de tamaño molecular 1 Kb Plus Ladder (Invitrogen); Carril 1, control positivo de amplificación con el plásmido pCR2.1-TOPO/*Clpnl2;* carril 2, control negativo de amplificación cepa X-33 *P. pastoris*; carril 3, producto de amplificación con la cepa X-33/*Clpnl2*-2. B) Amplificación por RT-PCR del fragmento correspondiente al ARNm de *Clpnl2*. Carril M, marcador de tamaño molecular 1 Kb Plus Ladder (Invitrogen); carril 1, control positivo de amplificación con ADNc de *C. lindemuthianum*; carriles 2-5, cepa X-33/*Clpnl2*-2 inducida por 24, 48, 72 y 96 h, respectivamente; carril 6, control negativo con la cepa X-33 de *P. pastoris*.

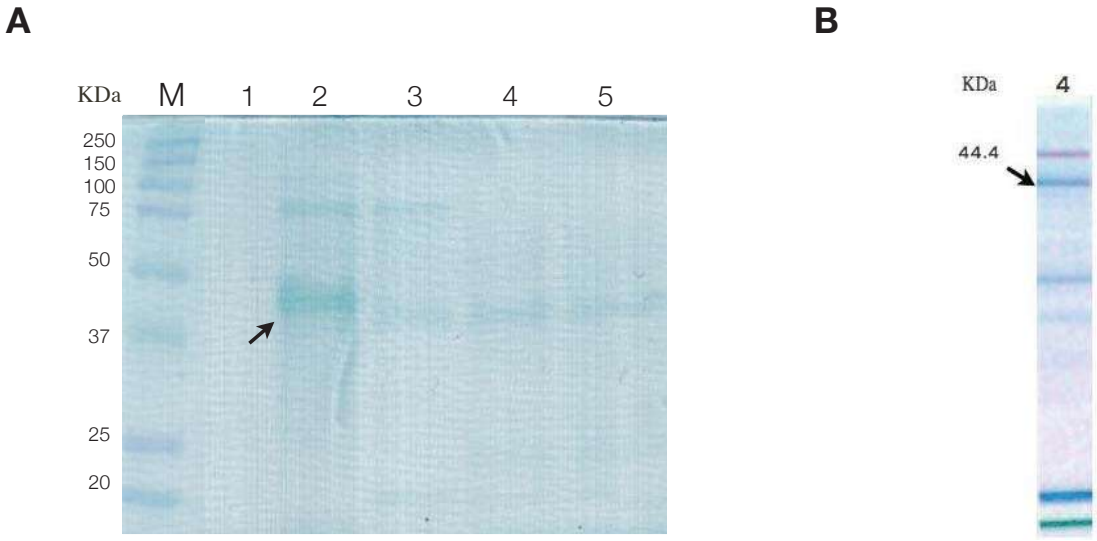

Figura 2. Análisis de expresión curso temporal de la proteína recombinante CLPNL2. (A) Extracto crudo fraccionado por SDS-PAGE al 15% teñido con Coomassie Brilliant Blue R-250. Carriles 1-5 extracto crudo de la cepa X-33/*Clpnl2*-2 inducida por 24, 48, 72, 96 y 120 h respectivamente con metanol al 0.5%; carril M, estándares de peso molecular (kDa) (Kaleidoscope Bio-Rad): miosina (216), β-galactosidasa (132), albúmina (78), anhidrasa carbónica (45.7), inhibidor de tripsina de soya (32.5), lisozima (18.4) y aprotinina (7.6). (B) Extracto de la cepa transformante X-33/*Clpnl2*-2 inducida por 48 h con metanol al 0.5%, analizado con un bioanalizador (2100 Agilent Bioanalyzer). La flecha indica la banda correspondiente a la PNL recombinante.

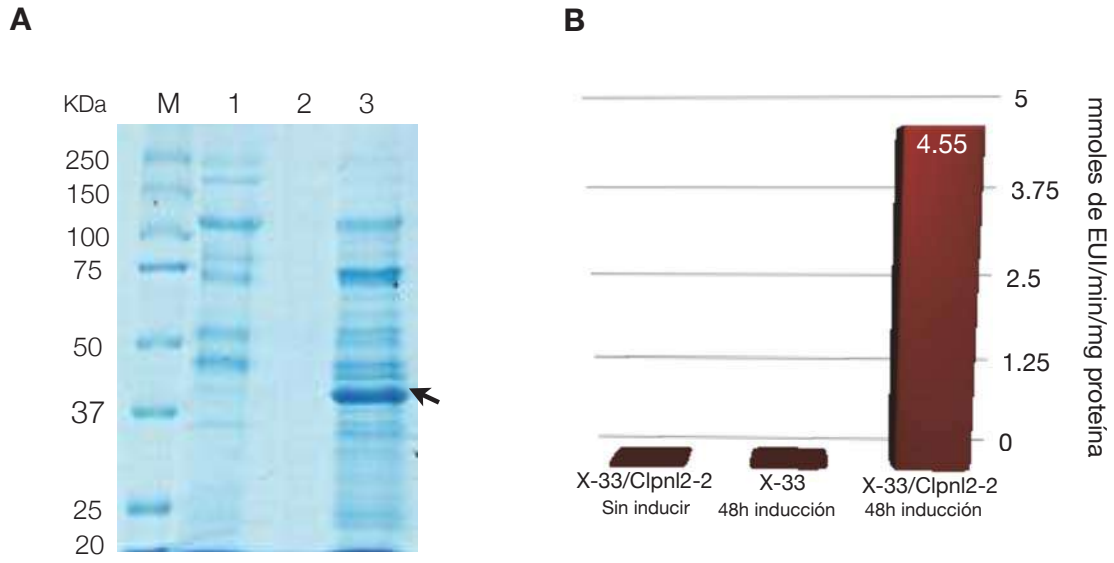

Figura 3. Análisis de expresión y actividad de la PNL recombinante. (A) Extracto crudo fraccionado por SDS-PAGE 15%, teñido con Coomassie Brilliant Blue R-250. Carril 1, extracto crudo de X-33/*Clpnl*2-2 sin inducir; Carril 2, extracto crudo de X-33 inducido por 48 h; Carril 3, extracto crudo de X-33/*Clpnl*2-2 inducido por 48 h. Carril M, estándar de tamaño molecular (Kaleidoscope Bio-Rad): miosina (216), -galactosidasa (132), albúmina (78), anhidrasa carbónica (45.7), inhibidor de tripsina de soya (32.5), lisozima (18.4) y aprotinina (7.6). La flecha indica la banda correspondiente a la PNL recombinante. (B) Actividad del extracto crudo de la cepa transformante X-33/*Clpnl*2-2 sin inducir, cepa X-33 inducida por 48 h y cepa X-33/*Clpnl*2-2 inducida por 48 h con metanol al 0.5%.

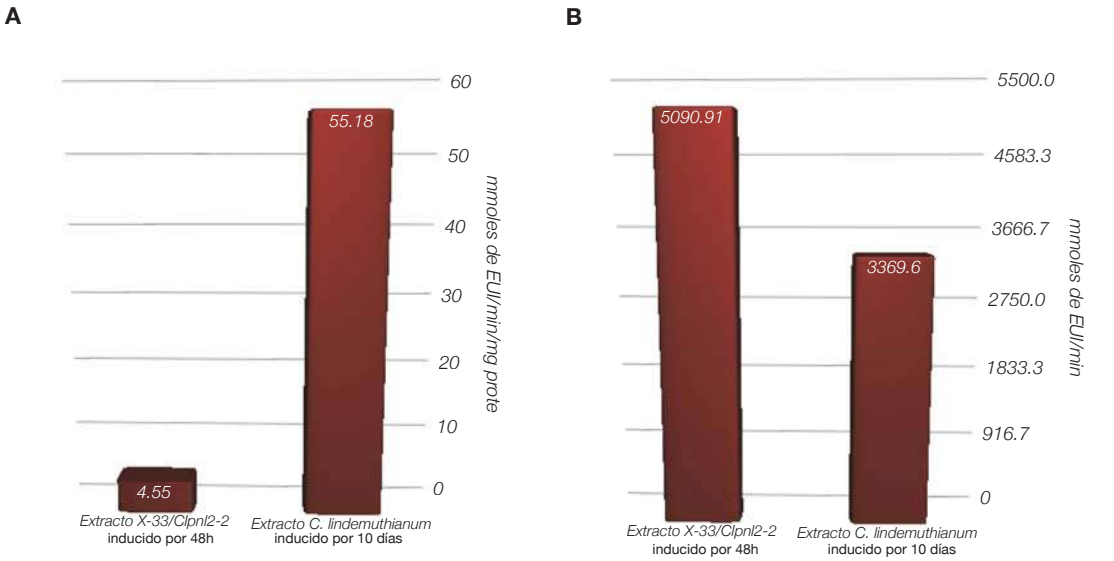

Fig. 4. Actividad específica y total de PNL. A) Actividad específica de extractos crudos de la cepa X-33/*Clpnl*2-2 de *P. pastoris* inducida por 48 h con 0.5% de metanol y *C. lindemuthianum* inducido por 10 días con 2.5% de pectina esterificada al 92%. B) Actividad total de extractos crudos de la cepa X-33/*Clpnl*2-2 de *P. pastoris* inducida por 48 h con 0.5% de metanol y *C. lindemuthianum* inducido por 10 días con 2.5% de pectina esterificada al 92%.

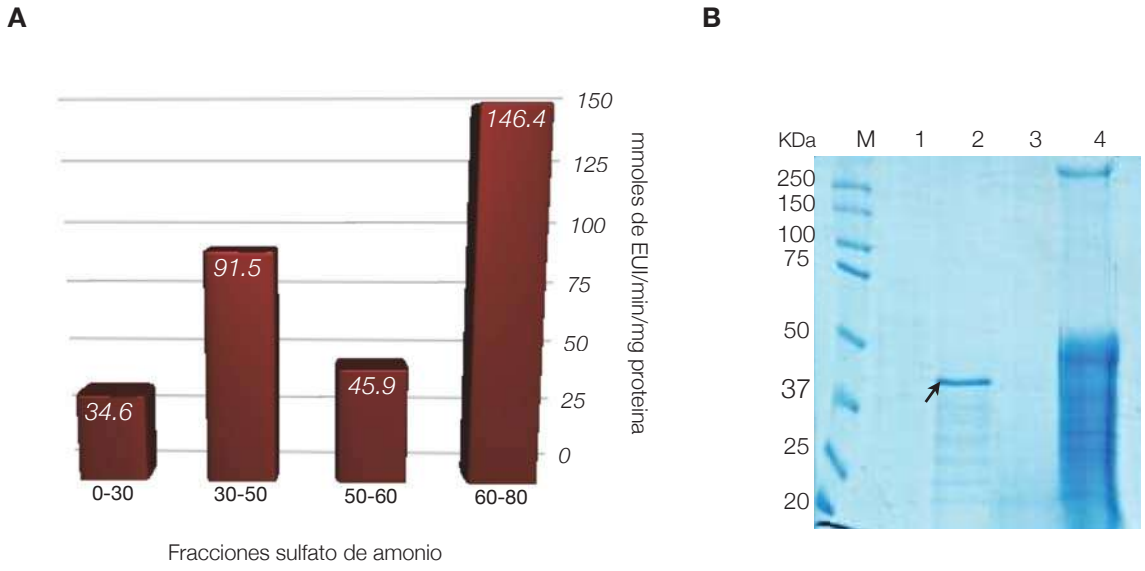

Figura 5. Fraccionamiento salino del extracto crudo de *C. lindemuthianum*. A) Actividad específica de PNL de las fracciones obtenidas del extracto crudo de *C. lindemuthianum* inducido durante 10 días con 2.5% de pectina esterificada. B) Fracciones analizadas por SDS-PAGE 15%, teñido con Coomassie Brilliant Blue R-250. Carril 1-4, fracciónes 0-30%, 30-50%, 50-60% y 60-80% respectivamente; Carril M, estándar de tamaño molecular (Kaleidoscope Bio-Rad): miosina (216), -galactosidasa (132), albúmina (78), anhidrasa carbónica (45.7), inhibidor de tripsina de soya (32.5), lisozima (18.4) y aprotinina (7.6). La flecha señala la banda del tamaño esperado para CLPNL2.

