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Universidad Autónoma Metropolitana
Unidad Iztapalapa

ESTUDIO DE LA PRODUCCIÓN Y ACTIVIDAD DE PROTEÍNAS TIPO HIDROFOBINAS Y QUITINASAS DE *Lecanicillium lecanii* EN CULTIVOS EN SUSTRATO SÓLIDO Y SUMERGIDO.

TESIS

QUE PARA OBTENER EL GRADO DE

DOCTORA EN BIOTECNOLOGÍA

P R E S E N T A:

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México D.F. a 3 de Julio de 2014

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Mientras más se aleje la realidad de lo que imaginamos,
más grande será la lección...Si la deseamos aprender.

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RESUMEN

Lecanicillium lecanii es un hongo entomopatógeno usado comercialmente como agente de control biológico en agricultura. Sin embargo, poco se sabe sobre su producción de proteínas tipo hidrofobinas (HfbLs) y quitinasas, las cuales son requeridas para su desarrollo y actividad patogénica. En virtud de lo anterior, en esta tesis de doctorado se estudió diversos factores que afectan a la producción de dichas proteínas, tales como, el tipo cultivo, de soporte y fuente de carbono. Asimismo, se determinó el efecto sobre la actividad superficial de HfbLs.

La hidrofobicidad resultado de factores extrínsecos, como el tipo de cultivo, tal es el caso del cultivo sumergido SmC (hidrofílico) y SSC (utilizando PUF, material hidrofóbico), la fuente de carbono (por ejemplo, la quitina que es insoluble en agua y la fructosa que es soluble en agua), influyeron significativamente sobre la producción de quitinasas e hidrofobinas de clase I de *L. lecanii*. El crecimiento de *L. lecanii* en SSC y el uso de quitina coloidal como fuente de carbono favoreció la producción de *β-N*-acetilhexosaminidasas e hidrofobinas ca. 3 y 10 veces, respectivamente, en comparación con el SmC. Interesantemente, en este estudio se observó que si bien la quitina es un inductor de quitinasas, también tuvo efecto significativo sobre la actividad superficial de hidrofobinas. La hidrofobina clase I obtenida de SSC con quitina mostró actividad superficial al reducir la hidrofobicidad del teflón (ca. 50 %), lo cual no ocurrió con las proteínas producidas en SSC adicionado con fructosa.

Las observaciones anteriores permitieron el planteamiento de la pregunta de investigación sobre cómo la hidrofobicidad del tipo del soporte y características químicas de la quitina empleada en SSC podrían afectar la producción y actividad de las hidrofobinas de *L. lecanii*. Los resultados mostraron que *L. lecanii* fue capaz de producir proteínas tipo hidrofobina clase I y clase II en cultivos en sustrato sólido (SSC) utilizando agrolita (P) o poliuretano (PUF) como soportes inertes adicionados con quitina coloidal como fuente de carbono. La pureza y las propiedades fisicoquímicas, así como el grado de acetilación (DA) de la quitina, influyeron significativamente en la producción de hidrofobinas clase I de *L. lecanii* en SSC sobre PUF. Además, se observó que el carácter hidrofílico de la agrolita y la hidrofobicidad del poliuretano fueron factores significativos para la producción y actividad superficial de las HfbLs de este hongo. La mayor producción de HfbLs clase I se obtuvo en

cultivos con soporte hidrofóbico, PUF, (302.1 ± 14.8 µg HfbL mL^{-1}), y mostraron tener actividad superficial al reducir ca. 50 % la hidrofobicidad del teflón. Las HfbLs clase II fueron producidas en ambos soportes, PUF y P, sin embargo su producción en cultivos con agrolita fue ca. 3 veces mayor en comparación al PUF. Las HfbLs clase II mostraron capacidad de reducir ca. 25 % la hidrofobicidad del teflón y ca. 50 % la tensión superficial del agua.

Se determinó que la hidrofobicidad de compuestos orgánicos volátiles (COVs) utilizados como fuentes de carbono en cultivos sumergidos tipo microcosmos también influyó significativamente sobre la producción de quitinasas y HfbLs. *L. lecanii* mostró capacidad de crecer y consumir n-hexano y tolueno con o sin quitina coloidal como fuente de carbono en SmC. La cepa L157 mostró el mayor consumo de n-hexano (55.6 %) y tolueno (52.9 %) al ser utilizados como única fuente de carbono. En SmC adicionados con quitina e hidrocarburos (MChT y MChH), la cepa L157 mostró capacidad de producir endoquitinasas y *N*-acetil hexosaminidasas e incrementó hasta 10 veces la producción de HfbL clase I (548.6 ± 26.3 µg Hfb mL^{-1} proteína) en comparación con lo observado en cultivos adicionados con quitina como única fuente de carbono (57.4 ± 4.7 µg Hfb mL^{-1} proteína). La hidrofobicidad de los COVs probados también afectó la actividad superficial de las HfbLs clase I. La mayor reducción de la hidrofobicidad del teflón fue obtenida con las HfbLs clase I obtenidas de MChT (ca. 48 %) en comparación con las obtenidas de MChH (ca. 10 %).

El presente estudio nos permitió observar la estrecha relación entre la producción de las quitinasas e hidrofobinas de *L. lecanii*. Es destacable observar que la hidrofobicidad del tipo de cultivo, el soporte y la fuente de carbono en el cultivo fueron elementos clave para la producción y actividad superficial de las proteínas tipo hidrofobinas, mientras que el uso de quitina fue esencial para la producción de HfbLs con actividad superficial. Determinar el efecto de la hidrofobicidad sobre la producción de quitinasas y HfbLs de *L. lecanii* resultó relevante para profundizar nuestro conocimiento sobre el desarrollo y patogénesis del hongo. Además, fue posible establecer un método de transformación de *L. lecanii* empleando fosfinotricina (PPT) como agente selectivo (gen *bar*) lo cual podría facilitar los futuros estudios sobresu desarrollo e interacción con el ambiente. Con base en lo anterior, este estudio podría continuar con la purificación, análisis estructural e interacción de este hongo sobre diferentes superficies y con el estudio de la participación de las HfbLs en el desarrollo fúngico.

ABSTRACT

Lecanicillium lecanii is an entomopathogenic fungus used commercially as a biopesticide in agriculture and horticulture. However, there is scarcity of information about how *L. lecanii* production of hydrophobins-like proteins (HfbLs) and chitinases are required for their development and enzymes related to pathogenic activity. Therefore, this study evaluated the effect of the hydrophobicity of the conditions culture, such as culture type, support type and carbon source type on the chitinolytic activity, and the production and surface activity of hydrophobins-like proteins (HfbLs) from *L. lecanii*.

The hydrophobicity as result of extrinsic factors such as the culture type, for example, submerged culture, SmC (hydrophilic) and SSC (using PUF, hydrophobic), as well as the carbon source (for example, the chitin, insoluble in water and fructose, soluble in water) had significant effect on the chitinases and class I Hfb production of *L. lecanii*. SSC added with colloidal chitin as carbon source increased the *β- N-* acetyl hexosaminidases and Hfbs production ca. 3- and 10-folds, respectively, compared with the submerged culture. Interestingly, in this study showed that the chitin as carbon source acts as inductor of chitinases, as well as also to hydrophobins, it is due to the hydrophobins obtained from SSC-chitin cultures showed surface activity to reduce the hydrophobicity of teflon (ca. 50 %), while hydrophobins from SSC added with fructose showed not surface activity on Teflon.

Based on these results, the investigation moved forward on the research question of how the hydrophobicity of support type and chemical caracteristics of chitin used in SSC could affect the production and activity of hydrophobins of *L. lecanii*. This doctoral thesis showed that *L. lecanii* was able to produce class I and class II HfbLs in solid substrate culture (SSC) added with colloidal chitin as carbon source, the inert supports tested were perlite (P) and polyurethane (PUF). The purity and physicochemical properties as the degree of acetylation (DA) of the chitin, it had significant effect on the production of class I HfbLs of *L. lecanii* in SSC with PUF. Furthermore, it was observed that the hydrophilic character of the perlite and the hydrophobicity of the polyurethane were significant factors for the production and surface activity of the fungal HfbLs. The class I HfbLs were produced only in cultures with hydrophobic support, PUF, (302.1 ± 14.8 µg HfbL mL^{-1}), and these showed surface activity to reduced ca. 50 % the hydrophobicity of teflon. The HfbLs class II were produced in cultures

with either both supports, PUF or P, however, HfbLs produced in SSC with P were ca. 3-folds higher than in SSC with PUF. The class II HfbLs were able to reduce ca. 25 % the hydrophobicity of teflon and to reduce ca. 50 % of the surface tension of water.

The hydrophobicity of volatile organic compounds (VOCs) used as carbon sources in submerged cultures of *L. lecanii* also showed significantly influence on the production of chitinases and HfbLs. *L. lecanii* showed be able to grow and consume n-hexane or toluene with or without addition of colloidal chitin as carbon source in submerged culture (SmC). L157 strain showed highest consumption of n- hexane (55.6 %) and toluene (52.9 %) as sole carbon source. In SmC cultures added with chitin and hydrocarbons (MCHT or MChH), the strain L157 showed ability to produce endochitinases and *N*- acetyl hexosaminidases, also, it increased ca. 10-folds their HfbLs class I production (548.6 ± 26.3 µg Hfb mL^{-1} protein) compared with cultures added with chitin as sole carbon source (57.4 ± 4.7 µg Hfb mL^{-1} protein). The hydrophobicity of VOCs tested. Also, it had significant effect on the surface activity of class I HfbLs. Class I HfbLs from MChT culture reduced ca. 48 % the hydrophobicity of teflon, in contrast to HfbLs from MChH (ca. 10 %).

This study allowed us to observe the close relationship between the chitinases and hydrophobins production of *L. lecanii*. Noteworthy, the hydrophobicity of the culture, the support and the carbon source type were key elements to the production and surface activity of hydrophobins-like proteins, while the chitin was essential for the production of HfbLs with surface activity. The information obtained about the hydrofobicity effect on the chitinases and HfbLs production, allows us to deepen our knowledge about the development and pathogenesis of *L. lecanii*. Furthermore, it was possible to establish a method of transformation of *L. lecanii* using phosphinothricin (PPT) as a selective agent (*bar* gene), which could facilitate for studies about their development and interaction with the environment. Based on the above, this study could continue the purification, structural analysis and interaction of this fungus on different surfaces and with the study the involvement of HfbLs in the fungal development.

ESTUDIO DE LA PRODUCCIÓN Y ACTIVIDAD DE PROTEÍNAS TIPO HIDROFOBINAS Y QUITINASAS DE *Lecanicillium lecanii* EN CULTIVOS EN SUSTRATO SÓLIDO Y SUMERGIDO.

INTRODUCCIÓN GENERAL

Lecanicillium lecanii es un hongo filamentoso entomopatógeno que se usa comercialmente como biopesticida (Gillespie y Claydon, 1989). El proceso infectivo del hongo sobre el insecto involucra la secreción de proteínas (hidrofobinas) que le permitan adherirse a la superficie del huésped y, posteriormente, de enzimas hidrolíticas, tales como las quitinasas que degradan la cutícula del insecto y permiten su invasión y lisis.

Las quitinasas son un grupo de enzimas capaces de hidrolizar la quitina y son reguladas por productos de degradación de dicho polímero, tales como oligómeros de diversos tamaños y su monómero, la *N*-acetil glucosamina. Durante el desarrollo del hongo, estas enzimas participan en la elongación de las hifas ya que hidrolizan parte de la quitina presente en la pared celular permitiendo el crecimiento del hongo. Durante la patogénesis, las quitinasas actúan sobre la quitina contenida en la cutícula del insecto liberando *N*-acetil glucosamina, la cual sirve como fuente de carbono para el hongo (Howard y col., 2003; Khachatourians y Qazi, 2008). *L. lecanii* ha sido reportado como productor de quitinasas en cultivo sumergido y sólido (Matsumoto y col., 2004; Quijano-Govantes y col, 2004; Marín y col., 2008).

Por su parte, las hidrofobinas son proteínas anfipáticas caracterizadas por ser pequeñas (*ca*. 100 aminoácidos) y contener ocho regiones conservadas de cisteína que forman puentes disulfuro (Zangi y col., 2002). Las hidrofobinas forman un ensamblaje con un arreglo en paralelo entre la interfase, ya sea de un sistema aire-líquido o aire-sólido. Este ensamblaje depende de sus características hidropáticas y solubles, por lo que se han dividido en dos grupos: las Hfbs clase I que forman ensamblajes llamados *rodlets*, resistentes a altas temperaturas (100 °C) e insolubles en agua, y las Hfbs clase II, las cuales forman ensambles de fácil disociación en presencia de surfactantes como el dodecil sulfato de sodio (SDS). Los hongos pueden producir más de una hidrofobina, las cuales pueden cumplir diferentes funciones o incluso funciones compensatorias entre unas y otras a fin de contribuir a impartir hidrofobicidad al hongo para facilitar su desarrollo (Sevim y col., 2012). En cultivo sumergido, los hongos secretan Hfbs que reducen la tensión superficial del medio, permitiendo que las hifas emerjan al aire. Asimismo las hidrofobinas pueden mediar la

adhesión de las hifas y esporas a superficies hidrofóbicas, ya sea en plantas o en la cutícula de insectos, como es el caso de *Schizophyllum commune* (Wösten y Willey, 2000) y *Beuveria bassiana* (Zhang y col., 2011). En otros casos, las hidrofobinas son componentes estructurales que recubren a los cuerpos fructíferos, como se ha descrito en *Agaricus bisporus* (Lugones y col., 1998). Tanto las hidrofobinas como las quitinasas son expresadas en altos niveles según son requeridas para el desarrollo y proceso infectivo del hongo. Por ello, factores extrínsecos pueden afectar a su expresión.

Se sabe poco sobre la producción de quitinasas e hidrofobinas de *L. lecanii*, por lo que este trabajo propone analizar el efecto de la hidrofobicidad de las condiciones de cultivo sobre la producción y características de las proteínas de tipo hidrofobinas y quitinasas de *L. lecanii* con el fin de profundizar en el conocimiento sobre la participación de estas proteínas durante el desarrollo del hongo y, a futuro, sobre el proceso de patogénesis del mismo.

Con la finalidad de cumplir con el objetivo principal del "Estudio de la producción y actividad de proteínas tipo hidrofobinas y quitinasas de *Lecanicillium lecanii* en cultivos en sustrato sólido y sumergido", los resultados de la tesis se presentan en el siguiente orden:

Inicialmente se presenta una introducción general, valiosa para entender el desarrollo de los siguientes capítulos, además de presentar la justificación, la hipótesis y los objetivos del trabajo.

El primer capítulo resume de forma general el proceso de patogénesis de los hongos entomopatógenos como *L. lecanii*, con la finalidad de conocer la interacción entre hongo-insecto y las proteínas y enzimas que requiere secretar para lograr exitosamente la infección del huésped. Asimismo, presenta una revisión bibliográfica sobre la producción y actividad de quitinasas e hidrofobinas reportadas.

En el segundo capítulo se analiza el efecto del tipo de cultivo y la fuente de carbono sobre la producción y actividad de quitinasas e hidrofobinas clase I de *L. lecanii*. Se determinó la producción de quitinasas e hidrofobinas como respuesta al crecimiento de *L. lecanii* en cultivos con sustrato sólido (SSC) utilizando poliuretano (PUF) como soporte inerte y en cultivos sumergidos, así como el efecto de la fuente de carbono, utilizando quitina coloidal o fructosa. Asimismo se evaluó el efecto de la fuente de carbono sobre la actividad de hidrofobinas producidas por *L. lecanii* en SSC.

El tercer capítulo presenta la evaluación de la hidrofobicidad del soporte utilizado en cultivos en sustrato sólido sobre la producción de proteínas tipo hidrofobinas (HfbL) de *L.*

lecanii. En este capítulo se analiza el efecto de la pureza de la quitina utilizada como fuente de carbono y la hidrofobicidad de el poliuretano y la agrolita utilizados como soportes inertes para el cultivo de *L. lecanii* sobre la producción de proteínas tipo hidrofobinas clase I y clase II. Además, expone el efecto de la hidrofobicidad del soporte sobre la actividad superficial de las HfbLs; en dicha evaluación se determinó la reducción de hidrofobicidad del teflón y la tensión superficial del agua debida a la presencia de HfbLs.

En el cuarto capítulo se estudia el efecto de la adición de hidrocarburos al cultivo de *L. lecanii* sobre la producción y actividad de quitinasas y proteínas tipo hidrofobinas. La evaluación de los cambios morfológicos del hongo, el consumo de hidrocarburos y la actividad quitinolítica fueron realizados por Marín-Cervantes (2008). Adicionalmente en este trabajo se evaluó la producción y actividad superficial sobre teflón de las proteínas tipo hidrofobinas clase I y II de *L. lecanii* incubado en cultivo sumergido, utilizando de forma individual o combinada el tolueno, el n-hexano y la quitina coloidal como fuentes de carbono.

En el quinto capítulo se llevó a cabo la transformación génica de *L. lecanii* mediante *Agrobacterium.* En la transformación se determinó la eficiencia de transformación y estabilidad de los genes de selección (resistencia a fosfinotricina) y la expresión de la proteína verde fluorescente. El estudio se realizó con la finalidad de utilizar el hongo para futuros estudios.

Finalmente, se presenta una conclusión general sobre los principales resultados obtenidos de estos estudios y las perspectivas sobre la temática abordada.

JUSTIFICACIÓN

Hoy en día existe un gran interés en investigar a fondo la producción, purificación y caracterización de enzimas, proteínas y microorganismos con potencial aplicación industrial. Tal es el caso de las Hfbs y quitinasas, elementos claves para el proceso de patogénesis de *L. lecanii*, el cual es utilizado comercialmente como biopesticida.

Las Hfbs permiten la adhesión del hongo a superficies hidrofóbicas como la cutícula de insectos, estas proteínas tienen potencial aplicación como biosurfactantes y para modificar la hidropatía de materiales. Por su parte, las quitinasas son enzimas que hidrolizan la quitina liberando mezclas de quitooligómeros y el monómero, *N*- acetil hexosaminidasa, los cuales cobran interés comercial debido a su aplicación biomédica para el tratamiento de la artritis. Se ha reportado, además, que los oligosacáridos con alto grado de acetilación presentan actividad antitumoral.

Durante el desarrollo y proceso infectivo del hongo, las Hfbs y las quitinasas son expresadas en niveles altos según son requeridas, de tal forma que algunos factores extrínsecos pueden modificar su producción y actividad. Sin embargo, existen pocos reportes sobre las condiciones de producción de las Hfbs de *L. lecanii*, su participación durante el desarrollo del hongo y cómo estas condiciones afectan a la producción de quitinasas. Por esta razón, este trabajo propone analizar la producción y características de las Hfbs y quitinasas de *L. lecanii* cultivado bajo condiciones de inducción hidrofóbica.

HIPÓTESIS

La hidrofobicidad debida al tipo de cultivo, el tipo de soporte y las fuentes de carbono afecta a la producción y actividad de quitinasas e Hfbs de *L. lecanii*.

OBJETIVOS

OBJETIVO GENERAL

Evaluar la producción y actividad de quitinasas e Hfbs de *Lecanicillium lecanii* utilizando cultivos y fuentes de carbono con diferentes niveles de hidrofobicidad.

OBJETIVOS PARTICULARES

- Determinar el efecto del tipo de cultivo y la fuente de carbono sobre la producción de quitinasas e hidrofobinas clase I de *L. lecanii*.
- Determinar el efecto de la hidrofobicidad del soporte sobre la producción de proteínas tipo hidrofobinas clase I y clase II de *L. lecanii*.
- Determinar el efecto de la hidrofobicidad de la fuente de carbono (compuestos orgánicos volátiles) sobre la producción de quitinasas e proteínas tipo hidrofobinas clase I y II de *L. lecanii* cultivado en medio sumergido.

CAPÍTULO 1:

Fundamentos y generalidades

1.1 Hongos entomopatógenos: *Lecanicillium lecanii*

Los hongos entomopatógenos son organismos capaces de invadir y lisar diferentes órdenes de artrópodos. Estos hongos se pueden desarrollar en ambientes hidrofóbicos, como es el caso de la cutícula de los insectos, la cual está constituida principalmente por ceras, proteínas y quitina (Kather y Martin, 2012).

Un representante importante de estos patógenos es *Lecanicillium lecanii,* hongo filamentoso perteneciente al grupo de los ascomicetos. Este hongo tiene capacidad para infectar insectos como la mosquita blanca, pulgones, cochinillas y trips (insectos patógenos de plantas) y cobra importancia debido a que ha sido utilizado comercialmente como una alternativa de biocontrol en agricultura y horticultura (Garraway y Evans, 1984; Gillespie y Claydon, 1989; Osborne y Landa, 1992; Butt y col., 2001).

1.1.1 Mecanismo de infección hongo-insecto

El ciclo patogénico de los hongos entomopatógenos inicia con la invasión del hongo a su huésped. Esto se lleva a cabo mediante la adhesión de las esporas, conidias o hifas del hongo a la cutícula del insecto (Boucias y col., 1988), mediante una interacción hidrofóbica entre ambos organismos. La hidrofobicidad del insecto es resultado de la presencia de ceras que recubren su cutícula (Kather y Martin, 2012), mientras que la hidrofobicidad del hongo ha sido atribuida a la presencia de proteínas llamadas hidrofobinas (Talbot y col., 1996; Tucker y Talbot, 2001). Posteriormente, el hongo germina y forma apresorios, los cuales son estructuras de adhesión mecánica entre el hongo y el huésped (Hajek y St. Leger, 1994). Asimismo, secreta enzimas para hidrolizar los compuestos poliméricos que conforman la cutícula del insecto (por ejemplo, la quitina y las proteínas) hasta compuestos simples que puedan ser asimilados por el hongo y sirvan como nutrientes. En este grupo de enzimas se encuentran las proteasas, que pueden ser de tipo subtilisinas, tripsinas, metaloproteasas y peptidasas (St. Leger y col., 1986; Bidochka y Kachatourians, 1988; St. Leger y col., 1998) y las quitinasas, que degradan la quitina (Deshpye, 1986; St. Leger y col., 1986 y 1987).

Una vez atravesada la barrera de la cutícula, el hongo prolifera mediante el desarrollo de sus hifas y blastosporas, invadiendo los tejidos y órganos internos del huésped hasta completar la lisis del mismo. Finalmente, el hongo esporula sobre el cadáver del insecto para iniciar nuevamente su ciclo patogénico (Hajek y St. Leger, 1994).

1.2 QUITINASAS

Las quitinasas son un grupo complejo de enzimas que llevan a cabo la hidrólisis de la quitina hasta liberar su monómero principal, la *N*-acetil glucosamina (2-acetamida, 2-desoxi-*β*-D-glucosa) (Figura 1.1a). Los hongos entomopatógenos, en presencia de sustratos quitinolíticos, secretan las quitinasas necesarias para la hidrolisis del polímero para así obtener *β*-*N*-acetil glucosamina, la cual es asimilada como fuente de carbono (Figura 1.1c) (Howard y col., 2003). Es por lo anterior que la expresión de estas enzimas se encuentra estrechamente regulada por los productos de degradación de la quitina.

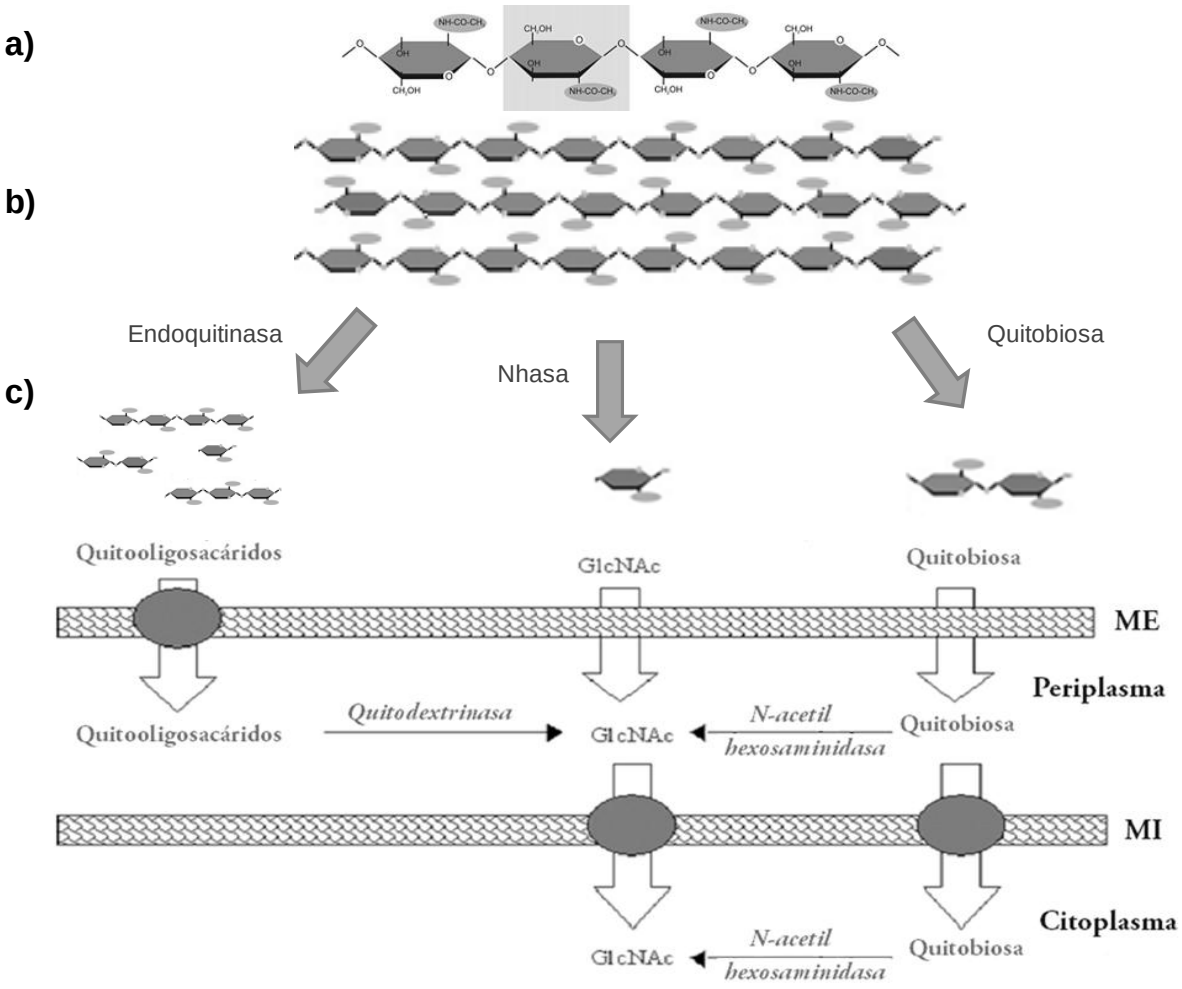


Figura 1.1. a) Estructura química de la quitina: a) Subunidad de *N*-acetilglucosamina enlazadas por uniones *β* (1-4) (recuadro en gris), b) α-quitina - arreglo antiparalelo de cadenas del polímero, c) Hidrolisis de la quitina mediante enzimas quitinolíticas y proceso de asimilación de la *β*-*N*-acetil glucosamina en hongos.

1.2.1 Clasificación de quitinasas

Las quitinasas tienen afinidad por cadenas de quitina altamente acetiladas. Su alta especificidad por el sustrato hace que estas enzimas liberen oligómeros quitinolíticos de tamaño específico, incluyendo al monómero (la *N*- acetil glucosamina). Por esta razónlas quitinasas se clasificancomo:

- Endoquitinasas (EC 3.2.1.1.4), cortan aleatoriamente enlaces internos de la cadena de quitina produciendo oligómeros de *N*-acetilglucosamina.
- Exoquitinasas (EC 3.2.1.14), cortan enlaces no reducidos al final de la cadena de quitina liberando diacetilquitobiosa (dímeros de *N*-acetilglucosamina), sin producir *N*-acetilglucosamina.
- Quitobiasas (EC 3.2.1.30), catalizan la liberación de, diacetilquitobiosa, mediante la hidrolisis de los enlaces no reducidos al final de la cadena del oligómero y sin producir monosacáridos u oligosacáridos.
- *N*-*β*-acetilglucosaminidasa o *N*-*β*-acetilhexosaminidasa (EC 3.2.1.52), corta el enlace no reducido de la quitina, aunque con preferencia utiliza como sustrato a la quitobiosa y puede actuar sobre quitotriosa o quitotetraosa liberando *N*-acetil glucosamina.

1.2.2 Producción de quitinasas de *Lecanicillium lecanii*

Factores extrínsecos como la humedad, la actividad de agua, los gases (CO_2, O_2), el pH, la concentración y tipo de nutrientes y el tipo de cultivo afectan a la fisiología y la producción de metabolitos de los hongos.

La quitina y la cutícula de insectos han sido las principales fuentes de carbono y nitrógeno empleadas para la inducción de quitinasas en los hongos entomopatógenos (St. Leger y col., 1986; Barranco-Florido y col., 2002; Iglesias y col., 2002; Quijano-Govantes y

col., 2004). Mientras que la glucosa, e incluso la *N*-acetil-*β*-D glucosamina, pueden actuar como represores catabólicos de las quitinasas (St. Leger y col., 1986; Bidochka y Kachatourians, 1988; Donzelli y Harman, 2001; Barreto y col., 2004).

La aireación, en conjunto con la agitación, contribuye a incrementar la solubilidad del oxígeno en cultivos sumergidos. Liu y col. (2003) reportaron que *L. lecanii* tuvo la mayor producción de quitinasas (18.2 mU mL^{-1}) en un reactor en lote de 5 litros al aplicar aireación de 0.6 vvm y agitación de 150 rpm, mientras que en un reactor airlift de 30 litros la mayor producción quitinolítica (19.9 mU mL^{-1}) se obtuvo con aireación de 0.9 vvm.

Por otra parte, se ha reportado que los cultivos en sustrato sólido (SSC) ofrecen ciertos beneficios para la producción de enzimas y proteínas, en comparación con los cultivos líquidos (SmC). Entre los beneficios, se menciona que se pueden obtener procesos con alta eficiencia biosintética (es decir, altos valores de conversión del sustrato a producto), debido a que este sistema permite mejor difusión del oxígeno y menor susceptibilidad del microorganismo a sufrir represión catabólica y estrés osmótico. Esto es debido a que en los sistemas sólidos el crecimiento del microorganismo se presenta en forma de agregados (sistema heterogéneo), formando gradientes de concentración de nutrientes y biomasa (Viniegra y col., 2003). Entre las restricciones del SSC se puede mencionar que, al ser un sistema heterogéneo en comparación con al cultivo SmC, es difícil controlar variables como la temperatura y el pH (Raimbault, 1998).

Diferentes soportes han sido utilizados en SSC para la producción de quitinasas, tal es el caso del bagazo de caña. Este material orgánico tiene la desventaja de tener exceso de nutrientes, baja porosidad y una estructura que dificulta la difusión de oxígeno, nutrientes y la remoción del calor. Además, dificulta la separación de la biomasa del soporte y favorece la contaminación del producto, complicando su purificación (Matsumoto y col., 2004; Hölker y col., 2004). Por el contrario, el uso de soportes inertes como la agrolita y la espuma de poliuretano (PUF) ofrecen ciertas ventajas, como alta porosidad, baja densidad y relativamente alta absorción de agua, de tal forma que dichas estructuras favorecen el crecimiento del microorganismo, la determinación directa de la biomasa, la extracción de productos limpios y una buena aireación y remoción del calor. El PUF presenta una estructura en forma de nido de abeja, por lo que la superficie de intercambio gaseoso puede ser hasta 400 veces mayor que en la interfase aire-líquido presente en un sistema SmC. Con este tipo de soporte, el crecimiento en SSC se presenta en forma de capas delgadas de

agregados celulares, con una superficie de intercambio gaseoso grande, favoreciendo la difusión de gases, sustrato y productos (Zhu y col., 1994; Auria y col., 1995; Viniegra y col., 2003; Marin-Cervantes y col., 2008).

En los cultivos SSC, la actividad de agua (aw) es de suma importancia para el desarrollo de los microorganismos, ya que un valor adecuado evita el alargamiento de la fase de adaptación (fase lag) y la disminución de la velocidad de crecimiento. Barranco-Florido y col. (2002) y Marin-Cervantes y col. (2008) han reportado que *L. lecanii* requiere de valores de aw entre 0.978 y 0.997 para un adecuado desarrollo.

También se han reportado diversos estudios sobre la producción de enzimas quitinolíticas de *L. lecanii* en SSC. Barranco-Florido y col. (2002) reportaron que *L. lecanii* incrementó la producción de quitinasas (1.3 a 1.7 veces) en SSC en comparación con SmC, diferencias que se atribuyen a una mejor adaptación de las cepas estudiadas al cultivo en medio sólido. Matsumoto y col. (2004) reportaron que el crecimiento de *L. lecanii* en SSC, utilizando bagazo de caña como soporte y desechos de camarón como sustrato, permitió incrementar un 40 % la actividad Nhasa con respecto al SmC. Marin-Cervantes y col. (2008) reportaron que la forma y tamaño de la espuma de poliuretano (PUF) afectó a la producción quitinolítica de *L. lecanii*, mencionando que el crecimiento fue disperso a lo largo del PUF, pero con agregados densos en las orillas del mismo y con una alta producción de quitinasas (Nhasa ca. 5000 mU g^{-1} PUF; endoquitinasa ca. 1200 U g^{-1} PUF) en poliuretano cortado (ca. 0.5 x 0.5 x 0.5 cm). Por su parte, Shi y col. (2009) reportaron la optimización de la producción de esporas de *L. lecanii* en SSC utilizando bagazo de caña como soporte. La producción de esporas fue determinada después de las 72 h de cultivo (25 °C, 97 % de humedad) obteniendo la máxima producción de 1 x 10^{10} esporas g de bagazo de caña $seco^{-1}$. Xu y col., 2011 reportaron el uso de soportes organicos e inorgánicos para la producción de esporas y quitinasas de *Verticillium lecanii*, los soportes probados fueron bagazo de caña, mazorca de maíz, paja de arroz, espuma de poliuretano y carbón activado. La mayor actividad quitinolítica se obtuvo con el bagazo de caña 3.3 U mg^{-1} seguido por la espuma de poliuretano 2.7 U mg^{-1} y con la mayor producción de esporas (10^{10} esporas g^{-1}) en cualquiera de los soportes.

1.3 HIDROFOBINAS

1.3.1 Actividad biológica de las hidrofobinas

Las hidrofobinas son proteínas anfipáticas que presentan actividad superficial, por lo que actúan como biosurfactantes. Su interacción en la interfase agua-aire provoca la reducción de la tensión superficial del agua, mientras que su interacción con superficies sólidas produce que la superficie cambie de hidrofóbica a hidrofílica y viceversa (Wösten y Wessels, 1997; Linder y col., 2005).

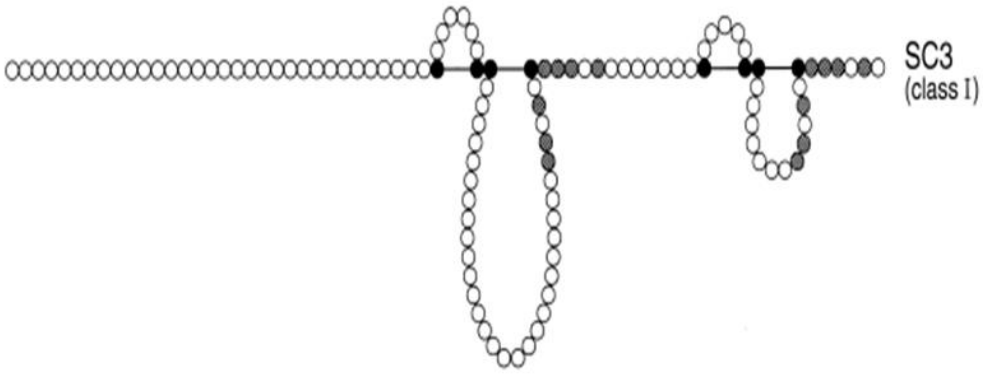
Las hidrofobinas son producidas por hongos para facilitar su crecimiento y desarrollo (Wösten, 2001; Kershaw y col., 1998). Por ejemplo, hidrofobinas clase I de *Magnaporthe oryzae* se encuentran relacionadas con la conidiogénesis y formación de apresorios (Talbot y col., 1996). Estas proteínas actúan como componentes estructurales que recubren a los cuerpos fructíferos, como ha sido observado en *Agaricus bisporus* (Lugones y col., 1998). Proporcionan hidrofobicidad a la superficie de los hongos, facilitando la dispersión de las esporas o bien para mediar la adhesión de esporas e hifas a superficies hidrofóbicas, tal como ocurre durante la infección de un hongo entomopatógeno a la cutícula del insecto (van Wetter y col., 1996; Wösten y col., 1994; Talbot y col., 1996; Tucker y Talbot, 2001). Asimismo, promueven la formación de estructuras aéreas: en cultivos sumergidos se ha observado que las hidrofobinas interaccionan con la superficie del líquido, disminuyendo la tensión superficial y permitiendo que las hifas aéreas emerjan del líquido (Wessels y col., 1991). Su presencia también contribuye a evitar la desecación del hongo y a formar canales proteicos sobre la superficie de las hifas para facilitar el intercambio gaseoso, como ocurre con la hidrofobina SC4 de *S. commune* (van Wetter y col., 2000).

1.3.2 Clasificación de hidrofobinas

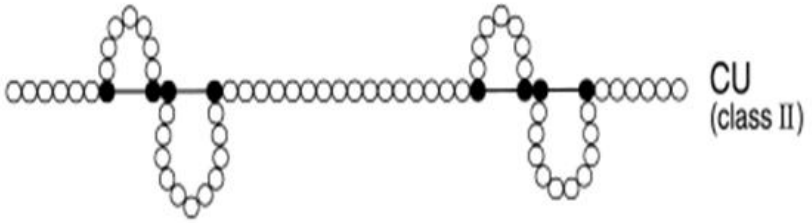
Las hidrofobinas se clasifican en dos grupos principales, de acuerdo a su hidropatía y solubilidad (Wösten, 2001):

- Hidrofobinas de clase I: son proteínas anfipáticas que pueden ser disueltas con tratamiento de trifluoroacético o ácido fórmico, y son insolubles en dodecil sulfato sódico (SDS) a 100 ºC (Schuren y Wessels, 1990). En interfases hidrofílica e hidrofóbica tienen la capacidad de formar películas estables de 10 nm de espesor, muy estables, llamadas *rodlets*. Estas estructuras son similares a las agrupaciones formadas por proteínas amiloides y se forman debido a un cambio estructura α a β (de Vocht y col., 1998; Szilvay y col., 2007). La hidrofobina clase I que ha servido como modelo de estudio es la hidrofobina SC3 del hongo basidiomiceto *Schizophylum commune*, la cual tiene la capacidad de reducir la tensión superficial del agua de 72 a 24 mJ m^{-2} (Calonje y col., 2002).

- Hidrofobinas de clase II: Son fácilmente solubles en 2 % de SDS o etanol al 60 % (Linder y col., 2005). No forman *rodlets*, como consecuencia las películas que forman son inestables en comparación con las de clase I. Algunos de los ejemplos más representativos y estudiados de esta clase de hidrofobinas son: la hidrofobina cerato-ulmin (CU) de *Ophiostoma ulm (*Richards y col., 1993; Bowden y col., 1994) y las hidrofobinas HFBI y HFBII de *Trichoderma reesei* (Askolin y col., 2005).



Clase I: $X_{26–85}$-C- $X_{5–8}$ -C-C- $X_{17–39}$ -C- $X_{8–23}$ -C$X_{5–6}$-C-C- $X_{6–18}$ -C- $X_{2–13}$



Clase II: $X_{17–67}$-C-$X_{9–10}$-C-CX_{11}-C -X_{16}-C-$X_{6–9}$-C-C-X_{10}- C-$X_{3–7}$

Figura 1.2. Distribución de los residuos de cisteína en la estructura primaria de las hidrofobinas clase I y clase II. X (○); indican los aminoácidos que conforman la proteína. C (●); indican las cisteínas contenidas en la proteína formando puentes disulfuro.

Cabe mencionar que recientes estudios han indicado que podría haber otras clases de hidrofobinas o proteínas tipo hidrofobinas, las cuales difieren en su secuencia de aminoácidos y de las cuales se ha reportado muy poco (Jensen y col., 2010; Seidl-Seiboth y col., 2011).

1.3.3 Ensamblaje e interacción de las Hfbs con interfases

La clasificación de las hidrofobinas (acorde a sus características estructurales e hidropáticas) también implica diferencias en su ensamblaje e interacción en interfases.

Los *rodlets* de las hidrofobinas clase I se ensamblan espontáneamente en interfases hidrofilicas-hidrofobicas, se encuentran asociados lateralmente, formando monocapas anfipáticas resistentes a la despolimerización por ácidos (Wösten, 2000). Las monocapas formadas por las hidrofobinas clase II son fácilmente despolimerizadas por el uso de detergentes y calor, sin embargo, su ensamblaje no forma *rodlets*, sino arreglos altamente cristalinos (Hakanpää y col., 2004a y 2004b). Ambos tipos de Hfbs son secretados por los hongos en su forma soluble, un estado estable hasta que entra en contacto con interfases, elemento que promueve el ensamblaje de estas proteínas (Yang y col., 2013).

Todas las Hfbs contienen un núcleo (β-barril) compuesto por cuatro cadenas β en antiparalelo, rodeado por otros elementos de estructura secundaria y limitado por bucles formados por cuatro puentes disulfuro, regiones relativamente flexibles y que dan estabilidad a la estructura (Linder, 2009). Sin embargo, las Hfbs clase II, por ejemplo, HFBI y HFBII de *T. reesei* exponen una estructura de núcleo barril cerrado y adicionalmente a los bucles, contienen una secuencia corta en α-hélice. Mientras, las Hfbs clase I Dewa y EAS (de *Aspergillus nidulans* y *Neurospora crassa*, respectivamente) tienen un núcleo relativamente abierto, llamado también núcleo "de medio cañón" rodeado por diversas estructuras secundarias, entre ellas estructuras β plegadas (Figura 1A). Parte de la superficie de las hidrofobinas consiste de cadenas laterales alifáticas hidrófobas, que forman un parche. El parche hidrofóbico se forma por dos regiones del bucle en la estructura central β- barril y contiene únicamente los residuos alifáticos, pero no los aromáticos hidrofóbicos. Sin embargo, representa cerca del 60 % de la superficie total accesible de la proteína, lo cual es notable ya que en el resto de las proteínas los aminoácidos hidrofóbicos se encuentran principalmente en el interior de la estructura, es por ello que las hidrofobinas poseen la habilidad de formar capas anfipáticas (Sunde y col., 2008) (Figura 1B).