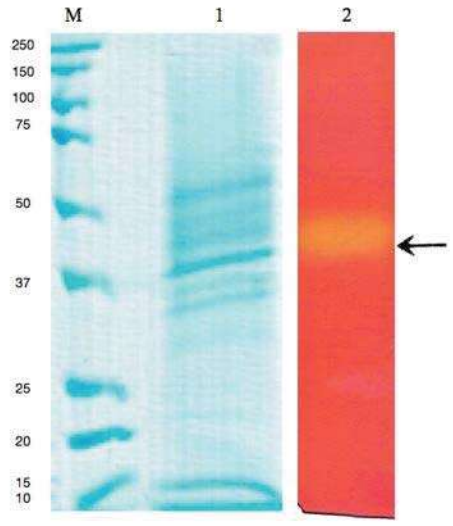

Figura 6. Análisis electroforético de la PNL de *C. lindemuthianum* por SDS-PAGE y zimograma. Carril 1, extracto concentrado de *C. lindemuthianum* inducido con pectina al 2.5% por 10 días; carril 2, zimograma en un gel de poliacrilamida al 5% incubado con pectina esterificada al 0.25% y teñido con Rojo de Rutenio. Carril M, estándares de peso molecular (kDa) (Kaleidoscope Bio-Rad): miosina (216), β-galactosidasa (132), albumina (78), anhidrasa carbónica (45.7), inhibidor de tripsina de soya (32.5), lisozima (18.4), aprotinina (7.6).

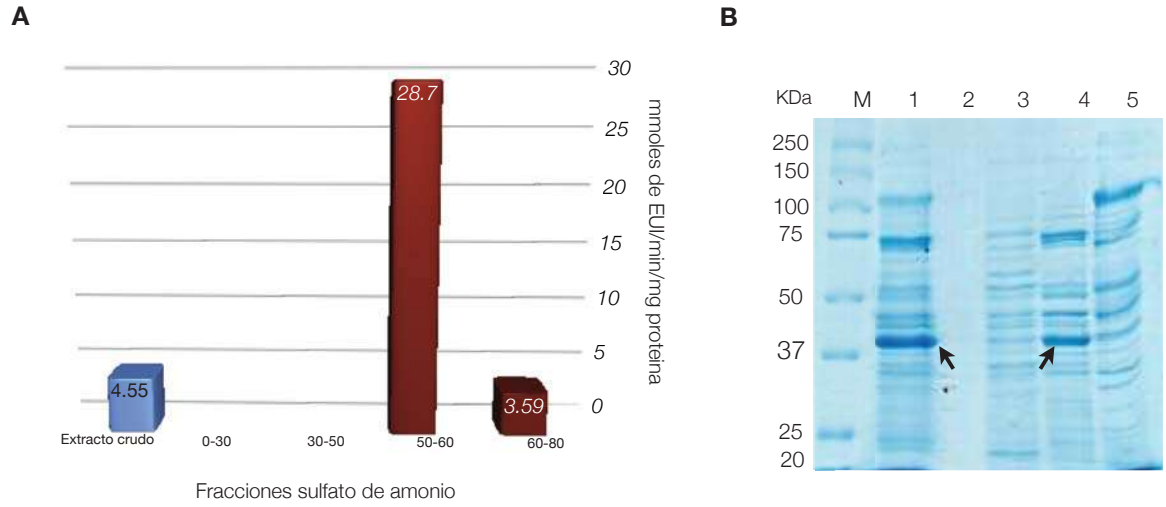

Figura 7. Fraccionamiento salino de extracto crudo de X-33/*Clpnl*2-2 de *P. pastoris*. A) Actividad específica de PNL de las fracciones obtenidas de extracto crudo de X-33/*Clpnl*2-2 inducido durante 48 h con 0.5% de metanol. B) Fracciones analizadas por SDS-PAGE 15%, teñido con Coomassie Brilliant Blue R-250. Carril 1 extracto crudo, carriles 2-5, fracciones 0-30%, 30-50%, 50-60% y 60-80% respectivamente; Carril M, estándar de tamaño molecular (Kaleidoscope Bio-Rad): miosina (216), -galactosidasa (132), albúmina (78), anhidrasa carbónica (45.7), inhibidor de tripsina de soya (32.5), lisozima (18.4) y aprotinina (7.6). Las flechas señalan las bandas de CLPNL2.

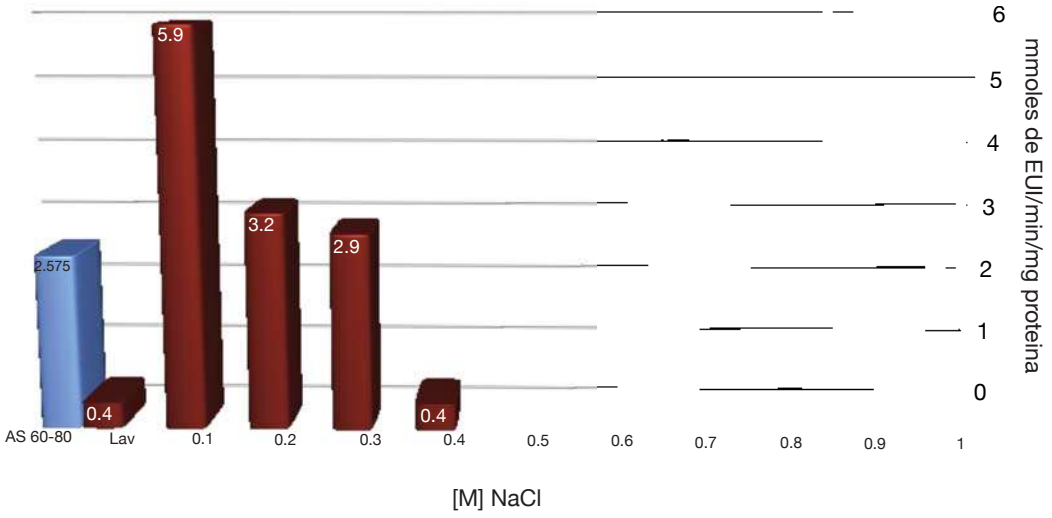

Figura 8. Cromatografía de intercambio iónico de la fracción 60-80% de *C lindemuthianum*. Se aplicaron 200 µl correspondentes a la fracción de saturación 60-80 % de *C. lindemuthianum* en una columna de 1.5 x 12 cm de DEAE-celulosa equilibrada con regulador de Tris-HCl 10 mM, pH 8.0. La proteína se eluyó con un gradiente de cloruro de sodio de 0.1 a 1M y se recuperaron fracciones de 5ml.

DISCUSIÓN GENERAL

La mayoría de la información disponible sobre enzimas pectinolíticas y los genes que las codifican proviene de hongos saprófitos como *A. niger* y *A. nidulans* (de Vries 2003). Sin embargo, es posible que a pesar de la conveniencia de utilizar estos microorganismos como modelos para la obtención de genes y/o producción de enzimas, el uso de enzimas provenientes de otros microorganismos que las producen como estrategia de nutrición y/o invasión, ofrezcan características diferentes, útiles a nivel biotecnológico. En este sentido, existen antecedentes que señalan a los hongos fitopatógenos 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 (King et al. 2011). Lo anterior sugiere una tendencia evolutiva de las enzimas que degradan los componentes de la pared celular, con el tipo y complejidad de los sustratos utilizados por los microorganismos de los que provienen.

De acuerdo a lo anterior, en este trabajo se consideró importante realizar la caracterización del gen *Clpnl2* que codifica la pectin liasa 2 (CLPNL2) del hongo patógeno de frijol *Colletotrichum lindemuthianum*. El análisis se realizó utilizando la secuencia genómica y la secuencia del ADNc de *Clpnl2* obtenida mediante amplificación por RT-PCR. Adicionalmente se analizó la secuencia de aminoácidos de CLPNL2 y las pectin liasas reportadas para microorganismos saprófitos/oportunistas y patógenos, así como la comparación de sus estructuras tridimensionales.

El análisis filogenético de las secuencias de aminoácidos de pectin liasas de bacterias, oomicetos y hongos saprófitos/oportunistas o patógenos de plantas realizado en este estudio, reveló una relación entre las pectin liasas y el estilo de vida de los microorganismos de los que provienen, incluyendo a la pectin liasa 2 de *C. lindemuthianum* (*Clpnl2*) con las enzimas de hongos patógenos. Estos resultados se reforzaron con los obtenidos del análisis comparativo de las estructuras tridimensionales de las pectin liasas. De nuevo, las proteínas se agruparon de acuerdo al estilo de vida de los microorganismos de los que provienen. Parece ser que la diversidad y la naturaleza del sustrato de estos microorganismos han jugado un papel determinante en la evolución de su estilo de vida.

Hasta donde sabemos, este es el primer estudio que utiliza un análisis comparativo de estructuras tridimensionales en combinación con un análisis filogenético para explorar la evolución de las enzimas pectinolíticas. El análisis estructural es una herramienta importante que puede ser utilizada para reforzar el análisis de agrupamiento de proteínas, así como las diferencias estructurales que pueden contribuir con la especificidad de sustrato, elementos que no pueden ser inferidos utilizando solamente las secuencias de amino ácidos.

En este estudio se utilizaron dos razas de *C. lindemuthianum* aisladas de cultivos de frijol en diferentes localidades de México: una de las razas más virulentas (raza 1472,) y una raza que es incapaz de infectar (raza 0), pero que de forma natural se comporta como un hongo saprófito del frijol (Rodríguez-Guerra et al. 2006). De acuerdo a los resultados del análisis, no existen diferencias entre las secuencias de núcleotidos del gen que codifica la pectin liasa 2 entre las dos razas. A pesar de que ambas producen una proteína activa, se han observado diferencias en el nivel de la actividad enzimática y la velocidad de aparición de la misma (Hernández-Silva et al. 2007), así como diferencias en la velocidad de expresión de *Clpnl*2 en las diferentes fuentes de carbono utilizadas en los ensayos de expresión realizados en este estudio. Este es un fenómeno que se ha observado en diferentes enzimas (xilanasas, celulasas 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, a saber, uno relacionado con la evolución de las enzimas y otro con la regulación de la expresión de las mismas. Para confirmar esta hipótesis será necesario comparar los complejos enzimáticos completos que degradan los polímeros de la pared celular de microorganismos con diversos estilos de vida.

Recientemente, un estudio demostró que la β-xilosidasa del patógeno de trigo *Mycosphaerella graminicola* ha estado bajo presión de selección positiva con un ritmo acelerado de evolución ancestral, mientras que esto no sucede en sus parientes cercanos colectados de pastos silvestres (Brunner et al. 2009). Es posible que la domesticación y las practicas agrícolas contribuyan a la evolución de enzimas únicas y muy activas en la degradación de los polisacaridos de su huésped que tienen un papel clave en la emergencia de

patógenos especializados, debido a la co-evolución antagónica con las plantas hospederas (Brunner et al. 2009; King et al. 2011).

Como se mencionó anteriormente, en México se han caracterizado diversas razas de *C. lindemuthianum* que difieren en su grado de virulencia en diferentes variedades de frijol. A pesar de que la raza 0 es incapaz de infectar a su huésped, es una de las más abundates de los aislados en cultivos de frijol en México (30 aislados de la raza 0 vs. 6 aislados de la raza patógena 1472) (Rodríguez-Guerra et al. 2006). Estos datos sugieren que la raza no patógena sobrevive como un microorganismo saprófito/oportunista de manera exitosa, posiblemente asociada con una o varias razas patógenas en los cultivos de frijol. Sería interesante entonces probar la existencia de esta asociación entre las razas de *C. lindemuthianum* en los cultivos de frijol en México.

En las aplicaciones de los complejos enzimáticos, una de las técnicas más utilizadas para la producción de enzimas de interés biotecnológico es la expresión heteróloga en levaduras y bacterias. Sin embargo, son pocos los reportes de expresión heteróloga de pectin liasas de hongos, tanto en sistemas de expresión bacterianos (Zhao et al 2007), como de expresión en levaduras (Templeton et al. 1994; Qiang et al. 2009). Es posible que esto se deba en parte a la dificultad que representa la expresión de algunas de ellas.