Las hidrofobinas clase I se autoensamblan en interfases hidrofílicas-hidrofóbicas como una membrana anfipática, con arreglo en paralelo alrededor de las esporas o del micelio. Se ha reportado que la cadena glicosilada de las hidrofobinas promueve la formación de la estructura en α-hélice y esta, a su vez, es inducida durante el ensamblaje de

la proteína sobre un sólido hidrofóbico. Por esto se ha relacionado con la alta insolubilidad del ensamblaje y la adhesión del hongo a sustratos hidrofóbicos, como la cutícula de insectos. La hidrofobina SC3, por ejemplo, en presencia de una interfase aire-agua muestra una mayor proporción de estructura β- laminar (65%) que de α-hélice (16%). Esta configuración proporciona mayor estabilidad en comparación a su forma soluble, que presenta un 23% de su estructura como α-hélice y un 41% β- laminar (Wösten y col., 1994; de Vocht y col., 1998).

La formación de monocapas de las Hfbs clase II en interfases aire-agua no requiere de cambios estructurales, como sí ocurrecon las de la clase I (Askolin y col., 2006). Sin embargo, se ha observado que las HFBI y HFBII forman agregados en solución que al secarse o al encontrarse en interfases muestran un empaquetamiento hexagonal y flexible a través de la superficie (Torkkeli y col., 2002; Lumsdon y col., 2005; Cox y col., 2007; Wang y col., 2010). Cabe mencionar que los bucles de las HFBs clase I son más largos que los de la clase II, a lo cual se puede atribuir las diferencias de ensamblaje.

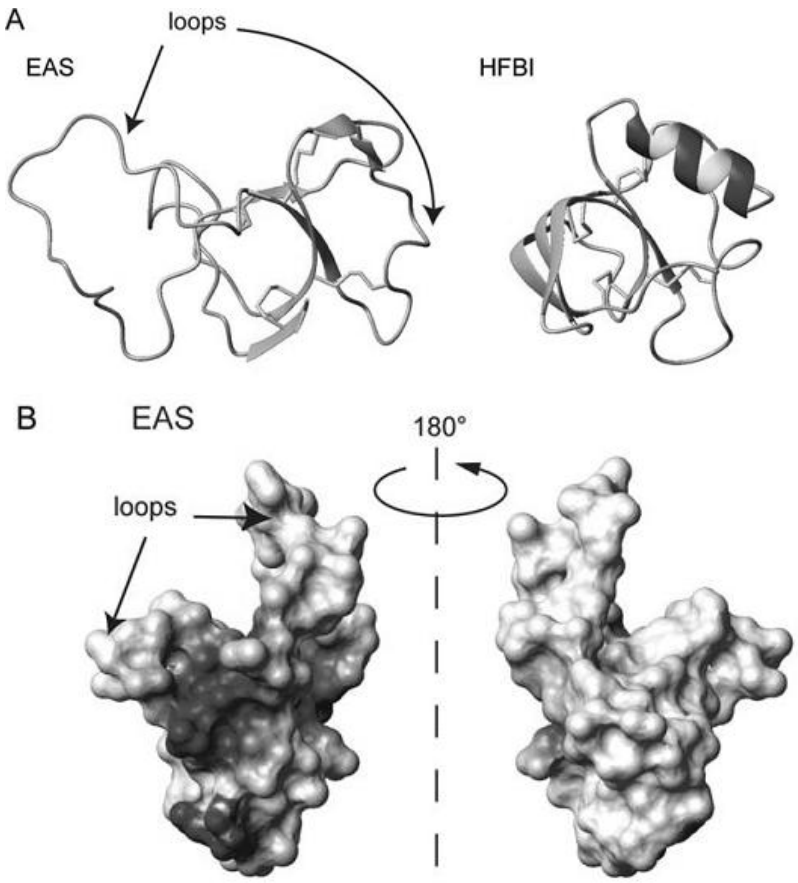


Figura 1.3. Comparación de la estructura de las hidrofobinas clase I y clase II A: Representación en liston de la Hfb clase I EAS y la clase II HFBI. Los blucles (loops) son marcados por la flecha. B: Representación de superficie de EAS muestra la superficie hidrofílica (izquierda) e hidrofóbica (derecha).

Se ha reportado que concentraciones muy bajas de hidrofobinas, cercanas a 1 mM, han sido suficientes para conseguir un efecto significativo en el cambio de la tensión superficial o cambio de la hidropatía de superficies sólidas. La actividad superficial de las Hfbs en interfases agua-aire se ha determinado mediante la prueba de tensión superficial, mientras que el cambio de la superficie de hidrofóbica a hidrofílica y viceversa se ha determinado mediante el ángulo de contacto (θ) de una gota de agua colocada sobre la superficie de un sólido. Un ángulo de contacto de 90° o mayor caracteriza a una superficie

como no-humectable (hidrofóbica) y un ángulo menor de 90° como humectable (hidrofílica). En la siguiente tabla se hace referencia a los valores determinados para hidrofobinas y otras proteínas en la literatura.

Tabla 1.1. Rangos de hidrofobicidad superficial de hidrofobinas y su efecto en la tensión superficial del agua.

Proteína/ superficie	Hidrofobicidad†	Hidrofilicidad†	Tensión superficial γ (mJ m^{-2})	Referencia
BSA	81.3	-	-	Jeffs, 1999
Soya	-	-	50*	Scholtmeijer y col., 2001
Hfb clase I	36-59	113-117	27-37	Askolin y col., 2006 Scholtmeijer y col., 2001 Wosten y col., 1994 Lugones y col., 1999 de Votch, 1998
Hfb clase II	22-60	90-105	32-45	Askolin y col., 2006 Scholtmeijer y col., 2001 Lumsdon y col., 2005 de Votch y col., 1998

†hidrofobicidad / hidrofilicidad de la proteína

*La soya logra la reducción de tensión superficial en un tiempo de 2000 s, mientras que las HFBII lo hacen en 200 s

Tabla 1.2. Ángulo de contacto reportado como actividad superficial de hidrofobinas en superficies sólidas.

Superficie	Angulo de contacto θ (°)			Referencia
	Sin Hfb	HFBII	SC3	
Teflón	108	-	48 ± 10	Wosten y col., 1994
Parafilm	105	-	36 ± 3	
Vidrio	15	-	23 ± 2	
Teflón	108	49	32	Lumsdon y col., 2005
Vidrio	39	45	55	

HFBII (clase II); SC3 (clase I)

La interacción de las hidrofobinas con gases también ha sido estudiada. Las Hfbs clase II actúan como sitios de nucleación para la formación de burbujas de CO_2, mediante la interacción del parche hidrofóbico de la proteína y el CO_2 y han mostrado mayor afinidad por este en comparación con las hidrofobinas clase I (Linder 2009; Deckers y col., 2012). Su afinidad con la formación de burbujas ha sido atribuida a la elasticidad del arreglo que forman, determinándose que HFBI mostró mayor elasticidad que HFBII y mayor que caseína y lactoglobulina (Lumsdon y col., 2005). Por su parte, Wang y col., (2005) han reportado que la membrana formada por la hidrofobina clase I SC3 de *S. commune* es permeable al vapor de agua pero no a moléculas mayores de 200 g mol^{-1}.

1.3.3 Producción de hidrofobinas en hongos

Se ha observado que las hidrofobinas son reguladas por diversos genes y señales ambientales. Son diferencialmente expresadas y aunque sus funciones pueden ser distintas, estas pueden actuar de forma compensatoria entre ellas. En algunos hongos se han encontrado más de un gen de hidrofobina, por ejemplo en *S. commune* se han aislado al menos cuatro genes que codifican hidrofobinas, los cuales pueden desempeñar diferentes

funciones: las hidrofobinas Sc1 y Sc4 son reguladas por el tipo de apareamiento y expresadas en los cuerpos fructíferos, mientras que Sc3 es expresada durante la formación de las hifas aéreas (Wessels, 1992).

En *Beauveria* se observó que las hidrofobinas en la pared celular de conidios variaron según las estructuras formadas con relación al tipo de cultivo en que se desarrolló el hongo. Se determinó que hay mayor presencia de hidrofobinas en conidios aéreos (cultivo superficial), que en blastosporas y conidios sumergidos (cultivo sumergido). Además, su presencia modificó la adhesión de dichas estructuras a superficies polares, hidrofóbicas e hidrofílicas, diferencias que repercuten en la especificidad de los hongos entomopatógenos a su huésped (Holder y Keyhani, 2005). Asimismo, las hidrofobinas clase I y clase II se encuentran involucradas con la conidiación, pigmentación, hidrofobicidad y virulencia del hongo (Zhang y col., 2011; Sevim y col., 2012).

Entre los factores ambientales que tienen incidencia en la producción de hidrofobinas se han reportado los siguientes. St. Leger y col. (1998) reportaron que el pH influye en la expresión de genes que codifican proteínas como las hidrofobinas, proteasas y quitinasas. En *Metarhizium anisopliae* estas proteínas fueron expresadas a pH de 5 a 8, valores que corresponden a los del sitio de infección en la cutícula de insectos. Ying y Feng (2004) correlacionaron la producción de hidrofobinas clase I con el tipo de sustrato y concentración, observando que se produjeron más hidrofobinas en concentraciones menores de 4% (p/v) de glucosa, sacarosa y almidón. Con ello mostraron que la termotolerancia de las conidioesporas de *B. bassiana* está determinada por el contenido de estas hidrofobinas. Asimismo, se han reportado diferencias entre las hidrofobinas de clase II producidas por *Rhinocladiella similis* incubado sobre un biofiltro, utilizando compuestos de polaridades opuestas. Si se utilizaba n-hexano como fuente de carbono la hidrofobina obtenida del micelio presentó un peso molecular de 15 kDa, mientras que al utilizar etanol como fuente de carbono la hidrofobina presentó un tamaño de 8.5 kDa (Vigueras y col., 2009).

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CAPÍTULO 2:

Production and activities of chitinases and hydrophobins from *Lecanicillium lecanii*

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ORIGINAL PAPER

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Abstract The production of chitinases and hydrophobins from *Lecanicillium lecanii* was influenced by the cultivation method and type of carbon source. Crude enzyme obtained from solid-substrate culture presented activities of exochitinases (32 and 51 kDa), endochitinases (26 kDa), *β*-*N*-acetylhexosaminidases (61, 80, 96 and 111 kDa). Additionally, submerged cultures produced exochitinases (32 and 45 kDa), endochitinases (10 and 26 kDa) and *β*-*N*-acetylhexosaminidases (61, 96 and 111 kDa). *β*-*N*-acetylhexosaminidases activity determined in solid-substrate culture with added chitin was *ca.* threefold (7.58 ± 0.57 U mg^{-1}) higher than submerged culture (2.73 ± 0.57 U mg^{-1}). Similarly, hydrophobins displayed higher activities in solid-substrate culture (627.3 ± 2 μg protein mL^{-1}) than the submerged one (57.4 ± 4.7 μg protein mL^{-1}). Molecular weight of hydrophobins produced in solid-substrate culture was 7.6 kDa and they displayed surface activity on Teflon.

Keywords Chitinases · Hydrophobins · *Lecanicillium lecanii* · Solid-substrate culture · Chitin oligomers

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Introduction

Chitin, the polysaccharide composed of *β*-1,4-*N*-acetyl-D-glucosamine repeat units found in insect cuticles, shells of crustaceans and fungal cell walls, is a target molecule for biological agents that might contain chitinases, chitin synthesis inhibitors or other microorganisms. Chitinases are present in chitin-containing microorganisms, bacteria and fungi with a diversity of roles, such as in the growing of hyphae, defence mechanisms in response to pathogens and abiotic stress, as well as their involvement in nutrition and pathogenesis [1]. Chitinases are extracellular cuticle-degrading enzymes also responsible for hydrolysis of macromolecular substrates (i.e. chitin, protein) to small molecule nutrients, which are required during fungus penetration to the host cuticle [1, 2]. These hydrolases are regulated by chitin degradation products and they are classified as endochitinases (EC 3.2.1.1.4), which randomly break down internal links of the chain; exochitinases (EC 3.2.1.14) that release diacetylchitobiose; chitobiases (EC 3.2.1.30) that split dimer of *N*-acetylglucosamine (chitobiose) and *β*-*N*-acetylhexosaminidase (HexNase) (EC 3.2.1.52) responsible for hydrolysis of chitobiose ($(GlcNAc)_2$), chitotriose or chitotetraose ($(GlcNAc)_4$) [1].

Hydrophobic proteins or hydrophobins (Hfb) have been reported as mediators for adhesion of hyphae and spores on host surfaces (i.e. insect cuticle) [3–5]. Hfb are small amphiphilic-secreted proteins characterized by the presence of eight conserved cysteine residues. These proteins are produced by filamentous fungi, such as the common button mushroom, *Agaricus bisporus* [6]. The first HFbs genes were found during the development of *Schizophyllum commune*. Based on the deduced protein sequences, Wessels [7] introduced the name hydrophobin for these relatively small fungal proteins of about 10 kDa in size.

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2.1 INTRODUCTION

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Later on, the Hfbs were classified as class I or II upon their hydropathy patterns and solubility characteristics. Class I form self-assemble rodlets, which are soluble in trifluoroacetic acid (TFA) and formic acid (FA), whereas class II are readily dissolved in ethanol or sodium dodecyl sulfate (SDS) [4–8]. Research on hydrophobins and chitinases are of great interest for their role in antagonistic mechanisms and biotechnological applications [1, 2, 9]. Chitinases have been used for production of bioactive chito-oligosaccharides and chitobiose [1, 10], while Hfb have been used for surface modification (e.g. immobilization of enzyme, biosensors and tissue engineering) as well as bio-surfactants [9].

Lecanicillium lecanii (former *Verticillium lecanii*) is an entomopathogenic fungus with a wide range of insect hosts, such as Homoptera, Coleoptera, Orthoptera and Lepidopthera, therefore, it is used as biocontrol agent as an alternative to the use of chemical pesticides [3]. *L. lecanii* has been studied in the production of chitinases in submerged (SmC) and solid-substrate cultures (SSC) [10–16]. SmC and SSC are systems frequently applied either for fungal sporulation or chitinase production. SmC is faster and easier to control the parameters

(i.e. pH and temperature); however, the products are more diluted than with SSC [10–16]. Herein, we are first to report Hfb production from this fungus in SSC and SmC.

2.2 EXPERIMENTAL

2.2.1 Microorganism and culture conditions

Lecanicillium lecanii strain 2149 was obtained from the Entomopathogenic Fungal Culture Collection (Ithaca, New York, NY, USA). *L. lecanii* was cultured on potato dextrose agar at 25 °C and maintained at 4 °C until needed. Spore suspension was prepared by agitation of cultures with a solution of 0.1 % (v v^{-1}) of Tween 80. Spores were counted with Neubauer chamber and the inoculum size was 5 x 10^7 spores per g of substrate for SmC and SSC.

2.2.2 SmC and SSC media

Mineral medium composition (g L^{-1}) was; K_2HPO_4 (2.8), $MgSO_4 \cdot 7H_2O$ (1.38), $CaSO_4 \cdot 2H_2O$ (0.48), $FeSO_4 \cdot 7H_2O$ (0.22), $(NH_4)_2SO_4$ (7.5) $CaCl_3$ (0.48), $NaH_2PO_4 \cdot 7H_2O$ (1.67), yeast extract (1), and either colloidal chitin (30) or fructose (22) was used to SmC and SSC [10]. Control cultures were carried out with fructose as carbon source. SmC was conducted in flasks incubated at 25 °C in rotary shaker at 180 rpm for 144 h [12]. Biomass was separated by centrifugation of the SmC at 4 °C and 14,000 g for 15 min (Beckman J2-MI, USA). The supernatant was filtered and ready to use as crude enzyme for further assays, i.e. protein determination and HexNase activity. SSC was carried out in glass columns packed with polyurethane foam (PUF) with size *ca.* 0.125 cm^3 within nutrient ratio of 1:15 (w v^{-1}) and aeration rate of 1.4 mL air min^{-1} per g of moist material. SSC was collected from each column, then mixed with its same weight in water and pressurized to 1,000 psi followed by centrifugation at 4 °C and 12,700 g for 20 min. Crude enzyme was prepared as described above.

2.2.3 Detection of chitinolytic activity on SDS–PAGE

Gels for SDS–PAGE [17] (mini Protean II Bio-Rad, Hercules, CA, USA) were prepared with added 0.01 % (w v^{-1}) of either glycol chitin or chito-oligosaccharides [Chitopentaose ($(GlcNAc)_5$), $(GlcNAc)_4$, $(GlcNAc)_2$] as substrates. Freeze-dried crude enzyme obtained after

144 h of culture was subjected to SDS–PAGE. Then, gels were treated in 0.1 M phosphate buffer (pH 6) and 1% (v v^{-1}) Triton X100 for 24 h at 37 °C. Further, gels were immersed for 5 min into a freshly prepared solution of Calcofluor white M2R (Sigma-Aldrich, St. Louis MO, USA) 0.01 % (w v^{-1}) in 0.5 M Tris–HCl buffer (pH 9). Gels were then rinsed with deionized water for 1 h. Chitinolytic activities appeared as fluorescent zones within homogenously dark background upon illumination using an UV transilluminator (Gel Doc Bio-Rad) [18]. Bands were analyzed with the ImageJ 1.41o program (National Institutes of Health, USA). All experiments were carried out by duplicate.

2.2.4 Determination of HexNase activity

HexNase activities were determined following the procedure reported by Tronsmo and co-workers [19]. Crude enzyme (200 µL) was added to 200 µL of a citrate–phosphate buffer (0.2 M, pH 5.6) and 200 µL of *p*-nitrophenyl-*β*-*N*-acetylglucosamine (2.9 mM) and then incubated with agitation (180 rpm) at 37 °C for 1 h. Reaction was halted by the addition of NaOH (1 mL, 20 mM). The released p-nitrophenol was monitored spectrophotometrically at k of 400 nm in a UV–Vis JENWAY 6305 (Essex, UK). One unit of HexNase was defined as the amount of crude enzyme required to release 1 lmol of *p*-nitrophenol per minute. Determinations were carried out every 24 h until 144 h.

2.2.5 Extraction of Hfb

Hfbs class I were extracted from mycelia after 144 h of cultivation either in SSC or SmC with the following procedure; PUF with the fungal mycelia were mixed with SDS (2 w v^{-1} %) in Tris–HCl (100 mM pH 9) buffer for 2 h at 25 °C and then centrifuged. Pellets were treated with concentrated FA (J. T. Baker, Mexico), neutralized and centrifuged (4 °C, 5,000 g) for 5 min. The supernatant was precipitated by electrobubbling [20]. Extracts were centrifuged and the pellet was solubilized with TFA and further evaporated with dry air stream. The biomass in SmC media was separated from the culture broth by centrifugation (14,000g) at 4 °C for 15 min.

2.2.6 SDS–PAGE analysis

Protein in the dried extract was determined by Bradford method [21] and subjected to SDS–PAGE [17]. Gels were stained with silver nitrate (Bio-Rad) and analyzed with the ImageJ 1.41o software (National Institutes of Health, USA).

2.2.7 Evaluation of Hfb on the surface activity of Teflon

Surface activities were evaluated as the ability to modify the surface hydrophobicity of Teflon (poly(tetrafluoroethylene)) by the Hfbs by measuring the contact angle [20]. Teflon was washed with water and ethanol and then dried. Hfb solution was prepared dissolving extract of dried protein in deionized water at a concentration of 50 µg protein mL^{-1}. Hfb solution was used onto 1.32 cm^2 of Teflon. Subsequently, 1 µL of deionized water was dropped on the treated Teflon surface and observed with a side-illumination horizontal light microscope Intel Qx3 (Intel Corporation, USA). Contact lengths (b) and heights (h) of the water droplet were measured by ImageJ 1.41o software (National Institutes of Health). Contact angle was calculated according to the Eq. 2.1 [22]. Analyses were conducted by quadruplicate, in three different random locations on each surface.

$$\theta = 2\tan^{-1}\left(\frac{2h}{b}\right) \qquad (2.1)$$

2.2.8 Statistical analysis

A randomized design was carried out in quadruplicate with HexNase activity as response variable and culturing condition, i.e. SSC and SmC, as source of variation. As well, statistical analysis was carried out on surface activity of Hfbs obtained from SSC with different carbon sources with contact angle measurements as response variable. Analysis of variance and multiple comparisons of means by Tukey– Kramer ($P \leq 0.05$) were performed using NCSS 2000 software (NCSS Inc., USA 2001).

2.2.9 Scanning electron microscopy

Samples of SSC and SmC with added chitin media were prepared by immersion in 5% (v v^{-1}) glutaraldehyde at 4 °C for 24 h and post-treated with OsO_4 1 % (w v^{-1}) for 2 h. Then, samples were dehydrated in a graded alcohol series and covered with carbon and gold

prior to scanning electron microscopy (SEM) examinations using a JEOL JSM-5900 LV (Japan).

2.3 RESULTS

2.3.1 HexNase activity from SSC and SmC media

Several factors have been reported as determinants in the production of chitinases in SmC and SSC of *Verticillium* such as pH, addition of chitin as carbon source and inducer, as well as culturing conditions i.e. type and size of support or agitation rate [10–16]. The increment of moisture content of sugar cane bagasse (support) and mycelia as inoculums in SSC of *L. lecanii* was reported to improve significantly the enzyme yield and to reduce the lag phase. In fact, the system of culture has impact not only on the enzyme yield, but also in the fungal metabolism as reported by Marin-Cervantes and co-workers [13]. Therein, catabolic repression depended on moisture level in SSC, being stronger for lower moisture contents for exo chitinases but milder or insignificant for endo-chitinases. In the present work, HexNases production was significantly higher in SSC than in SmC with added chitin media, whereas, insignificant HexNase activities were determined in SmC and SSC in the control media with fructose as sole carbon source (Fig. 2.1).

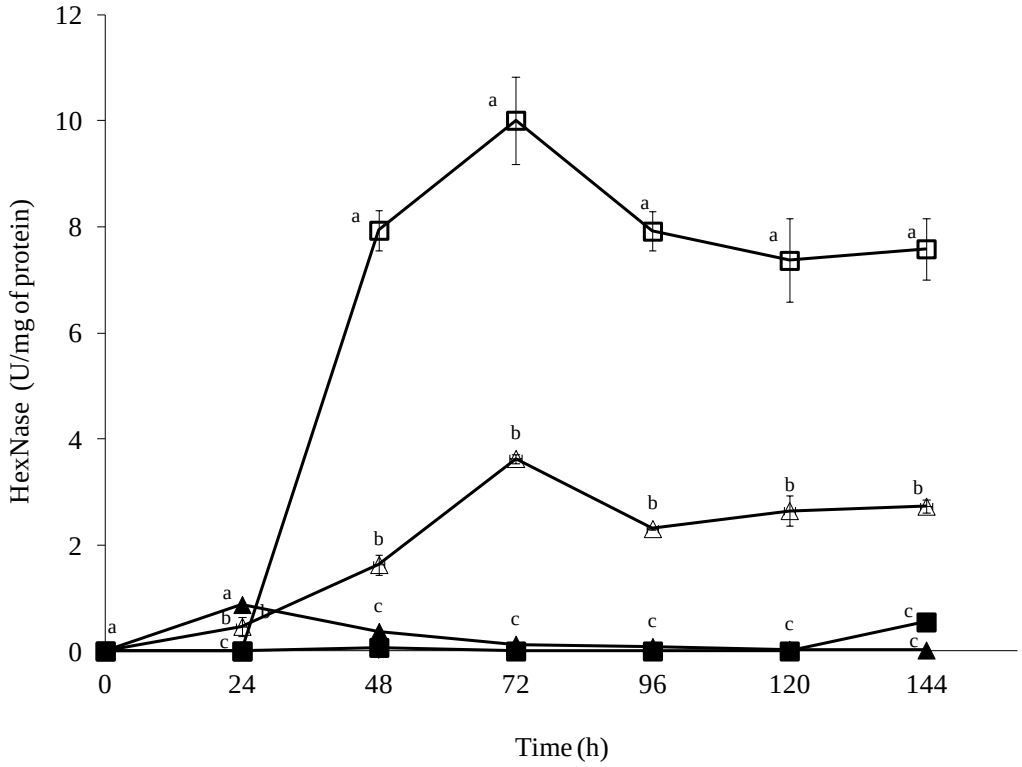

Figure 2. 1. Time course of HexNase activities by *L. lecanii* in SmC with added chitin (open triangle) or fructose (filled triangle) SSC with added chitin (open square) or fructose (filled square) as carbon sources.

2.3.2 Chitinolytic activity on SDS–PAGE

Solid-substrate cultures with added chitin showed two bands in SDS–PAGE with exochitinases activities of 32 and 51 kDa (Fig. 2.2). The endochitinases activity in this culture displayed a band of 26 kDa upon glycol chitin and chitopentaose $(GlcNAc)_5$. Additionally, HexNases, which are responsible for hydrolysis of chitobiose $(GlcNAc)_2$ and chitotetraose $(GlcNAc)_4$ [1] evidenced their activity with bands of 61, 80, 96 and 111 kDa. Similarly, SmC with added chitin media produced exochitinases of 32 and 45 kDa, which released diacetylchitobioses, further substrates for HexNases. Endochitinase activity was observed in 26 kDa, which acted on $(GlcNAc)_4$, whereas HexNase with bands of 61, 96 and 111 kDa were detected in the gel with $(GlcNAc)_2$ (Fig. 2.2).

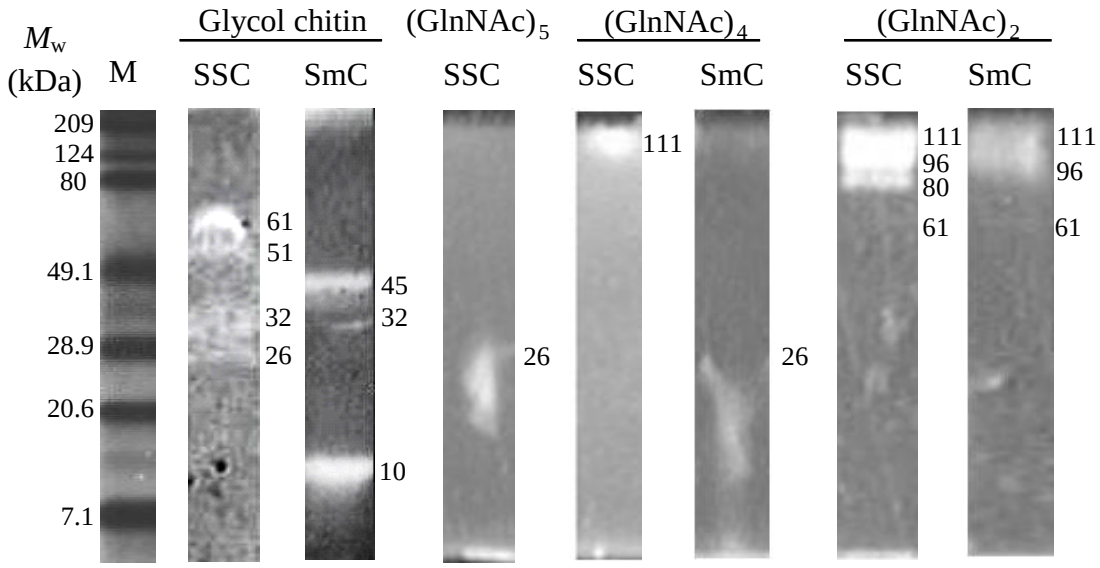

Figure 2. 2. SDS-PAGE with added glycol chitin and chitooligosaccharides as substrates for the crude enzyme obtained after 144 h of SSC and SmC of *L. lecanii* with added chitin mineral media. Lane M indicates the molecular weight standards.

2.3.3 Hfbs production and activity on Teflon surface

The amount of Hfbs produced in SSC with added chitin was 627.3 ± 2 µg mL^{-1}, which represents *ca.* 11-fold more Hfbs than those extracted from SmC (57.4 ± 4.7 µg mL^{-1}). Similarly, when fructose was added as carbon source, the extracted amount of Hfbs from SSC (612.4 ± 11.6 µg mL^{-1}) was significantly higher than that from SmC media (3.5 ± 0.4 µg mL^{-1}). Additionally, Hfbs extracted from SSC displayed a significant reduction (44 %) of the hydrophobicity of Teflon, while those obtained from SSC with added fructose was only 12 % (Table 2.1). Mw value of Hfb extracted from SSC with added chitin media was 7.6 kDa, whereas two bands with 12.7 and 16 kDa SSCF solid-substrate culture with added fructose, SSCC solid-substrate culture with added chitin were determined from this culture but using fructose (Fig. 2.3).

Table 2. 1. Contact angle measurement of Teflon upon treatment with Hfbs from SSC.

Sample	Contact angle (°)
Destilled water (control)	136.65 ± 1.29[a]
SSC with added fructose in media	120.96 ± 3.39[b]
SSC with added chitin in media	76.52 ± 1.12[c]

Mean of six independent observations and their standard errors. Different letters in the same column are significantly different (α ≤ 0.05) according to Tukey's multiple comparison test.

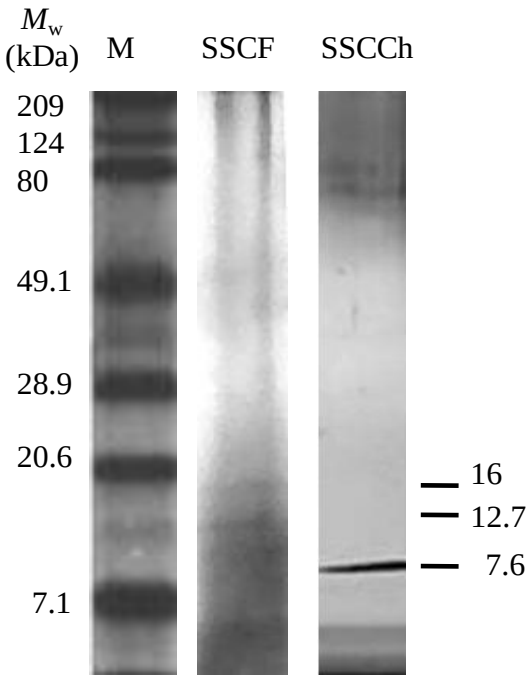

Figure 2. 3. SDS-PAGE of hydrophobin of *L. lecanii* extracted from solid substrate cultures.

Scanning electron microscopy observations of fungal growth after 144 h of SSC with added chitin media are shown in Fig. 2.4. Mycelia were observed as disperse and well

distributed through the PUF (Fig. 2.4a). The biopolymers covered the fungal cells forming a mucilaginous coat and mycelia abundantly colonized the PUF surface (Fig. 2.4b).

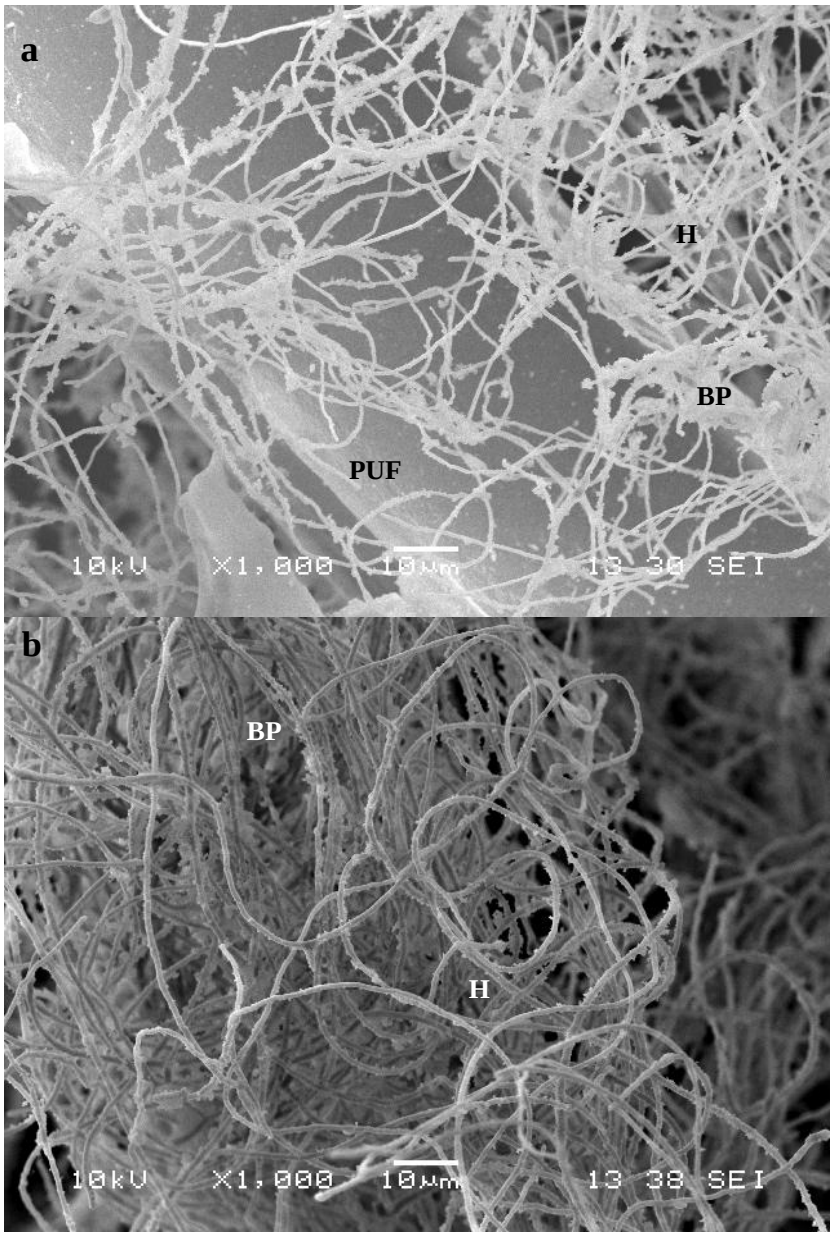

Figure 2. 4. SEM micrographs of PUF after 144 h of inoculation of *L. lecanii* (*H*) with added chitin media; producing mucilagenous coat (*BP*): a x 1,000, b x 2,000.

2.4 DISCUSSION

Experimental evidences pointed out that SSC produced more chitinolytic enzymes than SmC (Fig. 2.1), which might be attributed to the enhanced aeration, thus affecting the fungal metabolism on insoluble substrates, i.e. chitin [13]. This is in agreement with previous reports on chitinolytic enzyme production of *L. lecanii* in SSC using sugarcane bagasse [11, 12, 16] and PUF [13]. Barranco-Florido and co-workers [16] reported that the production of chitinases and proteases by *L. lecanii* was higher in SSC than SmC using cuticle *Sphenarium purpurascens* as inducer. In addition to the use of chitinous substrate as inducer and sole carbon source, other factors such as moisture content, mycelia aggregation in the support [13], and inoculum age have been evaluated in SSC of *L. lecanii* for enhancement of HexNases production [12]. We observed that fructose, as carbon source, showed catabolic repressive effects evidenced by negligible HexNases activities (Fig. 2.1). Similarly, it is reported that addition of glucose to the media in SSC of *L. lecanii* repressed HexNases production, despite of improved biomass production [13].

Although most of the reports dealing with chitinases detection and characterization use SmC [14, 15, 23], our study displayed chitinases in both SSC and SmC having wide Mw range as well as endo , exo- and HexNase activities (Fig. 2.2). Fenice and co-workers [14] reported a chitinase of *L. lecanii* in SmC of culture with Mw of 45 kDa and others detected two chitinases with Mw of 33 and 50 kDa under similar culture conditions [15]. Lu and co-workers [23] isolated and characterized two exochitinases from submerged cultures of *L. lecanii* in the presence of chitin, CHII and CHI2 with 40.93 and 45.95 kDa, respectively. In the present work, we found an exochitinase with Mw of 45 kDa in crude enzyme from SmC of *L. lecanii* (Fig. 2.2), which according to Mw was assigned to a basic chitinase also reported as extracellular and devoted to chitin digestion [23]. Zymograms displayed exochitinases of 32 and 51 kDa in SSC with added chitin as inducer and carbon source (Fig. 2.2) and additionally, several endochitinases and HexNases were produced both in SmC and SSC.

Regarding Hfb production, multiple genes of these proteins have been reported from microorganisms, which can be differentially expressed as a response to different developmental stages, thus Hfb class I is found in cell walls of *Trichoderma reesei*, whereas class II is present on spore walls [9]. Environmental condition also affected the production of

Hfb, such as the addition of compounds of opposite polarities for production of class II Hfbs of *Rhinocladiella similis* [24]. de Vocht and co-workers [25] reported that 1 mg of Hfb (class I) extracted from *S. commune* was enough to reduce the contact angle by its coating over 1 m^2 of a Teflon monolayer of *ca.* 10 nm of thickness. Herein, we focused on the determination of Hfbs class I, considering that fungi require Hfbs to allow growth of hyphae into the air. Our results displayed that the Hfb produced from SSC with added chitin media had significantly higher surface activity than that obtained using fructose. It is worth to note, that in the present work Hfb activity was influenced by the carbon source (Table 2.1). Furthermore, Hfbs produced by *L. lecanii* in SSC using chitin had similar Mw (*ca.* 7.6 kDa) to those reported from other sources, such as *Verticillium fungicola* [26].

Generally, fungal cells are relatively tolerant to stress when they growth in air by the production of Hfbs, which are key factors for development of fungi on solid or liquid surfaces [4, 5]. Additionally, a previous report showed that the production of mucus coat on the hydrophilic spore of *L. lecanii* facilitates dispersal and adhesion onto hydrophobic host surfaces [5].

Mucilaginous coat was also observed by SEM in our samples (Fig. 2.4) along with remarkable capacity of the FA extracted proteins (Hfbs) to modify the hydrophobicity (Table 2.1). This suggests that the solid culture requires the presence of Hfbs to enable the development of fungal cells attached to the support at contact to air. According to this successful result, further work is underway on Hfb identification and purification from *L. Lecanii*.

2.5 CONCLUSIONS

The production of Hfb was successfully achieved from *L. lecanii*. The type of culture and carbon source influenced results significantly as SSC with added chitin and fructose improved the Hfb production when compared with those from SmC herein and in other reports using other these and other fungi or carbon sources. In addition, the use of chitin in SSC media produced more chitinolytic enzymes than SmC.

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CAPÍTULO 3:

The hydrophobicity of the support in solid substrate culture affected the production of hydrophobins from *Lecanicillium lecanii*

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ENVIADO Y EN REVISIÓN
Process Biochemstry, Ref. No.: PRBI-D-14-00471, April 28th, 2014. Major revision received on 17th June, 2014.

The hydrophobicity of the support in solid substrate culture affected the production of hydrophobins from *Lecanicillium lecanii*

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Abstract

Lecanicillium lecanii has been successfully employed to produce hydrophobin-like proteins (HfbL) in solid substrate and submerged cultures varying the type of solid inert support. This study show the results on the effect of the hydrophilic Perlite and hydrophobic Polyurethane inert supports in the solid substrate cultures for production of HfbLs by *L. lecanii*. Class I HfbL was only produced using the hydrophobic polyurethane foam support, showing high surface activity that reduced ca. 50 % hydrophobicity of Teflon, whereas class II HfbLs were produced on both polyurethane foam and perlite supports, and they reduced ca. 50 % the surface tension of water ca. 25% reduction of the hydrophobicity of Teflon.

Keywords: Hydrophobins, chitin, *Lecanicillium lecanii*, solid substrate culture, polyurethane foam, perlite

3.1 INTRODUCTION

The hydrophobins (Hfbs) are amphipathic proteins produced by fungi with eight conserved cysteine residues forming disulfide bonds [1]. These proteins are classified according to their biophysical and hydropathy properties; Class I Hfbs are soluble in formic acid and self-assemble like amyloid proteins forming monolayers, named rodlets during their interaction at hydrophilic and hydrophobic interfaces. The rodlets are highly stable layers

which are dissolved in trifluroacetic acid and they have been observed at surfaces of aerial structures such as conidia, conidiophores and fruiting bodies [2]. Class II Hfbs are soluble in sodium dodecyl sulfate (SDS) or ethanol (60 %) solutions and present coat aerial structures and yeast-like cells but they assemble as flexible layers without rodlets [3]. The amphipathic properties and self-assemble of Hfb are related to their biological roles in fungal development by enabling the hyphae to migrate from submerged condition to air. In addition, Hfbs have been involved in pathogenic activity acting as toxins and allowing attachment of fungal structures, such as conidia or hyphae, to the host surface [4,5]. Hfb layers also covers fungal aerial structures, which confer hydrophobicity, wetting resistance and facilitating their dispersal in air, hyphae protection against desiccation and gas exchange [1,6]. The presence of Hfb like proteins were also reported as mediators of adhesion of on hydrophobic surfaces [7,8]. Some examples of these are the Hfbs class II from *Trichoderma reesei,* which are responsible of sporulation of the fungi in solid cultures [7]. Several authors reported class I Hfbs from *Schizophylum commune* (SC3) and the class II HFBI from *T. reesei,* which are secreted to liquid culture to interact at the air-water interface aiding the migration of hyphae into the air [1,7]. In another study, Hfb-like proteins were determined only with aerial conidia of entomopathogenic fungus *Beauveria bassiana* but not detected in blastospores and submerged conidia. Another entomopathogenic fungus used as biopesticide is *L. lecanii,* which is able to degrade n-hexane or toluene in submerged cultures (SmC), producing Hfbs like proteins with surface activity at the presence of these hydrophobic solvents [9]. The reported comparison among type of culture using this fungus displayed higher production of Hfbs class I in solid substrate culture (SSC) with added chitin or fructose as carbon source than in SmC with these substrates. Interestingly, the SSC-mediated Hfb reduced ca. 50 % the hydrophobicity of Teflon unlike to those obtained from SSC with added fructose, which showed no surface activity [10]. Despite of these reports, there are no reported studies on the use of inert supports on production of these proteins. In this regard, Perlite (P) and Polyurethane foam (PUF) have been employed as packed beds in SSC for several purposes including hydrocarbon degradation, enzyme and biomass production. The P is hydrophilic silicaceous material of volcanic origin while PUF is a synthetic polymer based on isocyanate polymers, which consist of polar urethane groups and soft non-polar segments. Both materials are considered as inert and did not contribute nutritionally to fungal growth, although they can hold several times its weight in water owing to their high porosity and they

are neutral in water without cation exchange capacity [11-13]. This study is first to report the use of PUF and P as solid porous matrices to support the fungal growth of *L. lecanii* in SSC for Hfb production. The effect of the solid support on the type and surface activities of Hfbs is described.

3.2 MATERIALS AND METHODS

3.2.1 Microorganism and culture conditions

Lecanicillium lecanii 2149 strain was provided by USDA-ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungi, USA) collection. *L. lecanii* was grown in SSC on two inert supports: i) PUF with a particle size ca. 0.125 cm^3 within nutrient ratio of 1:15 (w v^{-1}); ii) P with a particle diameter ca. 3.3 mm within nutrient ratio of 1:2 (w v^{-1}). Culture conditions were carried out according to Rocha-Pino et al. [10], the supports added with mineral medium pH 6, colloidal chitin (30 g L^{-1}) and inoculum of 5 x 10^7 spores g^{-1} of substrate were packed into glass columns and incubated at 25 °C. Aeration of 1.4 mL air min^{-1} per g of moist material was provided during 6 and 15 days. All the materials were sterilized at 121 °C for 15 min before inoculation.