Con el fin de contar con la proteína recombinante de pectin liasa 2 de *C. lindemuthianum* en este estudio se realizó la expresión heteróloga del gen *Clpnl2* en *P. pastoris*. El sistema de expresión es funcional ya que el gen *Clpnl2* se transcribe y la PNL recombinante se sintetiza y es secretada al medio de cultivo representando el 22.9% de la proteína total secretada por *P. pastoris*. La actividad específica de la enzima recombinante en el extracto crudo después de 48 h de inducción con metanol fue muy baja (8.24%) y aún después de un paso de purificación solo incrementó a la mitad (52.01%) comparada con la actividad detectada en el extracto crudo de *C. lindemuthianum* después de 10 días de inducción con pectina esterificada. Sin embargo, cuando se compararon las actividades totales de los extractos, la actividad total del sistema de expresión heterólogo fue 1.5 veces mayor que la detectada en *C. lindemuthianum*. Este resultado demuestra la ideonidad del uso de sistemas de expresión heterólogos en levaduras para la producción de PNLs y su caracterización con fines biotecnológicos, considerando que de acuerdo a los antecedentes es posible incrementar la producción de la

PNL producida por este sistema modificando las condiciones de crecimiento e inducción para alcanzar un porcentaje mayor al 30% de PNL en el medio extracelular (Qureshi et al 2010).

Se han encontrado resultados variables cuando se expresan genes que codifican enzimas hidrolíticas en sistemas de expresión en bacterias, levaduras y hongos, por lo que es posible que las diferencias entre el tamaño determinado para la PNL producida por *P. pastoris* y la calculada a partir de la secuencia de aminoácidos de CLPNL2 se deban a la glicosilación diferencial entre ambos sistemas (Benen et al. 2000; Nevalainen et al. 2005; Jeoh et al. 2008).

Se conoce poco sobre el papel de la glicosilación en la actividad de las enzimas, sin embargo se sabe que esta modificación puede influir en la termodinámica de las enzima y modular características cinéticas como la estabilidad, flexibilidad estructural y actividad (Nevalainen et al. 2005; Jeoh et al. 2008; Shental-Bechor y Levy 2009), y ya que la este es uno de los principales problemas de los sistemas de expresión heterólogos, el estudio de la glicosilación es un aspecto fundamental para hacer más eficiente la producción de enzimas y como una oportunidad para modificar características cinéticas y mejorar la estabilidad de enzimas con fines biotecnológicos.

El uso de sustratos complejos como residuos agrícolas, requiere del conocimiento de los sistemas de degradación de los componentes de la pared celular completos, detalles sobre los sistemas de regulación de los genes que las codifican, las propiedades bioquímicas de las enzimas y el efecto de las modificaciones post-traduccionales en sistemas de expresión heterólogos, etc., así como contar con una colección de genes de diferentes fuentes, asumiendo la relación propuesta por nosotros entre el estilo de vida y la eficiencia catalítica. Este tipo de información es importante ya que permitiría el desarrollo de nueva tecnología para la remoción selectiva de componentes de la pared celular sin dañar los polímeros deseados.

PERSPECTIVAS

Es importante estudiar el papel de la glicosilación en la actividad de la enzima, utilizando tanto la PNL nativa como la recombinante producida por *P. pastoris*. Contar con esta información permitiría establecer mejores estrategias de producción de la enzima con fines biotecnológicos.

Como se mencionó en la discusión, conocer las características bioquímicas de las enzimas es un factor importante en la toma de decisiones si se pretende emplearlas en procesos biotecnológicos, por lo que es fundamental concluir el protocolo de purificación de la PNL nativa y realizar su caracterización bioquímica.

Hemos comentado que la pared celular vegetal es el principal reservorio de carbono en la naturaleza y por lo mismo constituye una fuente de materiales de aplicación industrial, tales como los biocombustibles y otros biomateriales. Hasta la fecha, se han pretendido usar productos agrícolas como fuente de carbohidratos, sin embargo, este hecho es muy cuestionado por la sustitución de cultivos para la alimentación y su conversión en combustibles y otros materiales. Los llamados biocombustibles y biomateriales de segunda generación, buscando solucionar este hecho, han propuesto el uso de cultivos de plantas no alimenticias, como la Jatropha o la higuerilla. Sin embargo, la sustitución de cultivos comestibles para el cultivo de dichas plantas ha sido un factor limitante para el desarrollo de esta tecnológia. En este este contexto, ha cobrado importancia el uso de materiales agrícolas de desecho o malezas. Estos son los llamados biomateriales de tercera generación. A pesar de su atractivo de no sustituir cultivos, contener una biomasa de gran importancia y aparentemente no aumentar la tasa de CO_2 en la atmósfera, se trata de materiales que tienden a ser muy complejos en sus carbohidratos (en general, paredes celulares secundarias o tejidos altamente lignificados). Este punto ha detenido su expansión hasta encontrar una manera eficiente de movilizar sus componentes sin hacer uso de pretratamientos químicos o mecánicos que consumen energía convencional. En este punto, el uso de sistemas de degradación enzimática cobran importancia.

Dado que uno de los principales retos de la biotecnología es la remoción selectiva de componentes de la pared celular y controlar el proceso de degradación para generar los productos finales deseados, es importante:

- Conocer las enzimas que componen los sistemas de degradación de la parad celular vegetal.
- Conocer la forma en la que los genes que codifican dichas enzimas son regulados y clonarlos.
- Establecer sistemas de expresión de los genes que codifican estas enzimas y realizar la caracterización bioquímica de las mismas.

Teniendo la información anterior y tomando en cuenta los resultados obtenidos en este estudio, es importante que las enzimas a utilizar en una aplicación biotecnológica específica y el origen de las mismas se defina de acuerdo a la composición de la materia orgánica a procesar. Por lo que es fundamental:

- Conocer la composición de la pared celular vegetal.
- Contar con un complejo de enzimas con actividades específicas para la degradación completa de los polímeros que componen la pared celular vegetal.
- Diseñar sistemas de expresión de genes modulados de acuerdo a la composición de la materia orgánica y el producto final deseado.

A un nivel más básico y de acuerdo a nuestros resultados, al menos existen dos niveles que determinan el estilo de vida de los microorganismos; uno relacionado con la evolución de las enzimas y otro relacionado a la regulación de la expresión de las mismas, por lo que sería importante:

- Realizar el análisis filogenético y la comparación de estructuras terciarias de otras enzimas pectinolíticas (poligalacturonasas y pectato liasas) y enzimas que componen los sistemas de degradación de los polímeros de la pared celular vegetal, de microorganismos con diversos estilos de vida.

- Las enzimas pectinolíticas en muchos casos han sido consideradas como factores de virulencia, por lo que es posible que se encuentren bajo una fuerte presión de selección. Para estudiar esto, sería interesante analizar si los genes que codifican pectin liasas de microorganismos con diferentes estilos de vida han sido seleccionados positivamente.
- Cuantificar la selección de las pectin liasas en diferentes especies de *Colletotrichum* analizando los cambios sinónimos y no sinónimos de los genes que las codifican.
- Determinar los residuos de aminoácidos de las pectin liasas que han estado bajo selección positiva de diversificación y localizarlos en los alineamientos múltiples de estructuras tridimensionales para, de esta manera, determinar si estos sitios pueden estar contribuyendo a la especificidad de sustratos.
- Identificar y caracterizar los dominios catalíticos para modificarlos y obtener enzimas más activas y selectivas.
- Analizar la expresión de los genes que codifican para pectin liasa en diversas razas de *C. lindemuthianum* con diferentes grados de virulencia.
- Analizar la existencia de una asociación ecológica entre la raza no patógena de *C. lindemuthianum* con una o varias razas patógenas de *C. lindemuthianum* en cultivos de frijol para determinar la existencia de un posible modelo de ingeniero ambiental.

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ANEXO I

Artículo de revisión: Lara-Márquez, A., Zavala-Páramo, M. G., López-Romero, E., and Cano-Camacho, H. 2011. Biotechnological potential of pectinolytic complexes of fungi. Biotechnology Letters.

Biotechnol Lett (2011) 33:859–868
DOI 10.1007/s10529-011-0520-0

REVIEW

Biotechnological potential of pectinolytic complexes of fungi

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Received: 2 September 2010 / Accepted: 6 January 2011 / Published online: 19 January 2011

Abstract Plant cell wall-degrading enzymes, such as cellulases, hemicellulases and pectinases, have been extensively studied because of their well documented biotechnological potential, mainly in the food industry. In particular, lytic enzymes from filamentous fungi have been the subject of a vast number of studies due both to their advantages as models for enzyme production and their characteristics. The demand for such enzymes is rapidly increasing, as are the efforts to improve their production and to implement their use in several industrial processes, with the goal of making them more efficient and environment-friendly. The present review focuses mainly on pectinolytic enzymes of filamentous fungi, which are responsible for degradation of pectin, one of the major components of the plant cell wall. Also discussed are the past and current strategies for the production of cell wall-degrading enzymes and their present applications in a number of biotechnological areas.

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Keywords Aspergillus · Fungi · Pectin · Pectinases

Introduction

The cell wall of plants is the largest source of biomass in nature and is used in a wide spectrum of processes, most of which rely on mechanical or chemical methods and many are poorly efficient, highly complicated, aggressive to the environment and costly. Compared to commonly applied industrial processes, the use of enzymes exhibits attractive features such as increased stability and high activity, biocatalytic specificity and the reduction of undesirable byproducts. Likewise, the required temperature, pH, and pressure conditions are moderate as compared to those used in chemical procedures; furthermore, enzymes are biodegradable, a property that reduces the problems of chemical toxicity, thus being more environment-friendly and also a viable alternative in economic terms (Hoondal et al. 2002; MacCabe et al. 2002).

Due to biotechnological advances made during the past three decades, and because of the growing demand to replace and optimize some traditional processes involving microorganisms or their enzymes, enzymes such as cellulases, hemicellulases and pectinases are continuously been investigated as they exhibit potential applications in food, wine, animal

feed, textile, paper, fuel, and other industries (Bhat 2000; Hoondal et al. 2002).

Filamentous fungi exhibit characteristics that make them good models for industrial applications. Noteworthy, among some of these are their capacity for fermentation, the production of large quantities of extracellular enzymes (e.g. several grams per liter in strains of *Aspergillus*), the feasibility of cultivation, and the low-cost of production in large bioreactors (de Vries and Visser 2001; Aro et al. 2005). The filamentous fungi most frequently used for the production of polymer-degrading enzymes are *Trichoderma reesei* and a number of strains of *Aspergillus* and *Penicillium* (Aro et al. 2005; Cardoso et al. 2007). Here we emphasize the biotechnological importance of pectinolytic enzymes produced by filamentous fungi and the strategies for their application and improvement of efficiency in the food industry.