Solids (biomass and matrices) and supernatant (soluble matter in water) were separated by mixing the solids with water (50 wt%), then it was pressurized to 1,000 psi for PUF SSC, while the mixture was pulverized in a mortar for P SSC and centrifuged at 12,700 *g* at 4 °C for 10 min. Supernatants from both supports were separated using Whatman filter paper no. 40. 0.2 g (wet weight) of support with biomass was mixed with 5 mL of phosphoric acid (0.15 M) and heated in a water bath for 7 min. After centrifugation (12,700 *g*) the supernatant was used to determine the total protein from biomass [9]. Total soluble protein was determined from biomass and supernatant by Bradford [14].

3.2.2 Colloidal chitin preparation and characterization

Chitin was obtained from lactic acid fermentation of shrimp wastes and then purified by the following treatments: i) chitin was washed with distilled water (Ch1); ii) Ch1 was treated with HCl 0.5 N and NaOH 0.4 M (Ch2). Furthermore, Ch1 and Ch2 were treated with HCl 10 N and neutralized with water to obtain colloidal solutions. Ch1 and Ch2 were characterized on residual protein (%) by Kjeldahl method (K-435 BÜCHI, Switzerland) [15].

Degree of acetylation (DA) was determined with chitin treated with DCl/D_2O and analyzed by proton nuclear magnetic resonance (^1H NMR) spectroscopy (Bruker Advance III 500, Germany) at 200 MHz using 3-(trimethylsilyl) propionic acid as internal reference. DA was calculated from integration of assigned signals on the NMR spectra [16].

3.2.3 HfbLs extraction

The samples used for this analysis were class I and class II HfbLs fractions obtained from the mycelia of *L. lecanii* after 6 d of SSC. HfbLs were extracted from the biomass and the supernatant of *L. lecanii* in SSC. Class II HfbLs were extracted from mycelia following the methodology reported by Askolin et al. [3]. Supports with mycelia were washed with distilled water and incubated with SDS 2 % (w v^{-1}) in 100 mM Tris-HCl buffer, pH 9.0 for 2 h in cold water bath and soft stirring agitation. Then, mycelia and support were separated by compressing or centrifugation for PUFSSC and PSSC, respectively. Remaining SDS extract was precipitated with KCl (2 M) and centrifuged (12,700 *g*) at 6 °C for 20 min. Subsequently, supports were washed with water and Class I HfbLs were extracted with formic acid, followed by electrobubbling at 300 mA during 3 h. The foam obtained was centrifuged and the pellet solubilized with TFA and evaporated with dry air stream [10]. Class I HfbLs from supernatant were extracted by the following procedure: protein from supernatant was precipitated with trichloroacetic acid (TCA) 5 % (w v^{-1}) at 4 °C for 2 h and centrifuged (12,700 *g*) at 4 °C for 20 min. Pellet was washed with acetone and solubilized with performic acid at 4 °C h during 4 h, subsequently the acid was evaporated with air flow [17]. Protein precipitated (without addition of performic acid) was treated as class II HfbLs [17]. For each step of the HfbLs extraction, the protein was determined by Bradford [14]. Protein yield with respect to biomass ($Y_{HfbL/biomass}$) was calculated considering protein concentration of each step of purification and the total protein of either biomass or supernatant. Classes I and II HfbLs fractions were analyzed by denaturing electrophoresis SDS-PAGE according to Laemmli [18] using resolving gel of polyacrylamide in concentrations of 17 % and 15 % for class I and class II HfbLs, respectively. Gels were stained with coomassie blue or silver nitrate (Bio-Rad, USA) and analyzed with the image processing software (ImageJ 1.41o National Institutes of Health, USA).

3.2.4 Determination of surface activities of HfbLs by contact angle measurements

Surface activity of HfbLs at air-solid hydrophobic interface was measured by contact angle (θ) of a water drop (1 µL) on Teflon (poly (tetrafluoroethylene)) surface previously treated with an HfbL solution of 50 µg mL^{-1} [10]. Teflon treated with class I HfbL was washed with 2% SDS at 100 °C and then with deionized water, while class II HfbLs were washed only with deionized water at room temperature. In addition, class II HfbLs were subjected to a molecular weight cut off membrane of 30 kDa (Amicon Millipore, USA). Digital images of the water droplets were obtained in a horizontal light microscope Qx3 Intel with image processor (Intel Corporation, USA). Contact angles measurements were carried out by duplicate, randomly determined in six areas per sample and images analyzed with ImageJ 1.41o software.

3.2.5 Determination of HfbL surface activities by measurements of surface tension

Surface activity of HfbLs was determined at air-water interface by measuring the symmetry of a pendant drop of deionized water as reference and HfbLs solutions (50 µg mL^{-1}). Analysis was carried out using a Theta KSV optical tensiometer system (KSV Instruments, Finland) calibrated with a 4.00025 mm, grade 25 and tungsten carbide sphere. Images were analyzed through a Young-Laplace model employing Attention Theta software V 4.1.0 (Biolin Scientific, Finland). Surface tension determinations were carried out by duplicate with six determinations per sample.

3.2.6 Scanning electron microscopy of *L. lecanii* in SSC

L. lecanii grown on PUF or P and colloidal chitin were immersed in glutaraldehyde 5 % (v v^{-1}) at 4 °C for 24 h and subsequently treated with OsO_4 1 % (w v^{-1}) for 2 h. Then, dehydrated with alcohol and covered with carbon and gold for scanning electron microscope JEOL JSM-5900 LV (Japan).

3.2.7 Statistical analysis

A randomized design was applied using yield, contact angle and surface tension of HfbLs as response variables and chitin purity and support type as independent variables.

Analysis of variance (ANOVA) and multiple comparisons of means by Tukey-Kramer test ($p \leq 0.05$) were performed using NCSS 2000 software (NCSS Inc., USA 2001).

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of chitin purity on the HfbL production from *L. lecanii* in SSC.

Insoluble biopolymers, such as starch, cellulose, pectin, lignin or chitin have been used as substrates for SSC. Chitin has been studied as support and sole carbon source, as well as enzyme inducer for chitinases production [15, 16, 19-21]. The physicochemical properties of chitinous substrates might have influence when used for microbial growth and production of chitinolytic enzymes therefore the purity of this biopolymer on the class I and II Hfbs production from *L. lecanii* in SSC using PUF as support is prompted in this study. The chitins tested presented 8.6 ± 0.2 % w w^{-1} and 5.7 ± 0.3 % w w^{-1} of residual protein and DAs of 100 % and 89.8 % for Ch1 and Ch2, respectively. Ch2 used as carbon source increased *ca*. 14 % the class I HfbLs production from *L. lecanii* grown on PUF compared to Ch1 (Figure 3.1). However, the difference in purity of the chitin shows no significant effect ($p \leq 0.05$) on the class II HfbLs production, which might be ascribed to the residual protein bonded to chitin. This residual protein might form a physical interference between fungus-substrate interactions. Furthermore, the residual protein induces fungal proteases [21], which could reduce chitinases and the Hfbs production required to the adhesion on chitin thereby its hydrolysis.

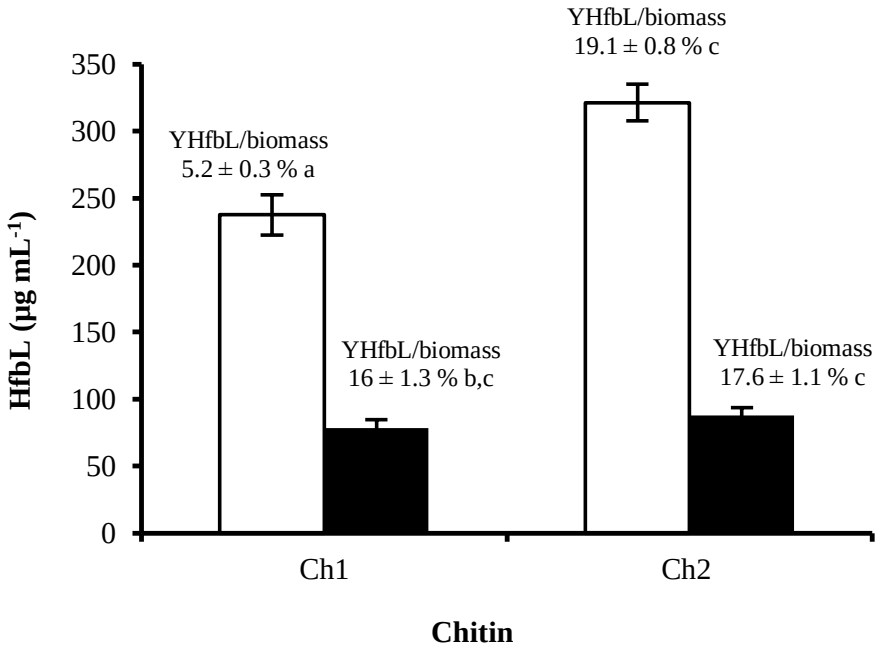

Figure 3. 1. Production of HfbLs associated to mycelia of *L. lecanii* cultured on PUF after 14 d. Class I (void), Class II (solid). $Y_{HfbL/biomass}$ (%) with each condition is indicated. Different letters in the same column are significantly different ($\alpha \leq 0.05$) according to Tukey-Kramer multiple comparison test.

On the other hand, Ch2 showed lower DA (89.8 %) than Ch1 (100 %) which might affect the interaction between fungus and biopolymer. According to Holder and Keyhani [8], Hfbs from *Beauveria bassiana* were adhered to specific surfaces upon their hydrophobicity. In this regard, the hydrophobic character in chitin is associated to the acetyl groups responsible for their interactions among chains, including the hydrogen bonding, electrostatic repulsion owing to positive charged amino group and crystallinity [22]. Therefore, the crystallinity reduction and the acetyl moieties increase the interaction among chitin and enzymes produced by *L. lecanii* during its hydrolysis [21]. It has been reported that Hfbs class II show high affinity to polar and cationic surfaces but not to anionic surfaces [23] and thus, positively charged amino groups as a consequence of deacetylation might influence the Hfbs production from *L. lecanii*.

L. lecanii produced classes I and II HfbLs, the former was determined in biomass from PUF cultures. The plausible explanation is that classes I and II Hfbs contribute to the hydrophobicity of fungal structures and they could have different or complementary functional roles during fungus development. In agreement to that, Sevim et al. [5] reported that Hfbs HYD1, HYD2, HYD3 from *Metharhizium brunneum* were differently expressed depending on the physiological stage of that fungus. In that study, class I HYD1 and HYD3 were important for conidiogenesis and pathogenicity, while class II hyd2 gene was less expressed on aerial mycelia but in mutant strain of hyd1 or hyd3 gene deleted, suggesting that HYD2 displayed a compensating role to confer hydrophobicity to fungus. Additionally, Askolin et al. [24] proved that a mixture of class I (SC3) and class II (HFBI or HFBII) Hfbs in solution would form mixed membranes that assemble independently and compete for available interface.

3.3.2 Effect of the support type on the production of class I and II HfbLs from *L. lecanii*

Fungi as *S. commune*, *B. bassiana* and *Trichoderma reesei* produce several Hfb according to their development on surfaces [6,8,24]. In this work, class I and II HfbLs production from *L. lecaniii* cultured in SSC was determined testing two inert supports, PUF and P, and Ch2 as a carbon source. The $Y_{HfbL/biomass}$ associated to mycelia were *ca*. 10-fold higher than that in the supernatants in both cultures of PUF or P supports (Table 3.1), which was attributed to the Hfbs affinity for the mycelium toward adhesion on surfaces and then the avoidance of desiccation [1,4-6]. Interestingly, the type of support displayed significant effect ($p \leq 0.05$) on the class I and II HfbLs production. The class I HfbLs associated to mycelia was 302.07 ± 14.8 µg mL^{-1} HfbL with $Y_{HfbL/biomass}$ of 10.7 ± 0.75 % produced by *L. lecanii* in PUF culture, which was 3-fold higher than that with P in SSC (69.1 ± 7.3 µg HfbL mL^{-1}) (Table 3.1).

Table 3. 1. Production of classes I and II HfbLs from *L. lecanii* in SSC with Ch2 at 6 d.

Sample	Support	Solvent employed for extraction of HfbLs	HfbL ($\mu g\ mL^{-1}$)	$Y_{HfbL/biomass}$ (%)
		Class I HfbLs		
Biomass	PUF	TFA	302.1 ± 14.8	10.7 ± 0.75^{c}
Supernatant	PUF	TCA-pF	61.7 ± 6	$0.3 \pm .02^{a}$
Biomass	P	TFA	69.1 ± 7.3	3.1 ± 0.3^{b}
Supernatant	P	TCA-pF	98.7 ± 14.3	0.7 ± 0.1^{a}
		Class II HfbLs		
Biomass	PUF	SDS/KCl	123.7 ± 8	2.2 ± 0.1^{b}
Supernatant	PUF	TCA	66.1 ± 5.7	0.3 ± 0.03^{a}
Biomass	P	SDS/KCl	118.7 ± 9.6	5.4 ± 0.4^{c}
Supernatant	P	TCA	180.4 ± 4.7	$1.3 \pm 0.03^{a,b}$

Mean of 3 independent observations. Different letters in the same column are significantly different ($\alpha \leq 0.05$) according to Tukey-Kramer multiple comparison test.

These evidences are attributed to hydrophobicity differences among solid supports, according to the criteria of wetting properties of surfaces [25]. PUF is a hydrophobic synthetic polymer derived from hydrocarbons with a water contact angle of 121.3 ± 1.3 °, while the P is a mineral origin material considered as hydrophilic, thereby contact angle could not be determined because the water drop was rapidly absorbed in P. In a previously reported work, the polarity of carbon sources in cultures of *Rhinocladiella similis* influenced significantly with the class of Hfbs produced, with ethanol, the Hfb displayed a molecular weight of 8.5 kDa while with n-hexane was 15 kDa [26]. Our results indicate that Hfbs class I were required to mediate fungi adhesion to PUF, whereas Class II Hfbs might be produced for protection

against desiccation. SEM analyses gave further evidences as in PUF culture, the *L. lecanii* hyphae evolved into the interstitial space of the support and showed a mucilage coat (Figure 3.2A). Contrarily, the mycelia in P cultures develop around the particle owing to the limited interstitial space and under this condition did not produce mucilage coat (Figure 3.2B). Thus, class II HfbLs could act as gas channels in order to avoid the hyphae desiccation and agglomeration. In this regard, earlier reports showed that specific class I Hfbs can modify the mucilage composition, thereby increasing the proportion of β-1,3-glucan in the mycelia of *B. bassiana* [4], which is in fact an additional factor for promotion of self-assembly of SC3 (class I Hfb) [27]. Additionally, the expression of genes of Hbfs class I has been reported by *M. brunneum* and *B. bassiana* in spores and mycelia during insect infection assays, which facilitated their adhesion to hydrophobic insect cuticle [4-5]. Similarly, the SC3 Hfb from *S. commune* is responsible for imparting hydrophobicity to the mycelia during adhesion to hydrophobic surfaces [1,6] and that n-hexane and toluene induced the production of Hfb class I in *L. lecanii* [9]. All this is in agreement with our results and indicates that the hydrophobicity of the solid support promote the production of class I HfbL of *L. lecanii* assembled in rodlets to act as adhesion mediator on PUF.

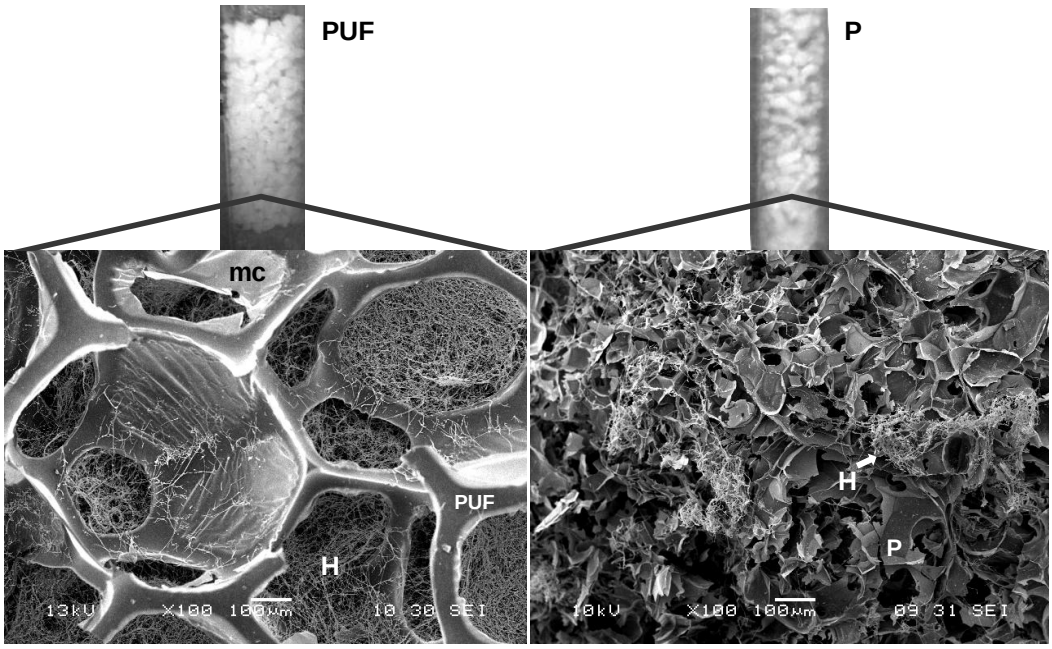

Figure 3. 2. Scanning electron microscopy (SEM) of *L.lecanii* grown in SSC using as support PUF or P. H- Hyphae, mc- Mucilage coat.

The concentration of class II HfbLs associated to mycelium of *L. lecanii* cultured on P and PUF were not significantly different with 118.73 ± 9.6 µg mL^{-1} HfbL and 123.7 ± 8 µg HfbL mL^{-1}, respectively. Although, the $Y_{HfbL/biomass}$ in P SSC was 5.4 %, that is, 2-fold higher than that determined with PUF ($Y_{HfbL/biomass}$= 2.2 %). This point out that the production of class II HfbLs is enhanced on P in SSC owing to the hydrophilicity of this support, and thereby the proteins could contribute to fungal development on the surface of P by means of formation of hydrophobic coat layer. This result is in agreement with the spores of *T. reesei* having the expression of class II Hfbs gene of hfbII [7].

3.3.3 Electrophoretic analyses of classes I and II HfbLs from *L. lecanii* in SSC.

The HfbLs from biomass at steady state, 6 d of culture, were subjected to electrophoretic analysis. Sevim et al. [5] have reported that Hfb expression from *M. brunneum* depends on growth stage, such as the formation of aerial mycelia and conidia. These authors observed that on solid glucose medium, 3 d-old colonies of strain with hfb1 delete were fluffier than colonies of wild type strain, however, after 7 d of growth, no morphological differences between strain mutant (hfb1) and wild type were detected, which was ascribed to the expression of other Hfb which could complement the hfb1 function.

The HfbL obtained from P were extracted only by the protocol of class II Hfb, and there were no protein bands assigned to Hfb class I (Figure 3.3). The class II HfbL obtained from both, PUF and P SSC, showed more than one protein band and agree with previous reported data, with Hfbs molecular masses in the range of 22, 11 and 7 kDa [1]. *L. lecanii* on PUF favored the production of a protein band of 22 kDa but using P SSC promotes small proteins of 11 kDa (Figure 3.3).

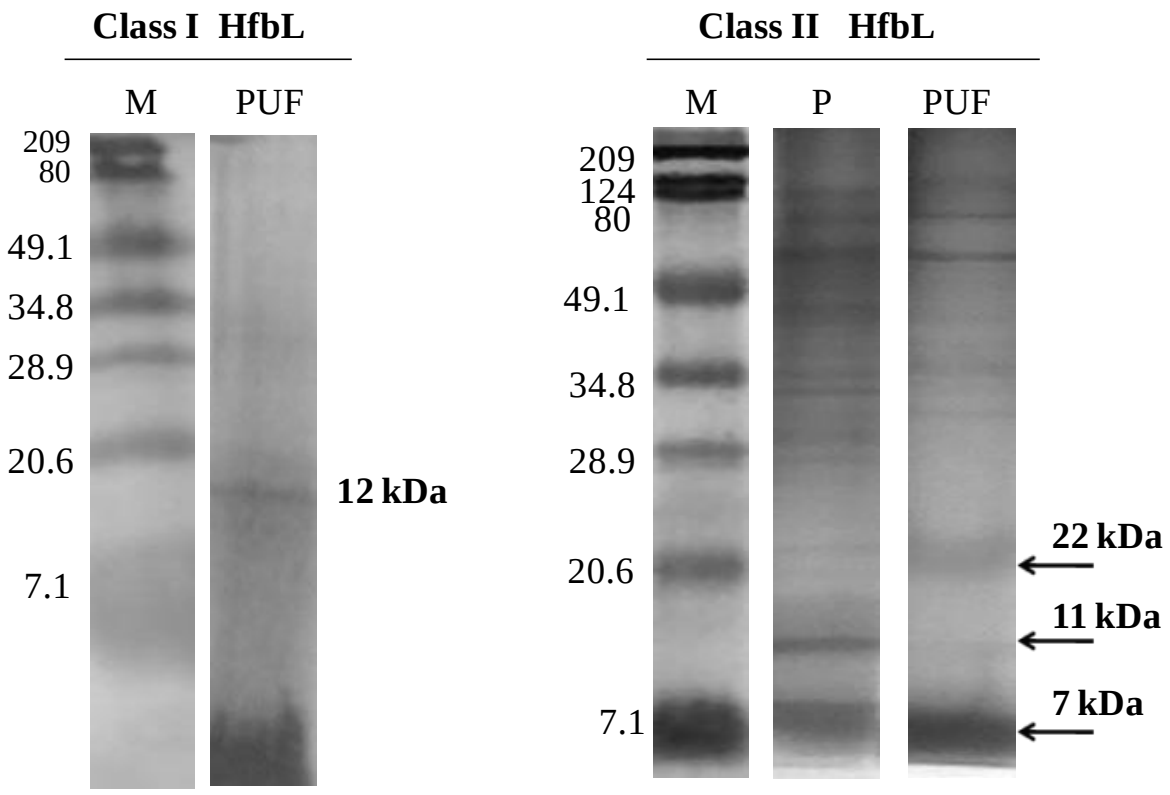

Figure 3. 3. SDS-PAGE of class I and class II HfbLs associate to mycelium from *L. lecanii* in SSC. M- Molecular weight standards; PUF as support; P as support.

3.3.4 Surface activity of class I and class II HfbLs from *L. lecanii* in SSC

In this study, the class I HfbL reduced the hydrophobicity of Teflon ca. 50 %, while, class II HfbLs showed only 25 % of reduction (Table 3.2). It has been reported that class I Hfbs are self-assembling as rodlet layers as result of exposure of its glycosylated zone, which promotes a structural change of the protein from α-helix to β-sheet, this configuration can be induced by hydrophobic environments, such as high concentrations of protein or the presence of β-1,3-glucan [2,27].

Nonetheless, class II HfbLs reduced the surface tension of water ca. 50 %, that is 10-fold higher than class I HfbL, which decreased the surface tension of water only 5 %. It has been reported that class II HFBI and HFBII from *Trichoderma* can reduce the surface tension of water in a few minutes as opposed to SC3 which needs several hours and this has been

explained for the structural change experienced by class I during its exposure to the air, while the class II only showed changes of orientation of their side chain in contact with the water-air without changes above its absorption or oligomers assembly [24].

Table 3. 2. Surface activities of class I and class II HfbLs.

	Surface tension		Contact angle	
Treatment	γ (mN m^{-1})	*Reduction of γ of water (%)	θ (°)	$^{+}$ Reduction of θ compared with water on Teflon (%)
		Class I HfbLs		
PUF	69.0 ± 0.2^{e}	4.6	56.1 ± 5.15^{a}	51.32
		Class II HfbLs		
PUF total extract	36.9 ± 0.1^{a}	48.9	119.5 ± 1.2^{b,c}	15.6
PUF > 30 kDa	40.5 ± 0.3^{b}	43.9	137.8 ± 2.7^{d}	1.7
PUF < 30 kDa	37.6 ± 0.1^{a}	48.0	106.9 ± 3.9^{b}	25.1
P total extract	37.1 ± 0.4^{a}	48.7	108.0 ± 2.5^{b}	24.33
P > 30 kDa	66.3 ± 0.9^{d}	8.2	121.7 ± 2.8^{c}	13.9
P < 30 kDa	57.7 ± 0.1^{c}	20.2	116.1 ± 2.5^{b,c}	18.1

HfbLs extracts at 50 µg mL^{-1}. (*) γ_{water} = 72.3 ± 0.2 mN m^{-1}. (+) Contact angle of drop of water on Teflon is 131.9 ± 0.6°. Different letters in the same column are significantly different (α≤0.05) according to Tukey-Kramer multiple comparison test.

The class II HfbLs showed proteins bands at 22, 11 and 7 kDa, these were favored according to the support used to *L. lecanii* culture, thus, the class II HfbLs were subjected to a molecular weight cut off at 30 kDa prior to surface tension measurements. The fraction class II HfbLs < 30 kDa showed greater surface activity, in both contact angle and tension surface than that of fractions > 30 kDa. The fraction < 30 kDa obtained from PUF SSC decreased from 72.3 ± 0.2 to 37.6 ± 0.1 mN m^{-1} the surface tension of water (ca. 48 % reduction) indicating higher surface activity compared to P SSC < 30 kDa fraction from 72.3 ± 0.2 to

57.7 mN m^{-1} (ca. 20.2 % reduction). According to previous reports on *Trichoderma,* the two reported proteins, HFBII and HFBI, showed significant differences in their surface activity because HFBI has a glycine at position 59 (gly59) in the amino acid sequence of the protein, thus, the steric and hydrophobic contribution of gly explains its high affinity to hydrophobic surfaces, compared to its association in solution. On the other hand, the HFBII showed Asp59, which reduces the contact area between the hydrophobic surface and Hfb [28]. It is worth noticing that the values of surface tension of water obtained in this work are within the values reported for other Hfbs class II [24].

3.4 CONCLUSIONS

The hydrophobicity of the supports used in SSC influenced the yield and surface activities of the class I and class II produced by *L. lecanii*. This highlights the high level of specificity and importance of the HfbLs and its biological function for the fungal development by surface adhesion with a direct implication in pathogenesis. Furthermore, the purity of the chitin can increase the HfbL production from *L. lecanii* in SSC representing a very important factor of process control.

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CAPÍTULO 4:

Morphological changes, chitinolytic enzymes and hydrophobin like proteins as responses of *Lecanicillium lecanii* during growth with hydrocarbon

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ORIGINAL PAPER

Morphological changes, chitinolytic enzymes and hydrophobin-like proteins as responses of *Lecanicillium lecanii* during growth with hydrocarbon

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Abstract *Lecanicillium lecanii*, *Verticillium chlamydosporium*, *V. fungicola* var *flavidum* and *Beauveria bassiana* were evaluated on their growth with pure *n*-hexane, toluene and *n*-hexane:toluene 17:83 (v:v) mixture. Another set of treatments were conducted with colloidal chitin as additional carbon source. All the strains of *Lecanicillium* were able to grow using hydrocarbons with or without the addition of chitin, although the presence of hydrocarbons showed significant inhibition evidenced by measured biomass, radial growth and microscopic analyses. Degradation of *n*-hexane ranged within 43 and 62 % and it was higher than that with toluene. The strains L460, L157 and L2149, which presented the highest growth, were further selected for determinations of hydrocarbon consumptions in microcosms. Strain L157 showed the highest consumption of *n*-hexane (55.6 %) and toluene (52.9 %) as sole carbon source and it also displayed activities of endochitinases, *N*-acetylhexosaminidase and production of hydrophobins class I and II.

Keywords Entomopathogenic fungi · Hydrocarbons · Chitinases · Hydrophobins

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Introduction

Entomopathogenic fungi such as *Lecanicillium lecanii* are able to produce hydrophobins (Hfbs) [1], which are small proteins capable to self-assemble in amphipathic membranes. Their role in nature consists in preventing desiccation of the hyphae or spores and decreasing the surface tension of water allowing the hyphae emerge from the liquid medium to in-air environment [2–5]. Hfbs are required for the successful development of filamentous fungi by the adhesion of hyphae in the hydrophobic cuticles of insects, which are covered by waxes and hydrocarbons [6], and subsequently, excreted cell wall degrading enzymes, chitinases, proteases and lipases, thus allowing host invasion [7].

On the other hand, filamentous fungi utilize hydrocarbons as sole carbon source. Isolates of *Paecilomyces* sp., *Verticillium* sp., *Beauveria* sp., and *Penicillium* sp. were tested for their ability to metabolize a variety of *n*-alkylbenzenes [8]. Fungi used in biological control, *B. bassiana* and *Metarhizium anisopliae*, can degrade *n*-Pentacosane, 3,11-dimethylnonacosane and *n*-hexadecane [9, 10]. Interestingly, the increase of entomopathogenic activity of *Beauveria bassiana* on the bean weevil was observed under cultivation with *n*-hexadecane as sole carbon source [9, 11]. With regard to the entomopathogenic fungi *Lecanicillium*, Krivobok et al. [12] showed 10 % degradation of anthracene, besides, Vroumsia et al. [13] claimed the degradation of 22 % of 2,4-dichloride phenol acid, but despite these reports, there is no information, at least to the best of our knowledge, on the tolerance of *Lecanicillium* to toluene or *n*-hexane and their effect on the production of Hfb and enzymes involved in the pathogenesis. Therefore, the aim of this work was to evaluate several strains of *Lecanicillium, Verticillium* and *B. bassiana* on tolerance, growth, morphological changes and production of

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4.2 MATERIALS AND METHODS

4.2.1 Microorganisms

Twenty strains of *Lecanicillium lecanii* were provided by the American type culture collection (ATCC26854 and ATCC46578), USDA-ARSEF collection of entomopathogenic fungal cultures (L157, L974, L991, L1029, L2009, L2149, L2460, L2832, L2858, L2916, L3909, L5129 and L5153) and from culture collection of the Basic Mycology Laboratory of the Department of Microbiology and Parasitology, Faculty of Medicine, Universidad Nacional Autonoma de Mexico (L348, L457, L458, L459 and L460). *Verticillium chlamydosporium* strain V2218, *V. fungicola var flavidum* strain V4519 and *Beauveria bassiana* (Bb) were propagated on potato dextrose agar slants at 25 °C and stored at 4 °C.

4.2.2 Media

Mineral media (g L^{-1}): K_2HPO_4 (2.80), $MgSO_4 \cdot 7H_2O$ (1.38), $CaCl_2$ (0.48), $FeSO_4 \cdot 7H_2O$ (0.22), $(NH_4)_2SO_4$ (7.50), $(NH_4)Cl$ (4.10), $NaH_2PO_4 \cdot 7H_2O$ (1.67) at pH 6.0. 15 g L^{-1} of bacteriological agar was added to mineral media for screening fungal strains. Carbon sources added to the mineral media were (g L^{-1}): glucose (25.68) as control; *n*-hexane (MH); toluene (MT); mixture of both solvents (17:83 molar ratio) (MTH) and chitin (10) combined with *n*-hexane (MChH); toluene (MChT) and mixture of both solvents (17:83 molar ratio) (MChTH).

4.2.3 Screening of fungal strains based on hydrocarbon tolerance

10^7 spores mL^{-1} of the fungal strains was inoculated in Petri dishes placed in hermetic containers. Each container was equipped with a tube of activated coal impregnated with the hydrocarbons, then, 1 mL of hydrocarbons was added throughout to the tube every 2 days. Each container was incubated at 25 °C during 30 days. Radial growth, hyphae diameters and halos of colloidal chitin hydrolysis were determined at the end of the incubation period. Agar was melted and filtered and fungal biomass determined by gravimetry.

4.2.4 Determination of consumption of toluene or *n*-hexane in microcosm experiments

10^7 spores mL^{-1} of *L. lecanii* strains L460, L157 and L2149 were placed in 125 mL flask containing 10 mL of mineral medium with added toluene (4.35 mg mL^{-1}) or *n*-hexane (3.3 mg mL^{-1}) as carbon sources. Flasks were sealed with inert Teflon valves (VICI Precision Sampling) and toluene or *n*-hexane was added by a microsyringe. All samples were incubated at 25 °C and 180 rpm for 60 days. Hydrocarbon concentrations were monitored by gas chromatography (GC) from samples withdrawn from headspaces. The experiments were performed by duplicate. VOCs consumptions were calculated considering the initial hydrocarbon concentration (µg mL^{-1}) and the concentration at time t.

4.2.5 Gas chromatography analysis

100 µL of each sample was injected (VICI Precision Sampling) into a gas chromatographer (Agilent Technologies 6890N) equipped with a flame ionization detector and an AT-WAX (Alltech Heliflex 25 m) column. GC settings were injector at 200 °C, oven 65 °C, detector at 250 °C and carrier (helium) flow rate of 0.6 mL min^{-1}. CO_2 concentration was determined by thermal conductivity detector with a Poropak 55-m column with operating

conditions: injector at 110 °C, oven 65 °C, detector at 110 °C and helium flow rate of 4.4 mL min^{-1} [14]. Measurements were carried out by duplicate.

4.2.6 Biomass determination from microcosms

Biomass was determined measuring the total protein by Bradford [15]. Culture medium was centrifuged (11,500 rpm) at 4 °C for 20 min. Then, 0.2 g of pellet was mixed with 5 mL of phosphoric acid (0.15 M) and heated in a water bath for 7 min and afterward centrifuged at 12,000 rpm. The protein from the pellet and supernatant were determined by Bradford. Bovine serum albumin was used as standard.

4.2.7 Assay of chitinolytic activity

N-acetylhexosaminidase (HexNase) and endochitinases (Endo) activities from the crude enzyme (supernatant after centrifugation) were assayed according to the methodology reported by Tronsmo and Harman [16]. Yield coefficients, $Y_{HexNase/x}$ and $Y_{Endo/x}$, were determined considering maxima enzyme activities on total biomass produced [17].

4.2.8 Hfb extraction and determination of surface activities

Hfb class I were extracted using formic acid following the procedure reported by Vigueras et al. [18] from biomass of the microcosm cultures. Hfb class II was carried out using SDS 1 % (p v^{-1}) as reported by Askolin et al. [19]. Surface activity was evaluated as the ability to modify the surface hydrophobicity of Teflon [poly(tetrafluoroethylene)] by the Hfbs by measuring the contact angle of a drop of water [18]. Teflon was washed with water and ethanol and then dried. Hfb solution was prepared dissolving extract of dried protein in deionized water at a concentration of 50 µg $protein^{-1}$. Hfb solution was used onto 1.32 cm^2 of Teflon. Subsequently, 1 µL of deionized water was dropped on the treated Teflon surface and observed with a side-illumination horizontal light microscope Intel Qx3 (Intel Corporation, Santa Clara, CA, USA). Contact lengths (b) and heights (h) of the water droplet were measured by ImageJ 1.41o software (National Institutes of Health, USA). Contact angle was calculated according to the Eq. (4.1) [1]. Analyses were conducted by quadruplicate, in three different random locations on each surface. The reduction of the hydrophobicity of Teflon was calculated as percentage considering the contact angles of water and the extracted Hfb.

$$\theta = 2\tan^{-1}\left(\frac{2h}{b}\right) \qquad (4.1)$$

4.2.9 Scanning electron microscopy

Samples were immersed in 5 % (v v^{-1}) glutaraldehyde for 24 h (4 °C) and treated with OsO4 1 % (v v^{-1}) for 2 h. Further on, they were dehydrated with ethanol and covered with carbon and gold prior examination in the scanning electron microscope (JEOL JSM-5900 kV, Tokyo).

4.2.10 Statistical analysis

A randomized design was carried out in sextuplicates for response variables of radial growth, biomass and halos of chitin hydrolysis determinations; as well the measurements of diameters of hypha were obtained from 60 to 90 observations with fungi and carbon source in the media as sources of variation. In microcosm experiments, determinations of biomass, HexNase, Endo and VOCs consumption were determined as response variables in octuplicate and fungi and carbon source as sources of variation. The surface activities of Hfbs were obtained by contact angle measurements in quadruplicates as response variable and carbon sources as source of variation. Analysis of variance and test of multiple comparisons of means by Tukey–Kramer ($p \leq 0.05$) were performed using NCSS 2000 software (NCSS Inc., USA 2001).

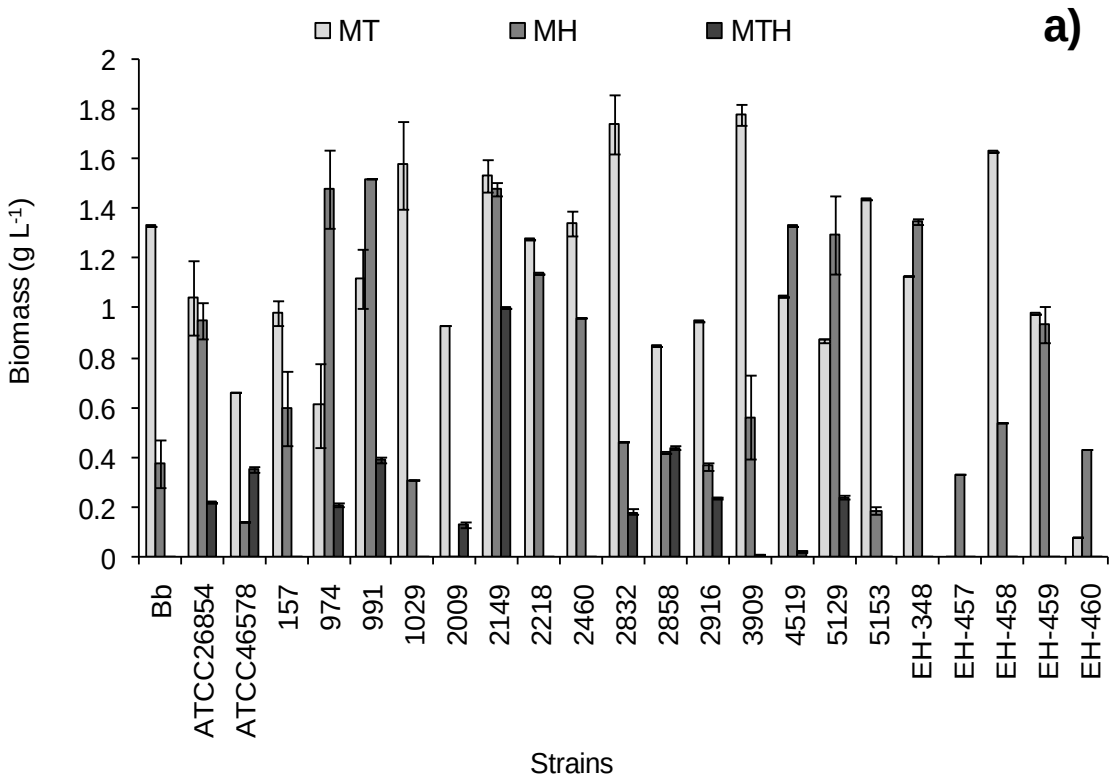
4.3 RESULTS AND DISCUSSION

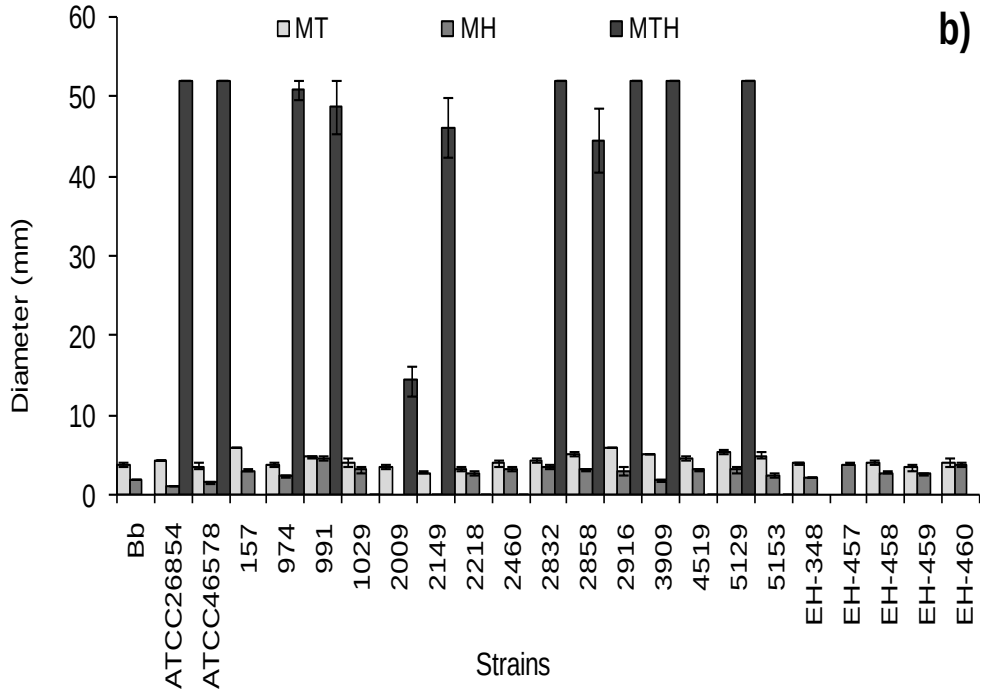
4.3.1 Selection of strains with capacity to grow with hydrocarbons

The fungal biomass determinations in hydrocarbon media with added VOCs were 5-fold in MT, 7-fold in MH and 11-fold in MTH lower than MG (control). The 75 % of the strains produced higher biomass with toluene than *n*-hexane or the mixture. *L. lecanii* strains with the highest biomass productions with MH were L991 (1.52 ± 0.01 g L^{-1}), L2149 (1.48 ± 0.03 g L^{-1}) and L974 (1.48 ± 0.16 g L^{-1}) and those with more biomass production in the MTH were L2149 (1.1 ± 0.01 g L^{-1}), L2858 (0.44 ± 0.01 g L^{-1}) and L991 (0.39 ± 0.01 g L^{-1}). For MT the strains were L3909 (1.78 ± 0.04 g L^{-1}), L2832 (1.74 ± 0.12 g L^{-1}) and L458 (1.63 ± 0.01 g L^{-1}) (Fig. 4.1a).