Pectin structure and depolymerization

Pectin is one of the major components of the primary cell wall of plants, the main constituent of the middle lamella and is found also in dividing cells and in the areas of contact between cells having a secondary cell wall including xylem and fibrous cells of woody tissue. Pectin comprises about 35% of the primary cell wall of dicots and non-graminaceous monocots. The content of pectin in secondary walls is greatly reduced; however, pectin plays an important role in the structure and function of both primary and secondary cell walls. The functions of pectin in cell walls are diverse and include plant growth and development, morphogenesis, defense, cell adhesion, cell wall structure, cellular expansion, porosity, ion bonding, hydration of seeds, leaf abscission and fruit development, among others (Willats et al. 2001; Mohnen 2008). Pectin comprises polysaccharides rich in galacturonic acid (GalA) that are present in the form of covalently-linked structural domains: homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Willats et al. 2001; Mohnen 2008). HG is the most abundant component in pectin (<65%). It is formed by nearly 100 GalA residues joined by α-D-1,4 bonds that can be modified by methyl-esterification in C-6, or by the presence of acetyl groups in O-2 and O-3 (Mohnen 2008). XGA is a HG backbone, where 25–75% of the GalA units are substituted at C-3 with one xylose residue and occasionally with a second xylose residue at C-4 (Coenen et al. 2007; Mohnen 2008). RG-I, comprising 20–35% of pectin, is a more complex polymer consisting of a backbone of repeating units of the -α-D-GalA-1,2-α-L-Rha-1-4-disaccharide, showing between 20 and 80% of the rhamnose residues substituted with lateral chains of arabinose and galactose oligomers of variable size. Occasionally, lateral chains of fucose and glucuronic acid are also present (Willats et al. 2001; Mohnen 2008). RG-II is the most complex structural domain of pectin and it is formed by a main chain of GalA residues joined by -α-D-1,4 bonds containing lateral chains made up of 12 different sugars (arabinose, galactose, xylose, fucose, aceric acid, glucuronic acid, apiose, etc.) (Mohnen 2008). The structural domains of pectin interact between themselves and with other molecules such as calcium, borate, polyamines, and phenolic compounds, which contribute to the properties of the pectin matrix. The polymers that make up the plant cell wall also interact by means of hydrogen bonds with microfibers of cellulose, hemicellulose, pectin and aromatic polymers of lignin, and with pectin and xyloglucans or xylans through covalent bonds (Popper and Fry 2008).

Fruits and vegetables are particularly rich in pectin which represents a large part of their biomass (>30%) (Table 1) while the amount of this polymer is lower in higher plants (1%) and in lignified tissues (Hoondal et al. 2002). In immature fruits, the insoluble pectin is bonded to the cellulose microfibers thus conferring rigidity to the cell wall. Along the maturation process, pectin is modified by plant pectinases which make it more soluble resulting in the softening of the cell wall (Kashyap et al. 2001). The degree of pectin esterification depends on the origin, growth and maturity stage of fruits and vegetables (Herron et al. 2000). Pectin types are classified according to their degree of esterification as low, medium/partial and high when the content of ester groups is less than 50%, between 50 and 70% and over 70%, respectively (Ralet et al. 2003).

The gelifying capacity of pectin is conferred by several factors such as temperature, pectin type (degree of methyl-esterification and acetylation), pH, and sugar and calcium concentrations. Gel is

Table 1 Percentage and degree of esterification of pectin in different fruits and vegetables

Fruit/vegetable	State of tissue	Pectin (%)	Esterification (%)	Reference
Golden apple	Dry	16–22	50–58	Koubala et al. (2008)
Banana	Fresh	0.7–1.2		Jayani et al. (2005)
Apricot	Fresh	0.1–0.9		Jayani et al. (2005)
Strawberry	Fresh	0.6–0.7		Jayani et al. (2005)
Cherry	Fresh	0.2–0.5		Jayani et al. (2005)
Pea	Fresh	0.9–1.4		Jayani et al. (2005)
Carrot	Dry	6.9–18.6		Jayani et al. (2005)
Orange pulp	Dry	12–28		Jayani et al. (2005)
Potato	Dry	1.8–3.3		Jayani et al. (2005)
Tomato	Dry	2.4–4.6		Jayani et al. (2005)
Sugar beet pulp	Dry	4.1–16	2–4.2	Yapo et al. (2007)
Maracuya skin	Dry	18.5	60	D′Addosio et al. (2005)
Soybean husk	Dry	7–16		Miyamoto and Chang (1992)
Sweet lemon	Dry	9–30	58–82	Koubala et al. (2008)
Peach pulp	Fresh	12	82	Pagán et al. (2001)
Peach pulp	Dry	18	38	Pagán et al. (2001)
Cacao husk	Dry	2.6–4.7	37.94–52.20	Barazarte et al. (2008)

formed when portions of HG link between them forming a crystalline network that traps water. In highly-esterified pectin, linkages are formed by hydrogen bonds and hydrophobic forces between the methyl groups of HG, whereas in low-esterified pectin bonds between calcium and free carboxyl groups are formed (Willats et al. 2006).

Due to its complex and heterogeneous structure, pectin degradation requires the combined action of different enzymes that are classified according to their mode of action and the substrate upon which they act. These include polygalacturonases (PG), which degrade HG by hydrolysis of glycosidic bonds and are classified as endo-PG (PGA; E.C. 3.2.1.15) and exo-PG (XPG; E.C. 3.2.1.67) (de Vries and Visser 2001; Benen and Visser 2003). Lyases such as pectate lyases (PLY; E.C. 4.2.2.9 and 4.2.2.2) and pectin lyases (PEL; E.C. 4.2.2.10) catalyze the degradation of polygalacturonate and esterified pectin, respectively, through β-elimination by removing a proton and generating an unsaturated bond between the C-4 and C-5 carbons of the nonreducing end of pectin (Fig. 1a). PLYs are specific for non-esterified pectin and depend on Ca^{2+}, while PEL's degrade methyl-esterified pectin and do not depend on this cation. Arg^{236} in PELs plays a similar role of that of Ca^{2+} (Herron et al. 2000; Jayani et al. 2005). The acetyl, methyl and feruloyl residues of pectin are removed by pectin methylesterases (PME; E.C. 3.1.1.11), acetylesterases (AE; E.C. 3.1.1.6), ramnogalacturonan acetylesterases (RGAE; EC 3.1.1.) and feruloyl esterases (FAE; EC 3.1.1.73). The activity of these enzymes is important for the complete degradation of pectin, as they promote the activity of other enzymes; for instance, the efficient of degradation of pectin by PGs and PLYs is largely dependent on the activity of PMEs (de Vries et al. 2000; de Vries and Visser 2001).

The endorhamnogalacturonan hydrolases (RHG; E.C 3.2.1.) degrade -α-D-GalA-1,2-α-L-Rha linkages in RG-I by hydrolysis, while rhamnogalacturonan lyase (RGL; E.C. 4.2.2.) degrade -α-L-Rha-1,4-α-D-GalA linkages by β-elimination (Fig. 1b). Rhamnogalacturonan rhamnohydrolase (RRH; E.C. 3.2.1.) and ramnogalacturonan galacturonohydrolase (RGH; E.C. 3.2.1.) degrade oligosaccharides from the non reducing end by an exo attack (de Vries and Visser 2001; Voragen et al. 2001). Also, two new pectinases were found capable of degrading XGA in *Aspergillus tubingensis*: an exogalacturonase able to remove a Xyl-GalA disaccharide and an endoxylogalacturonan hydrolase that cleaves 1,4-α-D-GalA linkages in XGA (Beldman et al. 1996; Van der Vlugt-Bergmans et al. 2000).

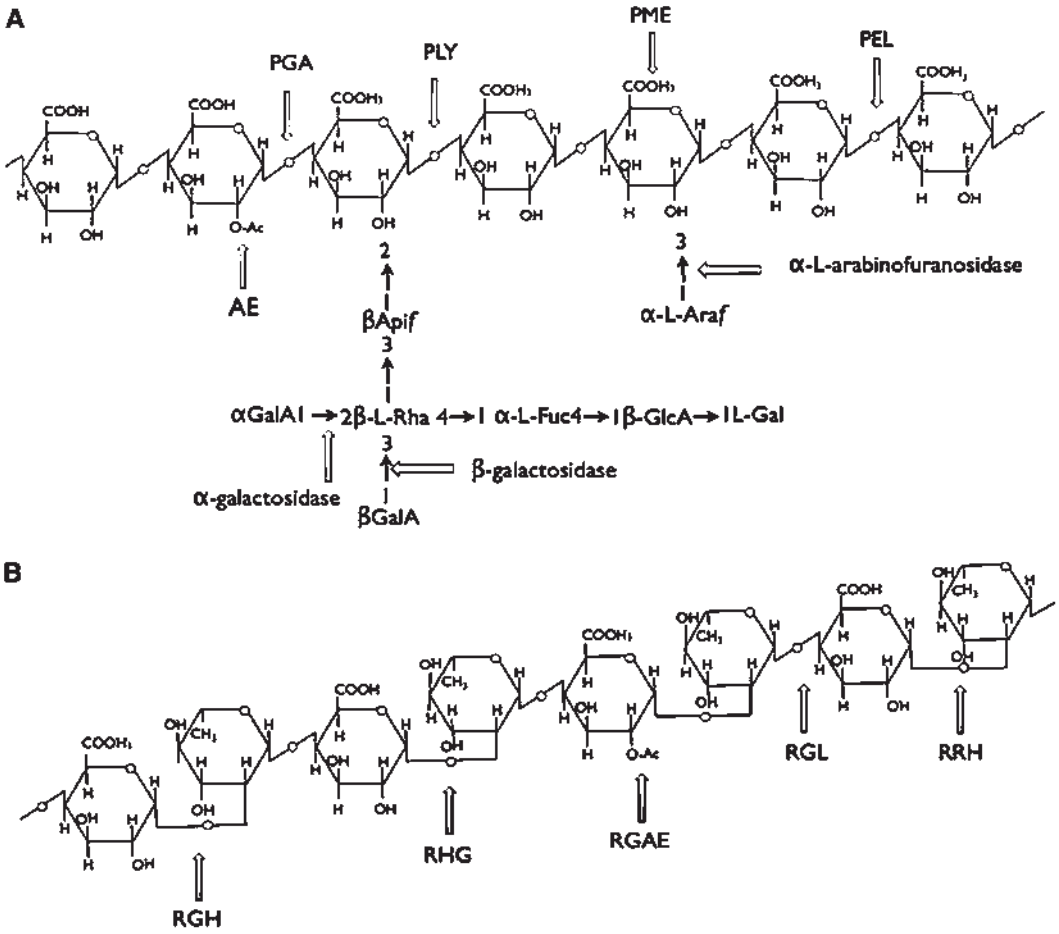

Fig. 1 Representative structure of pectin and site of action of enzymes involved in their degradation. **a** pectinolytic enzymes involved in the degradation of the HG backbone and lateral chains of RG-II, **b** depolymerases of the RG-I backbone (Voragen et al. 2001; Mohnen 2008)

Accessory enzymes acting on the lateral chains of RG-I and RG-II include endogalactanases (E.C 3.2.1.89), exogalactanases (E.C. 3.2.1.145), α- and *β*-galactosidases (E.C 3.2.1.22 and 3.2.1.23) (Fig. 1a), α-L-arabinofuranosidases (E.C. 3.2.1.55), endoarabinases (E.C. 3.2.1.99) and exoarabinases (E.C. 3.2.1.) (de Vries and Visser 2001; de Vries 2003).

Although many genes that encode pectinolytic enzymes have been cloned and several of the enzymes characterized (for a review see De Vries and Visser 2001; De Vries et al. 2005; Martens-Uzunova and Schaap 2009), many others have not been identified (De Vries et al. 2005). Currently, a large number of genomes of filamentous fungi including species of biotechnological and pathogenic importance have been sequenced. In particular, the analysis of genomes from *A. nidulans* and *A. niger* has proven the potential of this approach. De Vries et al. (2005) reported that more than two-thirds of the ORFs putatively involved in plant cell wall polysaccharide degradation that were found in the genome of *A. nidulans* encode novel enzymes; also, they identified ORFs that apparently encode putative intracellular oligosaccharide degrading enzymes and others with homology to oligosaccharide transporters of other organisms. The analysis of the *A. niger* genome revealed 21 genes belonging to family 28 of glycosyl hydrolases (Martens-Uzunova et al. 2006), 39 genes encoding enzymes involved in the depolymerization of the pectin-backbone and genes encoding enzymatic activities for the degradation of pectin side-chains (Martens-Uzunova and Schaap 2009). The

genomic sequences of these fungi and the use of bioinformatic tools have allowed a rapid progress in the study of pectinolytic genes, shown the complexity of pectin and how much we have still to learn about these processes.

Saprophytic and phytopathogenic fungi not only need to have the repertoire of enzymes required to degrade cell wall components, but also a well-coordinated regulation of their corresponding genes. de Vries (2003) proposed that since *Aspergillus* is not able to import polysaccharides; most likely that monosaccharides or small oligosaccharides, generated as products of the same enzymatic reactions, are the true inducers of the regulatory system. Some evidences supporting this notion include the observations that ferulic acid, L-arabinose/L-arabitol, D-mannose, GalA or compounds containing GalA, induce the expression of feruloyl esterases (Faulds et al. 1997), α-L-arabinofuranosidase (de Vries et al. 1994; Van der Veen et al. 1993), β-mannosidase (Neustroev et al. 1991) and a large number of genes encoding pectinolytic enzymes (de Vries et al. 2002), respectively. For this reason, several authors have proposed the existence of a regulatory system that activates the expression of a group of pectinolytic genes in response to GalA, and activates to others pectinolytic genes in response to L-arabinose, L-rhamnose, ferulic acid and D-xylose (Pařenicová et al. 2000; de Vries et al. 2002; Martens-Uzunova et al. 2006). Efficient degradation of the plant cell wall requires a synergistic interaction among wall component depolymerases (de Vries and Visser 2001). This was confirmed by de Vries et al. (2000) when they analyzed six accessory enzymes involved in the degradation of hairy regions of sugar beet pectin. At the same line, Martens-Uzunova and Schaap (2009) analyzed the expression of 46 genes encoding pectinolytic enzymes in three transcriptomes of *A. niger* by DNA microarray and proposed a cascade model for pectin degradation. This relies on a synergistic relationship between main chain-cleaving enzymes and accessory enzymes that result in the release of monomeric sugars, which in turn induce the activity of other enzymes.

As mentioned above, the composition of pectin and the overall plant cell wall is complex and fungi need to possess an efficient and well-coordinated degradation system to successfully confront this complexity. On this basis, the existence of a multifunctional control system for pectin degradation has been suggested (Martens-Uzunova and Schaap 2009). However, despite the progress achieved in the last two decades largely due to the genomic analysis, the regulation of pectinolytic genes in fungi is still poorly understood.

Much of the information on genes encoding pectinolytic enzymes and their regulation comes from studies carried out in *A. niger* and *A. nidulans,* which exhibit characteristics that make them good models for biotechnological purposes (de Vries 2003). Phytopathogenic fungi represent a good alternative source of pectinolytic genes and in general of genes encoding cell wall-depolymerases, since these organisms secrete a large variety of these enzymes during penetration and colonization of the plant tissue (Mendgen et al. 1996). A good example of these phytopathogens is the genus Colletotrichum which produces cutinase (Chen et al. 2006), glucoamylase (Krause et al. 1991), laccase (Guetsky et al. 2005), polyphenol oxidase (Singh 1968), cellulase, cellobiohydrolase, β-glucosidase (Acosta-Rodríguez et al. 2005) and several pectinases (Templeton et al. 1994; Wei et al. 2002; Hernández-Silva et al. 2007; Ramos et al. 2010) extracellularly.

Production of pectinolytic enzymes of biotechnological interest

Enzymes that degrade plant cell wall have been extensively studied for their biotechnological potential. The use of pectinolytic enzymes is being implemented in various production processes, mainly in the food industry. In juice and wine production, enzymes have been used to improve the yield, decrease the viscosity, clarify the juices and make them more stable (Kashyap et al. 2001; Wu et al. 2007). Unicellular products used for the preparation of nectars, baby food and yoghurt are generated through selective hydrolysis of the polysaccharides of the middle lamella to preserve the integrity of the cell wall (Kashyap et al. 2001). Preparations containing pectinases, cellulases and hemicellulases, used for the extraction of olive oil, increase the yield and the content of polyphenols and vitamin E (Kashyap et al. 2001). During coffee fermentation, the use of pectinolytic microorganisms reduces the fermentation

time (Amorim and Amorim 1977). In addition of their use in the food industry, pectinolytic enzymes are useful in textile processing (Hoondal et al. 2002), in the pulp and paper industries (Bajpai 1999), water treatment (Tanabe et al. 1986) and as tools for analysis of polysaccharides of plant cell wall (Bauer et al. 2006), among other applications.

In economic terms, the use of enzyme extracts is a viable option if it is taken into account that purification procedures significantly increase their cost of production. However, the use of enzymatic preparations has been questioned due to the presence of pectin methylesterases that releases methanol whose concentration and deleterious effects on the final product remain undetermined. Another objection is the presence in the commercial preparations of other undesired enzymatic activities that may be detrimental to the final smell of the product (Mantovani et al. 2005). Several alternatives have been suggested to obtain specific enzyme activities. These include the use of organisms producing a single type of pectinolytic enzyme, the search for fermentation conditions allowing the maximum production of the desired activity and fractioning of the enzymatic extracts to purify the desired enzyme (Mantovani et al. 2005). For the preparation of enzyme extracts, the solid state fermentation (SSF) system has been suggested for large-scale production of pectinases using a number of solid residues, many of which are agro-industrial residual byproducts such as soy (Castilho et al. 2000), bran and apple (Hang and Woodams 1994), cranberry and strawberry pulp (Zheng and Shetty 2000), sugar beet (Spagnuolo et al. 1997), coffee pulp (Boccas et al. 1994), lemon and orange peel (Mamma et al. 2008), tomato pomace (Freixo et al. 2008), and mixtures of orange and sugarcane bagasse with wheat bran as a substrate (Martin et al. 2004). In the past two decades, the use of SSF has been extended because of its advantages over the submerged fermentation (SmF) procedure, such as the larger amounts of biomass produced, a decreased proteolytic effect, a larger resistance to catabolic repression, the reduced energy and oxygen demands in comparison to the high expenditures needed by SmF systems, and an increased enzyme yield (Viniegra-González et al. 2003).

Cell immobilization offers numerous advantages over ordinary suspension cultures. These include cell stability, higher cell densities, enhanced fermentation productivity, feasibility of continuous processing, shorter fermentation times, increased tolerance to high substrate concentration, reduced end product inhibition, lower costs of recovery and recycling, elimination of the need to purify the enzymes and an increased resistance to environmental perturbations (Kourkoutas et al. 2004; Nighojkar et al. 2006). Various supports and immobilization techniques have been proposed and tested for application in wine- and cider-making, brewing, distillates, potable alcohol and novel beverages production (Kourkoutas et al. 2004). However, much information about immobilization of microbial cells for production of extracellular pectic enzymes is lacking (Nighojkar et al. 2006). Angelova et al. (1998) immobilized spores of *A. niger* in calcium alginate and the resulting immobilized mycelium was used for the batch production of polymethylgalacturonase. Nighojkar et al. (2006) also proved the immobilization of whole cells of *A. niger* in different systems, sodium alginate, glutaraldehyde-treated alginate and PVA-alginate gel, using dried orange peel powder as inducer for the production of PGs. Their results support the convenience of these systems for a semi-continuous production of PGs.

Alternatively, the immobilization of enzymes on solid matrixes has also been suggested. This strategy offers several advantages over the use of free enzymes, namely, high activity and stability, the ability to use them at high concentrations and increased accessibility to the substrate, and consequently a reduction of reaction times. Immobilization also allows recovery and reuses of enzymes, avoiding its presence in the final product, the ability to use multi-enzyme reactions, artificial metabolic pathways and the compartmentalization of catalytic reactions (Brady and Jordaan 2009). For immobilization of pectinases, several methods have been developed using different supports such as nylon (Vaillant et al. 2000; Diano et al. 2008), Eupergit C (Spagna et al. 1995), glass (Diano et al. 2008), chitosan and chitin (Vaillant et al. 2000), polyacrylonitrile (Diano et al. 2008), cellulose, XAD-amberlites, sulfides, γ-alumina (intermediate crystalline form of Al_2O_3), bentonite (Spagna et al. 1995) and CIM-disk epoxy monolith [epoxy columns composed of a disk of methacrylate-based porous polymer matrix a non-porous (monolith) (Delattre et al. 2008). Most research on immobilization has been carried out using commercial enzyme

mixtures for the co-immobilization of pectinolytic enzymes (Vaillant et al. 2000; Diano et al. 2008).

One of the most widely utilized techniques for the production of enzymes of biotechnological interest is their heterologous expression in filamentous fungi and yeasts exhibiting a high capability for the production and secretion of proteins. However, post-translational modifications, such as glycosylation, proteolytic cleavages and the formation of disulfide bonds, differ between filamentous fungi and yeasts and even between fungi such as *Aspergillus* and *Trichoderma* (Benen et al. 2000; Nevalainen et al. 2005; Jeoh et al. 2008). Glycosylation is the main post-translational modification of lytic enzymes that can affect their stability and activity (Nevalainen et al. 2005; Jeoh et al. 2008); yet, cases have been reported where no significant differences are observed between recombinant and native proteins (Benen et al. 2000).

Engineering approaches have allowed the development of systems for the overexpression of functional heterologous proteins in the yeast cell surface. This strategy has been used for the expression of amylolytic and cellulolytic enzymes to implement multiple enzymatic processes for the production of bioethanol (Kuroda and Ueda 2010) and could also be a good alternative for the production of pectinolytic enzymes.

The use of *Escherichia coli* as a heterologous expression system has given good results; however, the lack of translational modification and the inadequate secretion are the main drawbacks for its application in biotechnological purposes (Yoon et al. 2010). Nevertheless, there are examples of expression of fungal pectinase-encoding genes in this prokaryote in which the lack of glycosylation or other post-translational modifications do not affect enzyme activity (Zhao et al. 2007). Proteins expressed in *E. coli* are localized to the cytoplasm or periplasm; however, this bacterium has non-specific mechanisms that allow outer membrane and periplasmic proteins to be secreted. In some cases, the levels of secretion have been compared with those obtained in other expression systems (Yoon et al. 2010).

Another commonly used strategy is the overexpression of enzymes by the introduction of multiple copies of genes with strong promoters such as those of glyceraldehyde-3-phosphate dehydrogenase in *P. griseoroseum* (Cardoso et al. 2007), pyruvate kinase in *A. niger* (Benen et al. 2000) and the translation–elongation factor 1 alpha in *A. oryzae* (Kitamoto et al. 2001a, b). Accordingly, Cardoso et al. (2007) inserted multiple copies of the *plg1* gene encoding a PEL from *P. griseoroseum* under the control of the promoter *gpdA* and the terminator region of the tryptophan synthetase gene (*trpC*) from *A. nidulans* into *P. griseorosum* strain PG63. PEL production in carbon sources like sugar cane juice and its predominance over other extracellular proteins, make of this strategy a valuable option for the production of PEL for biotechnological purposes.

Finally, other reported strategies are the genetic transfer between fungal species by protoplast fusion (Solís et al. 2009) and mutagenesis and selection of overproducing strains of desired enzymes. For instance, after a single round of nitrous acid mutagenesis, wild type *P. occitanis* generates a hyperpectinolytic mutant, which constitutively secretes 50-fold higher activities of endo- and exopectinase than the parental strain (Hadj-Taieb et al. 2002).

Conclusions

Enzymes that degrade pectin are relevant in numerous biotechnological applications and even more so in food industry. The use of these enzymes on substrates such as citric peels and other materials containing large amounts of pectin is fundamental to obtain added value products. For these reasons, a big demand exists in the production of stable, highly active enzymes.

Several strategies have been developed for the production of biotechnological important enzymes mainly in *Aspergillus, Trichoderma* and *Penicillium*. The development of technologies that allow the use of organisms or enzymes in bioreactors in various industrial production systems promise to revolutionize fermentation technology. Despite progress, more studies are still needed to scale-up these applications efficiently to industrial levels. Once achieved, it is expected that costs and production times will be decreased, raw materials will be used more efficiently and products will be produced with better features. Selection of enzyme production systems are governed, however, by the characteristics of the target substrate; in other words, to achieve a better yield and quality of the products, the selection of enzyme(s) or

a combination of these, has to be appropriate for the specific biotechnological application as well as for the different phases of the production processes.

Despite the unquestionable advances into the knowledge of pectinolytic genes involved in pectin degradation, essential information is still lacking to develop efficient strategies for the production of specific enzymes. Further studies are required to have full information of the components of pectin degradation systems including all enzymes involved in their degradation, both main chain-cleaving enzymes and accessory enzymes, the details of their regulatory systems, their biochemical properties and the effect of post-translational modifications following their expression in heterologous systems.