The radial growth was not related with the fungal biomass production because fungi grew in aggregates or dispersed mycelia without significant difference ($p \leq 0.05$) in the biomass production among hydrocarbons (Fig. 4.1a, b). Agglomerated and dense mycelia were observed with toluene or *n*-hexane, where 70 % of the strains showed from 3- to 35-fold lower radial growth than the control. On the other hand, similar percentage of strains with the mixture displayed dispersed and spread mycelia, which were scarce and ramified covering completely the plate surface (Fig. 4.1b). This effect might be attributed to the hydrocarbon toxicity, as reported by Krivobok et al. [12], where *L. lecanii* was cultivated on agar medium using several anthracene concentrations as carbon source. In that report, the increase in the toxicity decreased the growth along with the observation of sterile and more disperse mycelia.

According to the microscopic observations, *n*-hexane was rather toxic than toluene or the mixture, since the 61 % of the strains showed decreased diameters of hypha, while the media added with the mixture or pure toluene, displayed only 30 and 8 % of the strains, respectively (Fig. 4.1c). The low solubility of the *n*-hexane in medium might explain the low fungal growth. Other toxic effect was the decrease in the diameter of hyphae, evidenced by morphological damages, such as the reduction of hypha diameter, which pointed to a fungal response to increase the surface area for gaseous substrate exchange. For instance, when hydrocarbons were used as sole carbon source, hypha diameters were significantly smaller ($p \leq 0.05$) (0.7 ± 0.19) than that of the strains in the control (1.6 ± 0.04 µm). The strains with the highest hyphae diameter in toluene were the strain ATCC26854, L157 and L2149 (1.1 ± 0.01 µm); in *n*-hexane was L460 (1.2 ± 0.01 µm) and with hydrocarbon mixture was L2009 (1.0 ± 0.03 µm) (Fig. 4.1c).


a)
MT
MH
MTH
Biomass (g L-1)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Bb
ATCC26854
ATCC46578
157
974
991
1029
2009
2149
2218
2460
2832
2858
2916
3909
4519
5129
5153
EH-348
EH-457
EH-458
EH-459
EH-460
Strains


b)
MT
MH
MTH
Diameter (mm)
60
50
40
30
20
10
0
Bb
ATCC26854
ATCC46578
157
974
991
1029
2009
2149
2218
2460
2832
2858
2916
3909
4519
5129
5153
EH-348
EH-457
EH-458
EH-459
EH-460
Strains

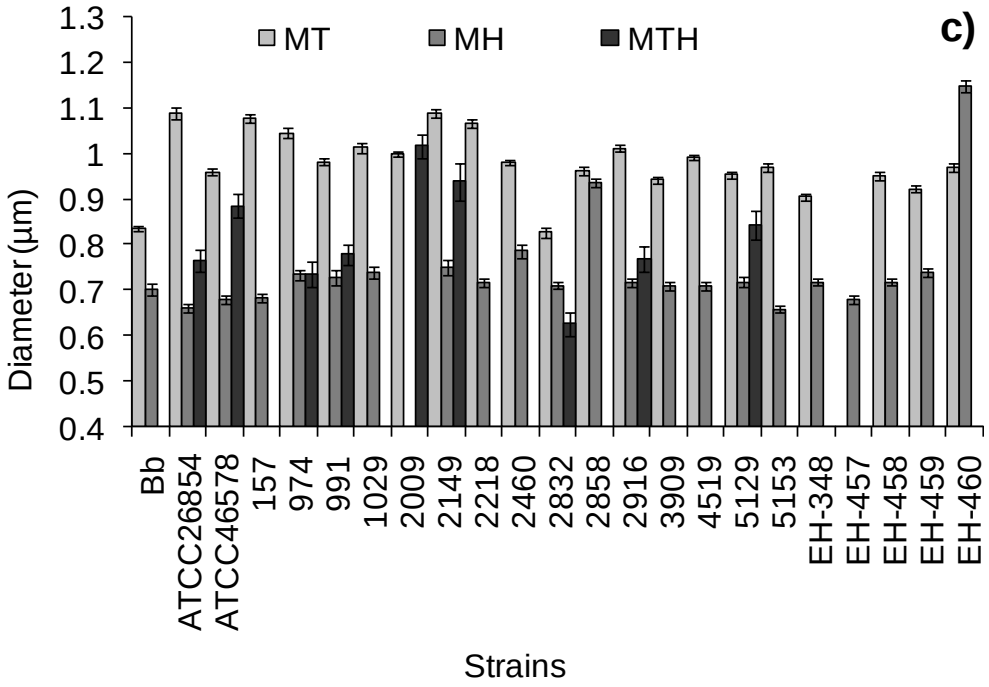


Figure 4. 1. a) Radial growth, b) biomass, c) diameters of hypha determinations of strains of *Lecanicillium*, *Verticillium* and *B. bassiana* determined in MH, MT or MTH as sole carbon source at 25 °C and 30 days of incubation. Radial growth and biomass data are shown as the average of six repetitions and their standard errors. The measurements of hypha diameter were carried out on micrographs at x100 and are the average of 70–90 observations and their standard errors.

In a previous report by Ramírez-Coutiño et al. [20], *Verticillium* and *Lecanicillium* strains were able to produce chitinolytic enzymes in media amended with colloidal chitin. Herein, the tested strains also displayed extracellular chitinolytic enzymes production by the formation of halos of hydrolysis in the VOCs and colloidal chitin agar media. Interestingly, all strains displayed activity in toluene media, and the largest halos of hydrolysis were determined with L2009 and L458. On the other hand, the strains L157 and L5153 presented halos of hydrolysis in MChH, whereas for the mixture of hydrocarbons only the strain L2009 presented activity (Fig. 4.2a). This evidence suggests that L2009 possesses higher tolerance

as it presented growth with the mixture of VOCs as sole carbon source (Fig. 4.1a) without significant diameter changes compared to the control (Figs. 4.1c, 2b).

The diameter of the hypha in the culture media with colloidal chitin and hydrocarbons was less damaged than those in the media with VOCs as sole carbon source (Fig. 4.2b). Indeed, the average of hypha diameter of medium with chitin and hydrocarbon varied from 1.4 to 1.1 µm, whereas in the medium with the hydrocarbon were 1.08 and 0.8 lm for toluene and *n*-hexane, respectively. The effect of toluene in the MChT medium was not remarkable in the structure of the hypha, neither in the ssimilation of the chitin, as the diameters were similar to those found in the control (0.9–1.2 µm). The *n*-hexane addition (MChH), however, reduced significantly the hypha diameter (0.7–0.8 µm) compared to control or MChT (Figs. 4.1c, 4.2b). The strains with the highest hyphae diameter with MChT were ATCC26854 (1.13 ± 0.01 µm), ATCC46578 (1.12 ± 0.02 µm) and L1029 (1.10 ± 0.01 µm); those with MChH were L460 (1.42 ± 0.02 µm), L2460 (1.39 ± 0.03 µm) and L2832 (0.84 ± 0.02 µm); and with MChTH were L974 (0.90 ± 0.02 µm), L2832 (0.82 ± 0.02 µm) and ATCC46578 (0.76 ± 0.02 µm) (Fig. 4.2b).

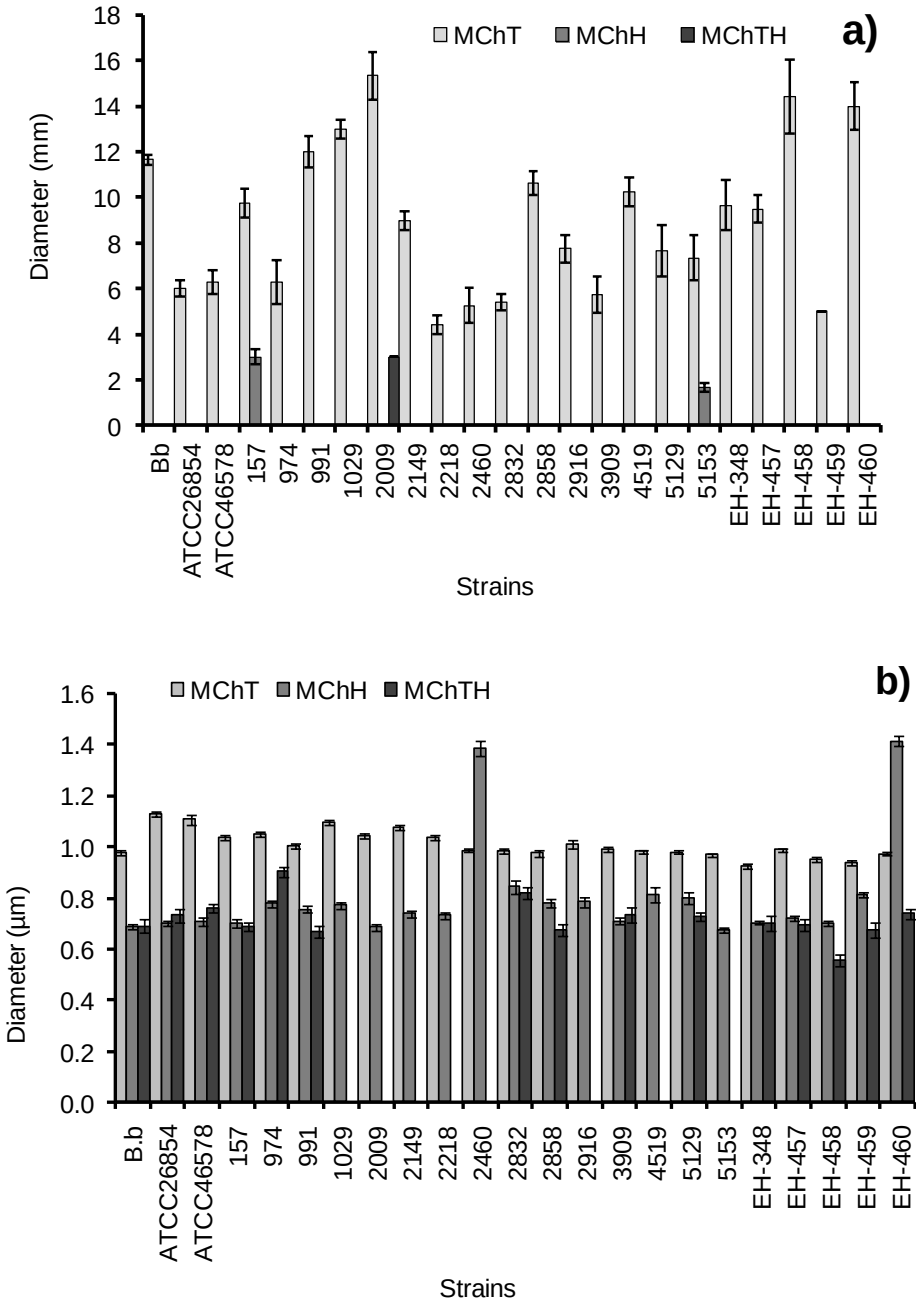


Figure 4. 2. Determination of halos of hydrolysis of chitin and b diameters of hypha of strains of *Lecanicillium*, *Verticillium* and *B. bassiana* grown in MChH, MChT or MChTH. Halos of hydrolysis data are the average of six observations and their standard errors. The measurements of hypha diameter were carried out on micrographs at x100 and are the average of 70–90 observations and their standard errors.

4.3.2 Consumption of toluene and *n*-hexane and chitinolytic activity for *L. lecanii* L460, L157 and L2149

In the degradation experiments, only pure hydrocarbon treatments were analyzed because the mixture showed lower biomass production than in the control (11-fold), and most of the strains did not show halos of hydrolysis of colloidal chitin. The strains of *L. lecanii* were chosen due to the highest hyphae diameters and growth (Fig. 4.1). The strain L460 displayed both highest radial growth (3.83 ± 0.31 mm) and diameter hypha (1.15 ± 0.01 µm) with *n*-hexane. In toluene, the strain L157 showed the highest radial growth (6.0 ± 0.01 mm) and hyphae diameter (1.08 ± 0.01 µm). However, the strain L2149 presented the highest biomass (1.48 ± 0.03 g L^{-1}) with *n*-hexane and the highest hypha diameter (1.09 ± 0.01 µm) with toluene.

The total biomass produced, chitinolytic enzymes activities and consumption of VOCs were determined during 60 days of incubation (Table 4.1). The protein measurements from the media with VOCs alone or in combination with colloidal chitin were conducted as an indirect estimation of biomass. *L. lecanii* L460, L157 and L2149 presented degrading activities of *n*-hexane and toluene with that of hydrocarbons from 43 to 62 % (Table 4.1). *L. lecanii* L157 showed the highest consumption of toluene (52.9 ± 0.11 %) and *n*-hexane (55.6 ± 0.30 %) used as sole carbon source. The strains L157 and L2149 degraded more *n*-hexane than toluene with ca. 2 and 5 %, respectively, whereas in the media with chitin showed ca. 18 and 17 %, respectively. The strain L460 did not show significant differences in the VOCs consumption (Table 4.1). These biodegradation results can be related to hyphae diameters (Fig. 4.1c), which decreased 5 % in the presence of *n*-hexane compared to the control.

The strains L460 and L157 had a positive correlation between biomass production and VOCs consumption ($p \leq 0.01$) in media with chitin owing to tenfold increase in biomass production, as well as higher degradation of *n*-hexane (6–19 %) and toluene (2.7–12.3 %) than in the media with hydrocarbons as sole carbon source. The exception was the strain 2149 in the medium MChT where consumption decreased 3.4 % compared to MT medium.

The three strains tested showed *N*-acetylhexosaminidase (HexNase) and endochitinase (Endo) activities in the media with or without chitin. The highest yield of HexNase was determined for L460 in hydrocarbon media with or without chitin with values between 0.26 and 0.8 mU µg $protein^{-1}$, while strains L2149 and L157 did not show significant

differences in production. Certainly, the addition of chitin to the medium increased the yield of HexNase and production of biomass compared to the media with only hydrocarbons.

However, the hydrophobicity of hydrocarbon affected the Endo activity, which was higher in MT than MH, this difference might be explained in terms of solubility as toluene is more soluble, 0.534 g L^{-1}, [21] than *n*-hexane, 0.0095 g L^{-1} [22]. The strain L157 had the highest yields of Endo in MT (17.1 U µg protein^{-1}), MH (5.12 U µg protein^{-1}) and MChT (1.63 U µg protein^{-1}). Ooki et al. [23] reported that the presence of polycyclic aromatic hydrocarbons (PAHs) competitively inhibit the activity of chitinases of *Aeromonas hydrophila* subsp. *anaerogenes*. These authors reported that inhibition is greater as the number of aromatic rings increase. Herein, there were no significant differences on chitinolytic activities among aromatic and aliphatic VOCs (Table 4.1).

Table 4. 1. Determination of biomass and chitinolytic enzymes productions as well as consumption of toluene or n-hexane for *L. lecanii* L460, L157 and L2149 in microcosms at 60 days.

Medium	Strain	Biomass (µg protein mL^{-1})	*N*-acetyl hexosaminidase $Y_{e/x}$ (mU $\mu g_{proteina}^{-1}$)	Endochitinases $Y_{e/x}$ (U $\mu g_{proteina}^{-1}$)	VOCs (%) consumption
MH	EH-460	$0.29 \pm 0.01^{a,b,c}$	$0.26 \pm 0.03^{a,b}$	4.75 ± 0.9^{b}	45.4 ± 0.54^{a}
	157	$0.59 \pm 0.06^{a,b,c}$	$0.04 \pm 0.01^{a,b}$	5.12 ± 1.11^{b}	55.6 ± 0.30^{d}
	2149	0.83 ± 0.02^{c}	$0.09 \pm 0.01^{a,b}$	1.82 ± 0.32^{a}	43.0 ± 0.78^{a}
MChH	EH-460	4.09 ± 0.17^{f}	0.64 ± 0.06^{c}	0.73 ± 0.09^{a}	$53.24 \pm 0.30^{c,d}$
	157	5.40 ± 0.03^{g}	$0.13 \pm 0.01^{a,b}$	0.53 ± 0.13^{a}	62.28 ± 0.47^{e}
	2149	4.29 ± 0.14^{f}	$0.20 \pm 0.01^{a,b}$	1.55 ± 0.15^{a}	62.21 ± 0.21^{e}
MT	EH-460	0.05 ± 0.02^{a}	$0.48 \pm 0.09^{a,b}$	20.24 ± 1.18^{d}	43.0 ± 0.17^{a}
	157	$0.16 \pm 0.07^{a,b}$	0.07 ± 0.02^{a}	17.07 ± 1.47^{c}	52.9 ± 0.11^{c}
	2149	$0.69 \pm 0.06^{b,c}$	$0.11 \pm 0.01^{a,b}$	$2.33 \pm 0.37^{a,b}$	48.6 ± 0.70^{b}
MChT	EH-460	5.05 ± 0.24^{g}	0.80 ± 0.03^{c}	0.51 ± 0.09^{a}	$55.26 \pm 0.92^{c,d}$
	157	2.50 ± 0.04^{d}	$0.24 \pm 0.02^{a,b}$	1.63 ± 0.48^{a}	44.27 ± 0.62^{a}
	2149	3.09 ± 0.10^{e}	0.28 ± 0.01^{b}	0.70 ± 0.20^{a}	45.42 ± 0.29^{a}

Values with different superscript letters in the same column showed significant differences ($p \leq 0.05$) according to Tukey–Kramer multiple comparison test.

4.3.3 Production of Hfbs-like proteins class I and II of *L. lecanii* L157 and their surface activities

Rocha-Pino et al. [1] reported recently the use of chitin as a carbon source to promote the production of Hfb-I in mycelia of L. lecanii cultured for 6 days. The production of Hfbs is regulated by environmental factors [2, 24] and depends on growth stage, such as the formation of aerial mycelia and conidia. Mulder and Wessels [25] reported that the expression of Hfbs of *Schizophyllum commune* reached the maximum at 4 days of culture on agar.

Herein, the production of proteins with Hfb activity was determined from 6-day culture of *L. lecanii* L157; this strain was selected owing to its VOCs consumption and its less inhibited chitinolytic activity.

L. lecanii produced 57.4 ± 4.7 μg protein^{-1} of Hfb class I in liquid media with chitin as sole carbon source [1]; however, in this work, the production of Hfb was 88.3 ± 4.6 μg protein^{-1} and 548.6 ± 26.3 μg protein^{-1} in media MChT and MChH, respectively. These results indicate that the addition of hydrophobic compound increased the Hfb production. In addition, the extracts of Hfb class I of the media MChT and MChH reduced the Teflon hydrophobicity to 47.8 ± 3.5 % and 10.1 ± 1.5 %, respectively. The differences on surface activity indicate that *L. lecanii* might be able to produce more than one Hfb depending on the hydrophobicity of media. It has been reported that *S. commune* expressed Hfbs SC3 depending on the biological function, for instance, SC3 covered the aerial hyphae reducing the surface tension and mediated the adhesion to hydrophobic surfaces, while Hfbs SC4 coated the fungal fruit bodies forming channels for gas transfer [26].

The media MChH significantly favored the production of proteins corresponding to Hfb class II (194.1 ± 11.6 μg Hfb mL^{-1}) and remarkably decreased the hydrophobicity of Teflon 27.2 ± 2.1 %, whereas in MChT, the Hfb class II was 69.9 ± 3.4 μg Hfb mL^{-1} with a surface activity on Teflon of 26.3 ± 2.6 %.

Figure 4.3 shows SEM micrographs obtained from strains cultivated in *n*-hexane and toluene compared to the media supplemented with chitin and VOCs. The micrographs showed the toxic effect of VOCs causing morphological changes in spores and hyphae. The damages were more evident at longer period of culture with VOCs (60 days) (Fig. 4.3a, b, e, f) compared to the strain cultivated in media with added chitin (Fig. 4.3c, d, g, h). Figure 4.3e depicts the strain cultivated with *n*-hexane with appreciable damage in the hyphae, having irregular shape with roughness instead of those flat and circular from the strain after 6 days of

incubation (Fig. 4.3g), besides higher number of non-germinated spores than those in toluene (Fig. 4.3f). The reduction of average diameter of spores was 0.57 ± 0.01 µm with *n*-hexane whereas that with toluene was 0.7 ± 0.02 µm (Fig. 4.3e, f). It has been reported that Hfbs interact with hydrophobic surfaces and triggered the appressorium formation [3] and that agrees with our findings as shown in Fig. 4.3c and d.

Crespo et al. [9] reported that the variation in the morphology is an indication of fungal cell wall composition changes in the presence of hydrocarbon as sole carbon source, specifically on the lipid fraction. Also according to Funtikova and Mysyakina [27], these modifications reduce the ability for the spore to germinate and that the change in the lipid proportion has some effect on the carbohydrates content on the surface, which has a positive correlation with the hydrophobicity. In such conditions, the mycelia surface is roughish, while under hydrophilic condition, it appears as flat and smooth [28]. In this work, similar effect was observed on the mycelia morphologies when fungi were cultivated within chitin and VOCs or pure hydrocarbons (Fig. 4.3e–h).