The use of complex substrates such as agricultural waste requires knowledge of the full set of cell wall-degrading enzymes. As in the case of the pectinolytic degradation system, it is important to have appropriate sources of genes encoding the necessary enzymes and to know their biochemical and regulatory characteristics. Such information is most valuable to develop more efficient and low-cost technologies aimed to the use of specific activities for the selective removal of cell wall components without damaging the desired polymers and to control the processes of degradation to generate the desired end products.

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ANEXO II

Artículo arbitrado: Lara-Márquez, A., Zavala-Páramo, M. G., and Cano-Camacho, H. 2008. Caracterización del gen *pnl* 2 que codifica la pectin liasa 2 de *Colletotrichum lindemuthianum*. Ciencia Nicolaita.

Ciencia Nicolaita, No. Especial, 2008: 110-117
Universidad Michoacana de San Nicolás de Hidalgo

Caracterización del gen pnl 2 que codifica la pectín liasa 2 de *Colletotrichum lindemuthianum*

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Resumen

En este trabajo se presenta la caracterización estructural del gen *pnl 2* que codifica una pectín liasa del hongo fitopatógeno de frijol *Colletotrichum lindemuthianum,* aislado a partir de una librería genómica (Lara-Márquez, 2004; Calderón-Cortés, 2004). De acuerdo con la secuencia del cDNA del mismo gen, la región codificante es interrumpida por cuatro intrones (60-87 pb). Las secuencias de las regiones 5´y 3´ muestran algunos posibles elementos regulatorios. La secuencia deducida de aminoácidos codifica una proteína de 379 aminoácidos, con un péptido señal de 20 aminoácidos. La comparación de la secuencia deducida de aminoácidos de *pnl2* con las de otros genes que codifican pectín liasas muestra alta homología con *pnl2* de *C. gloesporoides* y permitió realizar un fenograma en el cual se observa la formación de varios grupos. El análisis para la predicción de la estructura tridimensional confirma una conformación típica encontrada en pectinasas.

Palabras clave: Pectina, pectín liasas, ß-eliminación, *Colletorichum*

Abstrac

In this work we present the structural characterization of *pnl 2* gene that codes for a pectin lyase of the phytopathogenic fungus *Colletotrichum lindemuthianum*, isolated from a genomic bank (Lara-Márquez, 2004; Calderón-Cortés, 2004). According to the cDNA sequence the coding region is interrupted by four introns with a size ranging between 60 and 87 pb. The sequence of the 5´ and 3´ regions shows some possible regulatory elements. The pnl2 protein corresponds to 379 amino acids, with a 20 amino acids signal peptide. The comparison of the deduced amino acid sequence of pnl2 and other pectin lyases shows high homology with *pnl2* of *C. gloesporoides*. This allowed us to construct a phenogram in which the formation of several groups is observed. The prediction analysis of the three-dimensional structure confirms the typical conformation found in pectinases.

Keywords: Pectine, Pectin lyases, ß-elimination, *Colletorichum*

Introducción

Colletotrichum lindemuthianum es el agente causal de la antracnosis en frijol. Durante el proceso de infección a su hospedero, el hongo secreta una serie de enzimas que degradan los polímeros de las paredes celulares vegetales. Entre estas se encuentran las pectín liasas, las cuales son de las primeras enzimas secretadas por los hongos patógenos durante el proceso de infección y se ha visto que son capaces de macerar las paredes celulares por sí mismas (Ranveer *et al*., 2005).

Debido a su potencial biotecnológico, estas enzimas se estudian ampliamente, enfocándose principalmente en bacterias fitopatógenas. Se han aislado y caracterizado varios genes que codifican pectín liasas de algunos hongos saprófitos como *Aspergillus niger* y de hongos fitopatógenos como *C. gloesporoides*. A partir de algunos de estos genes se producen preparaciones comerciales que contienen mezclas de pectinasas, así como enzimas puras, que luego son utilizadas principalmente en la industria de los alimentos (Bussink, 1992; Kusters-van Someten, 1992; Annis y Goodwin, 1997). Sin embargo, es poco lo que se sabe sobre las estructuras de los genes de pectinasas de hongos fitopatógenos, así como sobre su regulación y la bioquímica de las enzimas que codifican.

En este trabajo se analizó una secuencia de 2,136 pb correspondiente al gen *pnl2* que codifica la pectín liasa 2 de *C. lindemuthianum*, aislado a partir de una librería genómica. También se analizó el cDNA correspondiente, aislado a partir de un cultivo de micelio inducido con pectina (Calderón-Cortés, 2004; Lara-Márquez, 2004; Lara-Márquez, 2007).

Materiales y Métodos

Aislamiento del DNA y cDNA de pnl2

La secuencia de 2,136 pb, correspondiente al gen *pnl2* de *C. lindemuthianum*, se obtuvo a partir de la secuenciación de ambas hebras de un fragmento de 4 Kb obtenido del escrutinio de la librería genómica de *C. lindemuthianum* raza 1247 y subclonado en pUC18 (Lara-Márquez, 2004; Calderón-Cortez, 2004).

EL cDNA del gen *pnl 2* de *C. lindemuthianum* se aisló a partir de RNA total de micelio crecido por 4 h con pectina como única fuente de carbono. El ensayo de RT-PCR se realizó de acuerdo con las instrucciones de un kit de origen comercial (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen) con oligo-dT, el oligonucleótido directo Pnl67 (5´ATGAAGTCTACCATCTTCTCCG3´) y el oligonucleótido reverso Pnl1569 (5´TTAGATCTTGCGAAACCGGC3´), los cuales fueron diseñados con base en la secuencia genómica del gen *pnl2* para la amplificación del cDNA.

Análisis de las secuencias

La región estructural del gen *pnl2* de *C. lindemuthianum* se analizó comparándola con la secuencia del cDNA de *pnl2* y la secuencia genómica del mismo con los programas ChromasPro Ver.1.42 para análisis de cromatogramas y edición de secuencias, BioEdit versión 3.6 y Mega4 para alineamientos por el método clustal y para la construcción del fenograma.

Para el análisis comparativo de las secuencias de núcleotidos y deducidas de aminoácidos se obtuvieron las secuencias del GenBank: *C. gloesporoides*

pnl2 (AF156984), *C. gloesporoides pnlA (*L22857), *A. niger pnlB* (X65552), *A. niger pelD* (M55657), *A. niger pnlA* (X60724), *A. oryzea pnl1* (AB029322), *A. oryzea pnl2* (AB029323), *E. nidulans pnl2* (DQ490478), *P. griseoroseum Plg1* (AF502279).

El análisis de las regiones no codificantes 5´y 3` se realizó comparando las secuencias UTR reportadas para genes de enzimas que degradan pared celular y de genes eucariotas en general.

La predicción del sitio de corte del péptido señal se realizó utilizando SignalP 3.0 (Dyrlov *et al*., 2004) y la predicción de la estructura tridimensional se obtuvo por el método Swiss-Model (Arnold, 2006).

Resultados

La comparación de la secuencia del cDNA con la del DNA de *pnl2* reveló la presencia una región no codificante 5´ de 151 pb, una región no codificante 3´ de 562 pb y un marco de lectura abierta de 1,422 pb. La región estructural del gen *pnl2* contiene 4 intrones con longitudes de 87 pb, 78 pb, 63 pb y 60 pb, y 5 exones de 304 pb, 51 pb, 122 pb, 263 pb y 394 pb. Las secuencias *Lariat* de los intrones son similares a los encontrados en otros genes de hongos; sin embargo, sólo el tercer intrón tiene una secuencia que coincide con la secuencia consenso (5´-GTGAGT-3´) correspondiente al sitio de empalme 5´ reportada para hongos filamentosos y una secuencia que coincide con la secuencia consenso de empalme 3´ (5´-YAG-3´) (Kupfer *et al*., 2004).

La región 5´ no codificante (UTR-5´) de *pnl2* contiene algunas secuencias que pueden ser posibles puntos de inicio de la transcripción en las posiciones -107 CAAC, -106 CAACT y -38 CAAC ó -37 CAACT (Gysler *et al*., 1990) (Figura 1), así como una posible secuencia Kozak para iniciación de ribosomas eucariotas (Kozak, 1986). Se localizaron varios elementos que se cree son esenciales para la terminación de la transcripción en la región 3´ no codificante (UTR-3´); una secuencia derivada de la secuencia consenso (5´-AATAAA-3´) encontrada en la mayoría de los ARNm eucariotas, una secuencia similar a la secuencia propuesta como terminal en eucariotas (5´-TTTCACTGC-3´) (Benoist *et al*., 1980). También se localizaron dos de las tres secuencias consenso de las regiones Y-terminal de levaduras (5´-TAGT-3´) y (5´-TIT-3´) (Gysler, 1990).

El gen codifica para una proteína putativa de 379 aminoácidos con una masa molecular deducida de 39.7 kDa y un punto isoeléctrico de 9.1. El posible sitio de corte del péptido señal se localiza entre los aminoácidos en las posiciones 20 y 21 de acuerdo a SignalP 3.0 (Dyrlov *et al*., 2004), lo cual coincide con otros genes que codifican pectín liasas de hongos (Figura 2) (Von Heijne *et al*., 1983). Se localizaron dos sitios de posible N-glicosilación en las posiciones 101 y 130.

Cuando se compararon las secuencias deducidas de aminoácidos de *pnl2* de *C. lindemuthianum* con las de otras pectín liasas de hongos (Figura 2), la mayor identidad se encontró con *pnl2* de *C. gloesporoides* 90.7%, seguido por *pnlA* de *C. gloesporoides* con 80.7%, 59.8% con *pnl2* de *E. nidulans,* 59.0% con *pnl1* de *P. occitanis,* 58.1% con *pel1* de *A. oryzar*, 56.9% con *pelB* de A. niger, 55.1% con *pelA* de *A. niger* y 53.2% con *pel2* de *A. oryzae*. Los sitios

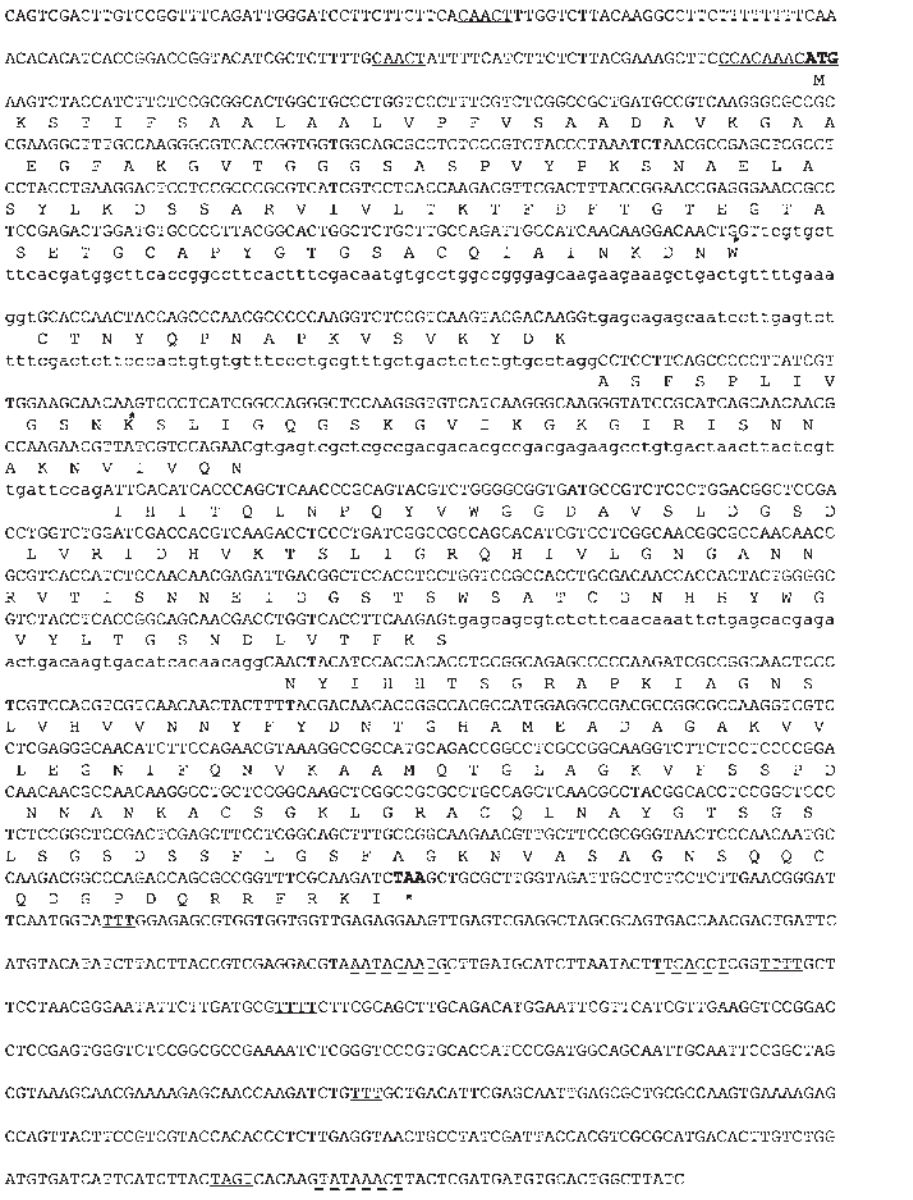

Figura 1. Secuencia de nucleótidos y deducida de aminoácidos del gen *pnl2* de *C. lindemuthianum*. Secuencias correspondientes a intrones en letras minúsculas, a exones en letras mayúsculas. (___) Posibles secuencias de inicio de la transcripción caac ó caact (Gysler *et al*., 1990), así como una posible secuencia Kozak para iniciación en ribosomas eucariotas (Kozak, 1986). En la región no codificante 3´ se señalan algunos posibles elementos involucrados en la terminación de la transcripción; (_ _) una secuencia derivada de la consenso AATAAA, encontrada en la mayoría de RNAs mensajeros eucariotas, así como una secuencia derivada de la propuesta como terminal eucariota TTTCACTGC (Benoist *et al*., 1980). (___) Dos de las secuencias consenso encontradas en regions Y-terminal de levaduras TAGT y TIT (Zaret y Sherman, 1982; Gysler 1990). (*) Posibles sitios de N-glicosilación.

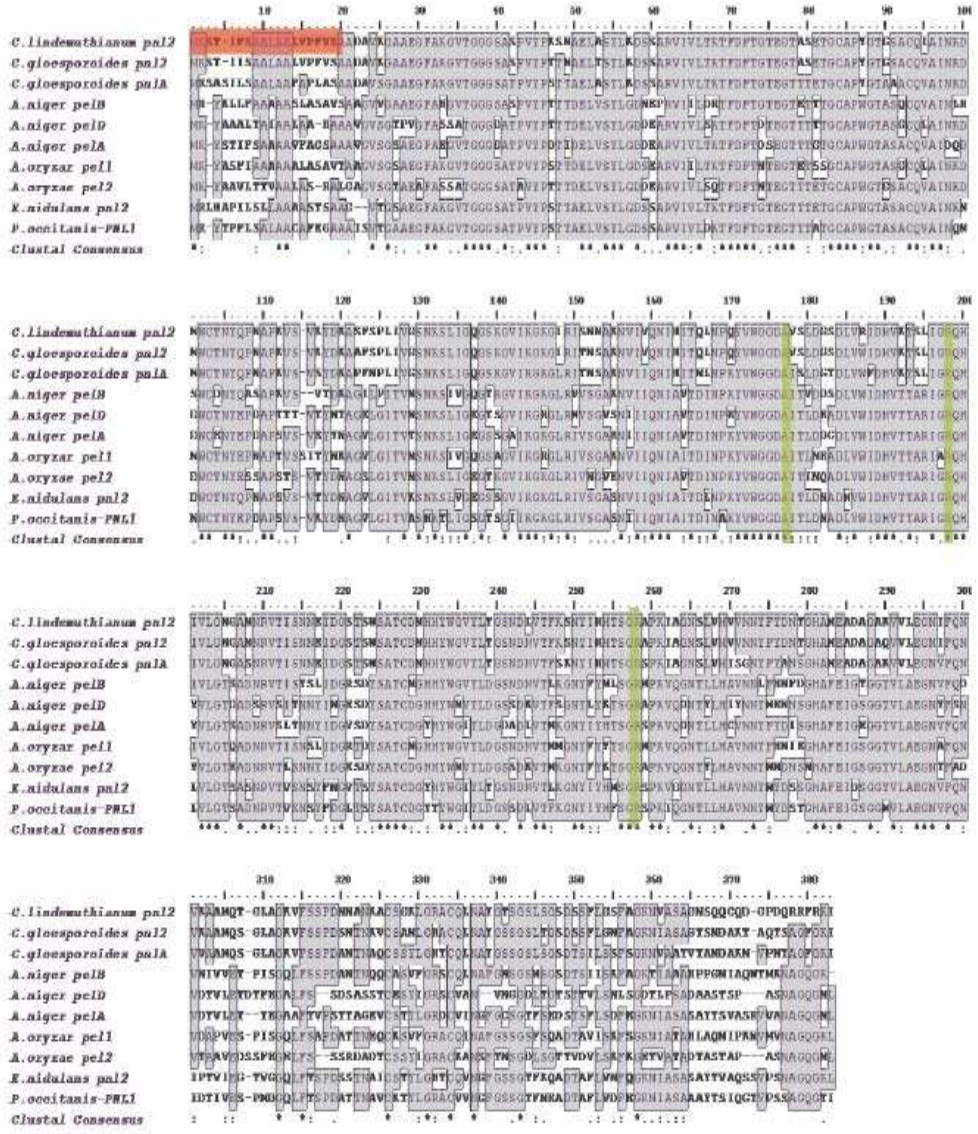

Figura 2. Alineamiento de la secuencia deducida de aminoácidos del cDNA de *pnl2* de *C. lindemuthianum* con las de otros hongos. *C. gloesporoides pnl2* (AF156984), *C. gloesporoides pnlA (*L22857), *A. niger pnlB* (X65552), *A. niger pelD* (M55657), *A. niger pnlA* (X60724), *A. oryzea pnl1* (AB029322), *A. oryzea pnl 2* (AB029323), *E. nidulans pnl2* (DQ490478), *P. griseoroseum Plg1* (AF502279). La secuencia de aminoácidos correspondiente al péptido señal resaltada en rojo, sitios catalíticos resaltados en verde.

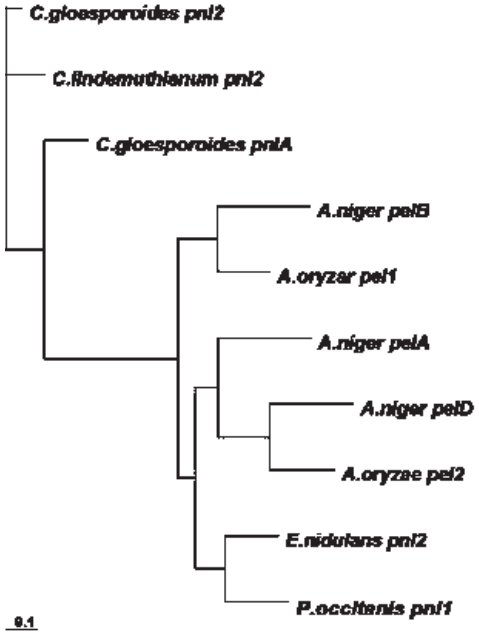

Figura 3. Fenograma de *pnl2* de *C. lindemuthianum* y enzimas pnl de otros hongos con alta similitud de secuencia deducida de aminoácidos. El árbol se realizó en BioEdit por el método de Neighbor-joining versión 3.6ª2.1.

catalíticos, representados por la triada DRR se observan conservados en todas las enzimas (Vitali *et al*., 1996).

El fenograma construido con las secuencias deducidas de aminoácidos de *pnl2* de *C. lindemuthianum* y de otras pectinasas de hongos muestra el agrupamiento de *pnl2* de *C. lindemuthianum* con *pnl2* y *pnlA* de *C. gloesporoides*. Además, se observa que *pnlA* de *C. gloesporoides* se encuentra estrechamente relacionado con el resto de las secuencias de aminoácidos comparadas, las cuales forman otro grupo que se separa a su vez en tres cladas (Figura 3). Es interesante notar que las enzimas de una misma especie se encuentren en distintos grupos, lo que refleja relaciones de funcionalidad más que relaciones de ancestro descendencia de las enzimas.

La estructura tridimensional de *pnl2* de *C.lindemuthianum* fue determinada por el programa de modelaje de proteínas Swiss-Model (Figura 4). Los resultados muestran una similitud del 62% con *pel B* de *A. niger* (Vitali *et al*., 1996). La predicción estructural confirmó la conservación de la topología característica de las pectinasas en β-hélice paralela (Mayans *et al*., 1997; Pickersgill y Jenkins, 2003).

Discusión

El análisis de la secuencia del cDNA de *pnl2* de *C.lindemuthianum* permitió establecer la estructura del gen e identificar secuencias consenso para corte y empalme (splicing) en hongos

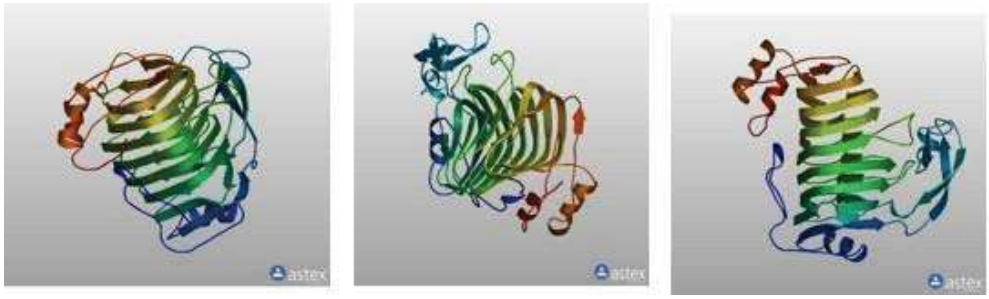

Figura 4. Estructura tridimensional de *pnl2* de *C.lindemuthianum* determinada por el programa Swiss-Model.

filamentosos, así como secuencias de empalme no reportadas anteriormente. Se identificaron también algunos posibles elementos regulatorios en las regiones no codificantes 5´y 3´del gen reportados para genes eucariotas. Sin embargo, sería importante obtener una porción mayor de la región no codificante 5´ con la finalidad de encontrar otros posibles elementos regulatorios y en un futuro realizar un análisis funcional del promotor que permita obtener más información sobre la regulación de estos genes.

La secuencia deducida de aminoácidos del cDNA de *pnl2* de *C. lindemuthianum* muestra alta similitud con las pectinasas utilizadas en la comparación, en particular con los genes *pnl2* y *pnlA* de *C. gloesporoides* un hongo muy relacionado con *C. lindemuthianun*. Además, fue posible localizar residuos de aminoácidos críticos para la actividad catalítica (Vitali *et al*., 1996), los cuales se encuentran conservados en todas las enzimas comparadas.

En el fenograma, *pnl2* de *C.lidemuthianum* se ubicó en una clada con *pnl2* de *C. gloesporoides* y cerca de estas dos se encuentra otra clada con *pnlA*, también de *C. gloesporoides,* evidenciando cercanía entre las dos especies. Por esta razón es posible que *C. lindemuthianum* tenga una enzima correspondiente a *pnlA* en *C. gloesporoides*.

El resto de las enzimas se observan en tres grupos formados por *pelB* de *A. niger* y *pel1* de *A. oryza*; *pelA* de *A. niger, pelD* de *A. niger y pel2* de *A. oryzae*; *pnl2* de *E. nidulans* y *pnl1* de *P. occitanis*. Es interesante notar que las enzimas de una misma especie se ubican en grupos distintos, reflejando posiblemente relaciones de funcionalidad más que relaciones de ancestro de descendencia de las enzimas.

No obstante, no se descarta la posibilidad de que todas estas enzimas evolucionaron a partir de un posible ancestro común, como se ha planteado por varios grupos de investigadores (Yoder, *et al*., 1993; Mayans, *et al*., 1997; Pickersgill y Jenkins, 2003).

Se ha visto que las pectín liasas y pectato liasas comparten una topología similar en β-hélice paralela, la cual fue encontrada inicialmente en *pelA* de *A. niger.* Los resultados obtenidos con el programa de modelaje de proteínas, indican que *pnl2* de *C. lindemuthianum* conserva la misma topología característica de las pectinasas, con una similitud del 62% con *pel B* de *A. niger* (Mayans *et al*., 1997; Pickersgill y Jenkins, 2003).

Agradecimientos

Los autores agradecen el apoyo financiero de la Coordinación de la Investigación Científica de la Universidad Michoacana de San Nicolás de Hidalgo (clave CIC 14.8).

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ANEXO III

Artículo de revisión: Conejo-Saucedo, U., López-Romero, E., Horacio-Cano, C., Lara-Márquez, A., and Zavala-Páramo, G. Z. 2011 Hemicellulases of fungi: A vision of their function in the coordinated degradation of polysaccharides of plant cell walls. Current Trends in Microbiology.

Current Trends in Microbiology

Review

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

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ABSTRACT

Hemicellulases digest 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 the development in biotechnological applications, the food, paper, textile, drink and juice industries. The industrial application of hemicellulases has typically been restricted to the use of a single enzyme in digestive processes, but current research is directed to 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 hemicellulolytic complexes in fungi and their potential in biotechnological applications.

KEYWORDS: hemicellulolytic systems, fungi, 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 enzymes [4].

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 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

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hemicellulolytic complexes and the potential integrated use of these enzymes for the functional 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 taxonomical group to which the plants belong and the degree of development of the cell wall. Cellulose comprises between 35% and 50% of dry weight of plant cell walls, whereas the content of hemicellulose and lignin ranges from 20% to 35% and 5% to 30%, respectively [5, 9]. Hemicellulose, cellulose and lignin form networks of microfibrils with covalent and non-covalent linkages that confer 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/glucuronic acid ramifications [9, 11]. Since these complexes are insoluble in water, 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 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%), while glucomannan is the most abundant 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 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 bind to the ends of pectic polysaccharides and 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, thus greatly affecting 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 xylose) and the xylan of herbal plants [19].

α-D- glucuronic acid and/or 4-O-methyl-α-D-glucuronic acid, bound to the C2 of β-D-xylopyranose in both hard wood xylan (10% xylose) and soft wood xylan (20% xylose) 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 is 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, glucuronic acid, ferulic acid and *p*-coumaric acid [11, 19].

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

Glucuronoarabinoxylan, the xylan of soft woods, which is 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 breakdown 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: 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*-coumaric 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) release 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 breakdown 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 their 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 hydrolytic activity, there are reports of transglycosylation activity for endo-1,4-β-D-mannanases in *A. niger* [23], β-mannosidases in *A. niger* and *Penicillium wortmanni* [24], α-galactosidases in *A. nidulans* [25] and 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 mechanisms) (Table 2).

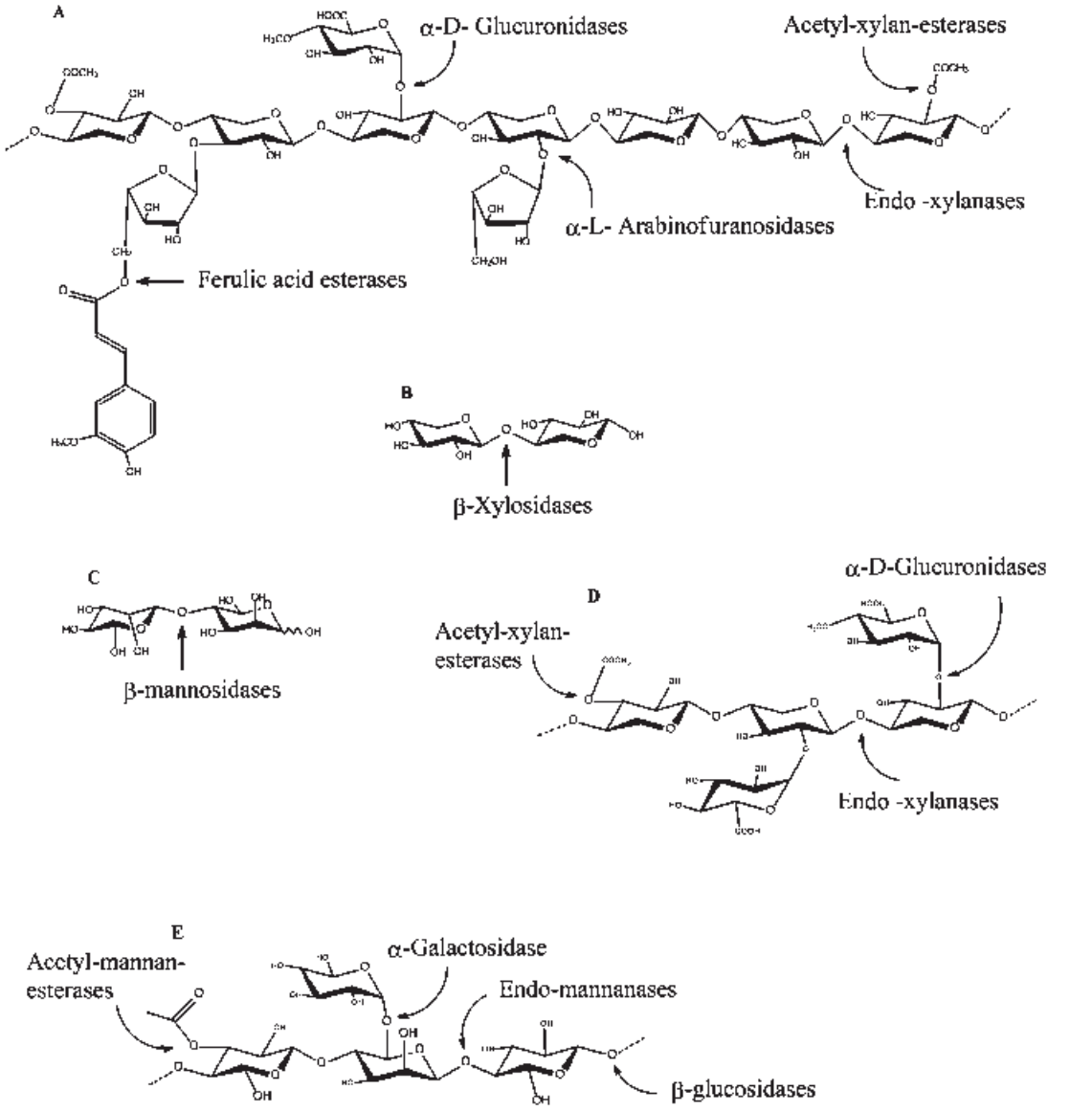

Figure 1. Structural components of hemicelluloses and their breakdown by hemicellulases. A) breakdown of xylan backbone by endo-xylanases and removal of side chains by debranching enzymes. B, C, D and E, hydrolysis of xylobiose, mannobiose, glucoronoxylan and galactoglucomannan, respectively.

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.

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

Microorganism	Enzyme	Reference
Thermomonospora fusca BD25	β-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 Trichoderma reesei	α-L-arabinofuranosidase GH 43, α-L-arabinofuranosidase GH 51, endo-1,4-β-xylanase GH10, β-xylosidase	[63]
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.

Enzyme	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]

The diversity of 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 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 by arabinofuranosidases involves the synergistic collaboration 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 depolymerizes lignin to access cellulose and hemicellulose, thus suggesting an adaptive difference that favors microorganisms that can breakdown any polymer [35].

Although several genes that encode hemicellulolytic enzymes have been cloned and the enzymes have been characterized [5, 36], many have not been identified [36]. To date, many filamentous fungal genomes, including those from 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] reported that more than two-thirds of the ORFs putatively involved in the breakdown of plant cell wall polysaccharides and found in the genome of *A. nidulans* encode novel enzymes. They also identified ORFs that apparently encode putative enzymes for intracellular oligosaccharide breakdown and others with homology to oligosaccharide transporters in other organisms. The genomic sequencing of *A. niger* and the use of bioinformatic tools have allowed a 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 have been described for *Fusarium graminearum* [37, 38].

Regulation of hemicellulolytic systems of fungi

Saprophytic and phytopathogenic fungi need to have not only a repertoire of enzymes required to breakdown cell wall components but also a 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 major 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 it is strongly induced in the absence of D-glucose and in the 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 to negative regulation of the 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 modulate not only the expression of more than 20 genes involved in the breakdown of xylan but also the expression of genes required for cellulose depolymerization [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 cellulose and hemicellulose, including processes of mutation, co-culture and heterologous expression 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 (Thr2 and Thr28) with cysteine 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 obtaining of fungal 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*. A great success has been achieved in yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* [57]. These strategies have revolutionized the use of fungal 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 releases more phenolic acid 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 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 among the enzymes [58].

A biotechnology application of hemicellulases that is becoming increasingly attractive 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*, increases 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, cellulose and lignin by up to 75%, 50% and 40%, respectively [69].

Bifunctional enzymes

The current trend is to use enzymatic complexes capable of fully breaking down of any cell wall substrate; such an approach 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 concertedly, breakdown different hemicellulolytic substrates in a more efficient manner and at a lower cost [70] (Table 3). In the seek to develop new and efficient

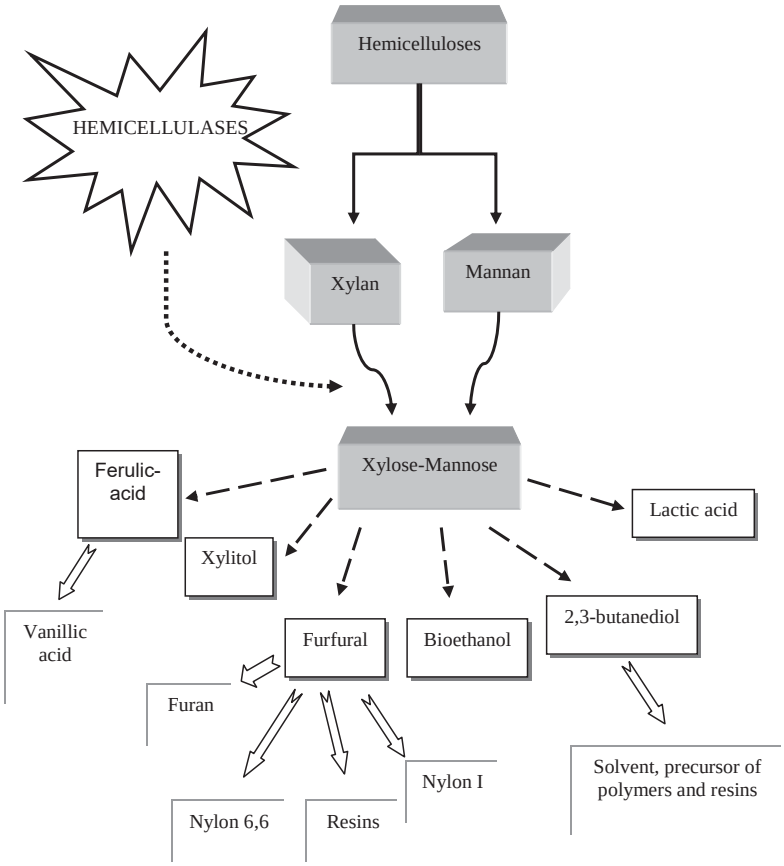

Figure 2. Biotechnological applications of hemicellulases and their role in the generation and production of various compounds.

strategies, yeasts bearing enzymes on the cell surface that are capable not only of breaking down the structures of plant cell walls have also fermenting these products in a single step are also been 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, catalyzing the direct conversion of xylan to ethanol and the potential use of these microorganisms on lignocellulose products. The characteristics of bifunctional enzymes have revealed new properties. For example, the product of the first reaction is also the substrate of the second one. The state of the active site of the first may influence the properties that of the second enzyme, 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 the efficient bioconversion to sugars. Fungi produce

Table 3. Construction of engineered multifunctional enzymes.

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

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 feed 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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