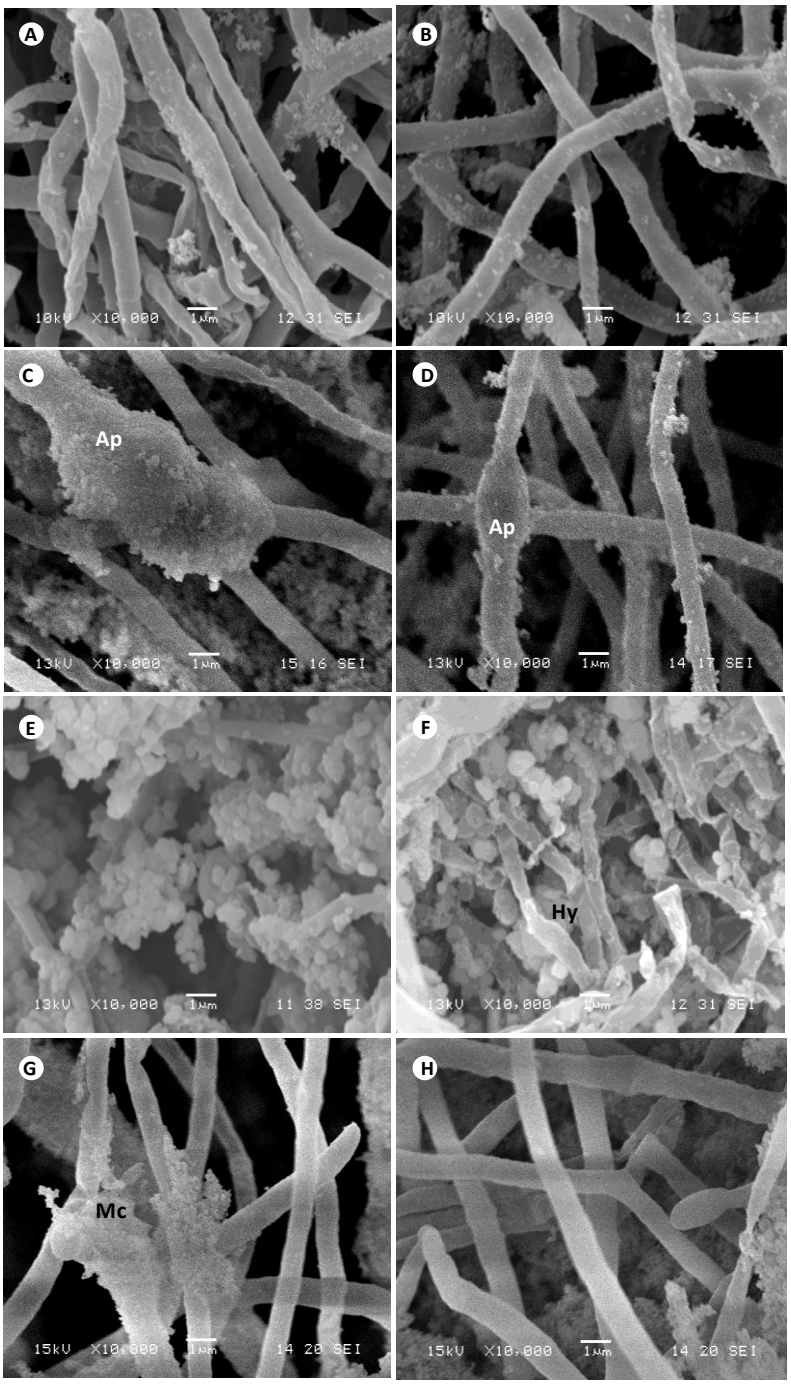


Figure 4. 3. Scanning electron micrographs (x10,000) of *L. lecanii* L157 in microcosms with mineral media after 6 days of incubation with added VOCs and chitin: a) MH; b) MT; c) MChH; d) MChT. After 60 days of incubation: e) MH; f) MT; g) MChH; h) MChT. Ap- appressorium formation, Hy- hyphae, S- spore, Mc- mucilaginous coat.

4.4 CONCLUSION

Lecanicillium and *Beauveria bassiana* cultivated in liquid cultures and agar that contained *n*-hexane, toluene, or the mixture of both hydrocarbons, presented agglomerates of spores as well as fine, scarce and ramified mycelia, showing their ability to grow in media with VOCs. Generally, the inhibition with *n*-hexane was higher than toluene or mixture of hydrocarbons *n*-hexane:toluene 17:83 (v:v). The strains of *Lecanicillium* L460, L157 and L2149 degraded from 43 to 55 % of *n*-hexane or toluene in liquid medium and they produced chitinolytic enzymes and Hfbs-like proteins with surface activity as response of the presence of hydrophobic substrates.

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CAPÍTULO 5:

Phosphinothricin as a new selectable marker entomopathogenic fungus *Lecanicillium lecanii*

Zaizy Rocha-Pino, Israel Padilla-Guerrero, Keiko Shirai, Michael Bidochka

En preparación para su publicación

Phosphinothricin as a new selectable marker entomopathogenic fungus *Lecanicillium lecanii*

Zaizy Rocha-Pino, Israel Padilla-Guerrero, Keiko Shirai, Michael Bidochka

EN PREPARACIÓN PARA SU PUBLICACIÓN

Abstract

Lecanicillium lecanii is an entomopathogenic fungus used commercially as a biological pesticide. In this work *Agrobacterium tumefaciens*-mediated transformation method was applied to *L. lecanii*, using phosphinothricin (PPT) as a selectable marker (*bar* gene), and green fluorescent protein. The transformation showed an efficiency of 17 ± 2 transformants per 5 x 10^{-5} target conidia of *L. lecanii* and stability of green fluorescent proteins. This transformation method is a useful tool to study *L. lecanii*, its development and interaction with the environment.

Keywords: *Lecanicillium lecanii*, *Agrobacterium*, Glufosinate ammonium, GFP

5.1 INTRODUCTION

The biological pesticides used commercially are object of great interest due to their potential for development of environmentally friendly technologies, for this reason is important to know more about impact on the ecosystem where they are applied. An example of this is *Lecanicillium lecanii*, entomopathogenic fungus, able to infect a wide variety of insects (whiteflies, aphids and others insects) which are causing economic losses in agriculture and horticulture (Butt et al., 2001; Goettel et al., 2008).

In the other hand, the transformation technique is a useful tool for studying genes to obtain strains with increased benefits to development more efficient insect control agents (Moon et al., 2008; Donzelli et al., 2010; Tseng, Chung and Tzean 2011). Thus, Hasan et al.,

2011, has report the genetic transformation of protoplast of *L. lecanii* using nitrate reductase gene.

Currently, there is no much information about the effects caused by the *L. lecanii* when it is applied as biopesticide in an open field. For this reason, we require the tools to study and improve the morphological and biochemical interaction of the fungus with the environment. The aim of this study was to apply a method to transforming *L. lecanii* via *Agrobacterium*-mediated that serve as a simple tool for following and analysis of its development and interaction with the environmental as well as for genetic analysis in future studies.

5.2 MATERIAL AND METHODS

5.2.1 Microorganism

Lecanicicillium lecanii strain 313 was provided by United States Department of Agriculture Collection of Entomopathogenic Fungal Cultures (USDA-ARSEF), Ithaca, NY. It was maintained on potato dextrose agar at 27 °C for 10 days to obtain conidia suspension. *Agrobacterium tumefaciens* AGL-1 was used for *L. lecanii* transformation according to Fang, Pei and Bidochka (2006).

5.2.2 Plasmid

We used a casset containing phosphinothricin resistance gen (BAR) and green fluorescent protein gen (eGFP) constructed for Padilla-Guerrero (unpublished).

5.2.3 Transformation of *L. lecanii* mediated by *Agrobacterium*

The transformation of *L. lecanii* via *A. tumefaciens* AGL-1 was performed according to the procedure described by Fang, Pei and Bidochka (2006) with the following modifications: one milliliter of conidia suspension (5×10^5 conidia mL^{-1}) of *L. lecanii* was centrifuged at 5000 rpm during 5 min, the pellet obtained was mixed with one milliliter of culture of *A. tumefaciens* (AGL1:pBAR-GFP) previously grown and induced with 200 µM acetosyringone. One hundred microlliter of the mixture was spread on a black filter paper (Ahlstrom, 0.18 mm thickness) deposited on IMAS agar plate and incubated at 27 °C in darkness conditions during 2 days. After, the black filter was transferred onto M-100 agar (1.5 % w w^{-1}) plates added with 100 µg

mL^{-1} cloramphenicol to kill *Agrobacterium* remaining and 250 µg mL^{-1} PPT (previous concentration tested) as marker selection; these plates were incubated during 1 day at 27 °C, after the black filter was overlaid with M-100 agar (1 %) plates containing 100 µg mL^{-1} cloramphenicol and 250 µg mL^{-1} PPT and incubated at 27 °C for 10 days. The transformant method was carried out by triplicate. After, the transformants *L. lecanii* were cultured on non selective medium M-100 by 3 generations. The transformants were confirmed by resistence to glufosinate and with a fluorescent microscope to observe the green fluorescent protein expression.

5.3 RESULTS AND DISCUSSION

In this work we transformed *Lecanicillium lecanii* using the cassett BAR-GFP as a new selectable marker. The tansformant efficiency was 17 ± 2 colonies for 5 $x10^{-5}$ target conidia. The transformants of *L. lecanii* were visualized after 5 to 6 days and these were collected after 10 days of incubation and subculture 3 times on selective medium (250 PPT) for mitotic stability. The transformants were stable after 3 generations of subculture on non-selective media. All *L. lecanii* transformants obtained were available to express gfp (Figure 5.1), this may be suitable to simultaneously detect of different proteins for multiple labeling in vivo studies of *L. lecanii.*

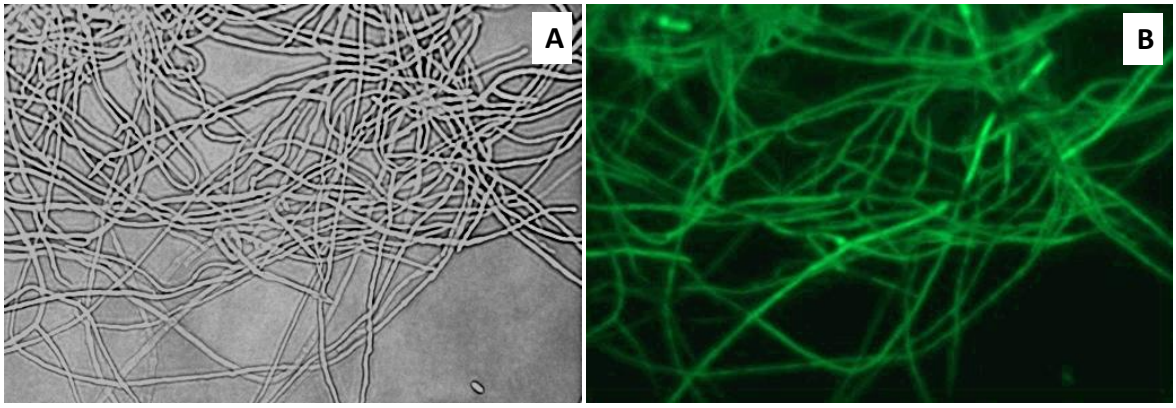

Figure 5. 1. Micrography of *L. lecanii* grown in M-100 at 25 °C,10 days. A) Strain 313 wild type under white light and B) *L. lecanii*:pBAR-GFP under fluorescent light. 40X objective, exposure time 2s.

5.4 CONCLUSION

We observed that *Agrobacterium*-mediated transformant system may be applied to *L lecanii* successfully. The dual property of the selectable marker, PPT resistance and florescent proteins (GFP) of this system is a useful tool to future applications and studies of distribution and dynamics interaction with the environmental of *L. lecanii*.

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CONCLUSIÓN GENERAL

Estos son los primeros estudios sobre la producción y la actividad superficial de proteínas tipo Hfbs de *L. lecanii*, lo que resulta importante debido a las aplicaciones biotecnológicas que tienen las Hfbs y quitinasas. Asimismo, los resultados de este estudio permiten profundizar en el conocimiento sobre la función biológica de las Hfbs y su estrecha relación con las quitinasas, así como las implicaciones que esto puede tener en el desarrollo y patogénesis del hongo. Finalmente, todo lo mencionado anteriormente abre la posibilidad de mejorar las formulaciones para la aplicación de *L. lecanii* como biopesticida.

Production and activities of chitinases and hydrophobins from *Lecanicillium lecanii*

El uso de quitina coloidal como fuente de carbono en cultivos en substrato sólido, SSC, incrementó ca. 10 veces la producción de quitinasas y HfbL clase I de *L. lecanii*, en comparación con cultivos sumergidos y el uso de fructosa. Se determinó que solo las Hfbs clase I producidas por *L. lecanii* en SSC sobre PUF con quitina mostraron actividad superficial sobre el teflón, a diferencia de las Hfbs obtenidas de los cultivos en PUF con fructosa, las cuales no mostraron tener actividad superficial. Esto sugiere una estrecha relación entre la producción de quitinasas e hidrofobinas con actividad superficial de *L. lecanii.*

The hydrophobicity of the support in solid substrate culture affected the production of hydrophobins from *Lecanicillium lecanii*

Continuando con el análisis sobre la producción de porteínas tipo hidrofobinas de *L. lecanii* en cultivos con sustrato sólido se determinó que este hongo tiene capacidad de producir HfbLs clase I y clase II. Se observó que la hidrofobicidad del tipo de soporte, la agrolita y el PUF, utilizados como soporte inerte en SSC e incluso se determinó que las características fisicoquímicas de la quitina empleada como fuente de carbono influyeron significativamente sobre la producción de HfbLs y su actividad superficial. Esto evidenció la importancia de las hidrofobinas para la interacción de *L. lecanii* con las superficies en que se desarrolla, incluyendo su sustrato el cual es naturalmente la quitina obtenida de insectos.

Morphological changes, chitinolytic enzymes and hydrophobin-like proteins as responses of *Lecanicillium lecanii* during growth with hydrocarbon

Por su parte, la hidrofobicidad debida a la presencia de compuestos orgánicos volátiles como el tolueno y el n-hexano, promovieron la producción de las HfbLs de *L. lecanii* en cultivos sumergido tipo microcosmos. La diferencia de hidrofobicidad entre los hidrocarburos probados influyó significativamente sobre la actividad superficial de las HfbLs. Un compuesto de alta hidrofobicidad como el n-hexano favoreció la producción de HfbLs, sin embargo, las HfbLs obtenidas de los cultivos con el tolueno (menos hidrofóbico) mostraron mayor actividad superficial que las anteriores, lo que sugiere una alta especificidad de las Hfbs de *L. lacanii* para favorecer el desarrollo del hongo.

Phosphinothricin as a new selectable marker entomopathogenic fungus *Lecanicillium lecanii*

Actualmente, el estudio de hongos entomopatogenos como *L. lecanii* sigue siendo de gran relevancia debido a su uso como biopesticida, el entender mejor su desarrollo e interacción con el medio nos permitirá utilizarlo de forma cada vez más eficaz. Es por esta razón que surge la importancia de contar con herramientas de transformación génica que faciliten su estudio. Con el método de transformación empleado en este trabajo se obtuvo una cepa de *L. lecanii* que expresa el gen *bar* el cual otorga resistencia a fosfinotircina, principio activo de algunos pesticidas, y con la expresión de la proteína verde fluorescente, GFP. La expresión de GFP podría ser de gran ayuda para determinar su interacción con el ambiente, siendo esta una herramienta de facíl monitoreo.

PERSPECTIVAS

La importancia de las Hfbs y quitinasas en el desarrollo de hongos entomopatógenos has sido ampliamente investigadas, sin embargo, como se ha observado en este trabajo las características y funciones biológicas de las Hfbs muestran tener un alto grado de especificidad, lo cual tendría implicaciones significativas para la patogénesis del hongo y que deberían ser consideras para la aplicación de *L. lecanii* como biopesticida. Incluso, las propiedades de estas HfbLs podrían ser aprovechadas en aplicaciones biotecnológicas como la modificación de materiales o su aplicación como biosurfactante.

Es por esto que se sugiere profundizar en el estudio de las Hfbs de *L. lecanii*. El análisis genómico y proteómico de estas proteínas nos permitiría determinar su regulación génica, estructura tridimensional y mecanismo de ensamblaje para entender mejor su mecanismo específico de interacción con diferentes moléculas y superficies. Lo anterior nos permitiría determinar las funciones biológicas que llevan a cabo HfbLs de *L. lecanii* y a entender la interacción del hongo en ambientes complejos como ocurre durante su aplicación como biopesticida en campo.

TRABAJOS DERIVADOS DE ESTA TESIS

PUBICACIONES EN REVISTAS INDIZADAS (ISI).

1. Z. Rocha-Pino, MC. Marín-Cervantes, M. Martínez-Archundia, E. Soriano-Blancas, S. Revah, K. Shirai. 2013. Morphological changes, chitinolytic enzymes and hydrophobin-like proteins as responses of *Lecanicillium lecanii* during growth with hydrocarbon. Bioprocess and Biosystems Engineering. 36(5): 531-539.

2. Z. Rocha-Pino, G. Vigueras and K. Shirai. 2011. Production and activities of chitinases and hydrophobins from *Lecanicillium lecanii*. Bioprocess and Biosystems Engineering. 34: 681-686.

3. Z. Rocha-Pino, G. Vigueras, JD. Sepúlveda-Sánchez, M. Hernandez, J. Campos-Terán, FJ. Fernández and K Shirai. 2014. The hydrophobicity of the support in solid substrate culture affected the production of hydrophobins from *Lecanicillium lecanii.* Process Biochemstry, Ref. No.: PRBI-D-14-00471 (En revisión).

4. Zaizy Rocha-Pino, Israel Padilla-Guerrero, Keiko Shirai, Michael Bidochka. Phosphinothricin as a new selectable marker entomopathogenic fungus *Lecanicillium lecanii.* (En preparación)

PARTICIPACIÓN EN CONGRESOS

INTERNACIONALES

1. Israel Enrique Padilla-Guerrero, Zaizy Rocha-Pino, Keiko Shirai and Michael J Bidochka. Overlapping gene functions in the endophytic insect-pathogenic fungus *Metarhizium*. 46th Annual Meeting of the Society for Invertebrate Pathology Conference on Invertebrate Pathology and Microbial Control & NEMASYM RCN Nematode-Bacterium Symbioses Research Coordination Network 5th NEMASYM Meeting. 11 to 15 August, 2013. Pittsburgh, Pennsylvania, USA.

2. Zaizy Rocha-Pino, Israel Padilla-Guerrero, Keiko Shirai, Michael J. Bidochka. Phosphinothricin as a new selectable marker entomopathogenic fungus *Lecanicillium lecanii.* 12th International Symposium on the Genetics of Industrial Microorganisms (GIM-2013). June 23 to 28, 2013, in Cancun QR, México.

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5. Zaizy Rocha y Keiko Shirai. Producción y actividad de quitinasas e Hfbs obtenidas de cultivos sólidos y líquidos de *Lecanicillium lecanii*. V Simposio Iberoamericano de Quitina, Sociedad Iberoamericana de Quitina. Santiago de Chile, Chile, 6 al 9 de Junio, 2010.

NACIONALES

1. Zaizy Rocha-Pino, Gabriel Vigueras, Maribel Hernandez, José Campos-Terán, Sergio Revah, Francisco J. Fernández y Keiko Shirai. Producción de hidrofobinas de clase I y II de *Lecanicillium lecanii* cultivado en sólido utilizando diferentes quitinas como fuente de carbono. 1er Simposio: Perspectivas en Biotecnología. Distrito Federal, México, 13 de abril de 2012.

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ANEXOS

A1. Electroburbujeo

El electroburbujeo fue llevado a cabo en comlunmas de vidrio equipada con alambre de platino a la cual se le hizo pasar una corriente de 300 mA (Figura A.1). Las hidrofobinas se ensamblan alrededor de los iones de hidrógeno que se generan através de este sistema y suben a la superfie del líquido en forma de espuma, donde son colectadas para su análisis.

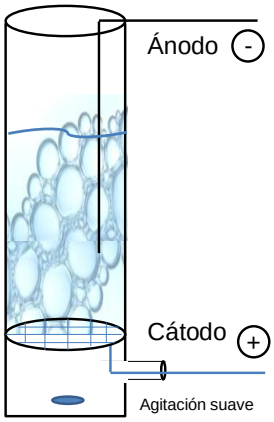


Figura A1 1. Esquema de columna de electroburbujeo.

A2. Estudio de tolerancia de *L. lecanii* a fosfinotricina (PPT)

La tolerancia de *L. lecanii* 313 a PPT fue determinada mediante la observación del crecimiento del hongo cultivado en medio M-100 con agar y adicionado con PPT en concentraciones de 50, 100, 150, 200, 250, 300 y 400 µg mL^{-1}.

La concentración elegída fue de 250 ug mL^{-1} PPT donde no se observó crecimiento de *L. lecanii* 313 después de 15 dias de incubación a 27 °C.

Casa abierta al tiempo
UNIVERSIDAD AUTÓNOMA METROPOLITANA

ACTA DE DISERTACIÓN PÚBLICA

No. 00131
Matrícula: 210180367

ESTUDIO DE LA PRODUCCION Y ACTIVIDAD DE PROTEINAS TIPO HIDROFOBINAS Y QUITINASAS DE *Lecanicillium lecanii* EN CULTIVOS EN SUSTRATO SOLIDO Y SUMERGIDO.

En México, D.F., se presentaron a las 15:00 horas del día 3 del mes de julio del año 2014 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. FRANCISCO JOSE FERNANDEZ PERRINO
DR. JUAN GABRIEL VIGUERAS RAMIREZ
DR. ISRAEL ENRIQUE PADILLA GUERRERO
DRA. CLAUDIA PATRICIA LARRALDE CORONA

Bajo la Presidencia del primero y con carácter de Secretaria la última, se reunieron a la presentación de la Disertación Pública cuya denominación aparece al margen, para la obtención del grado de:

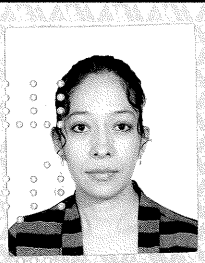
DOCTORA EN BIOTECNOLOGIA

DE: ZAIZY ROCHA PINO

y de acuerdo con el artículo 78 fracción IV del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

APROBAR

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.



ZAIZY ROCHA PINO
ALUMNA

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DIRECTOR DE SISTEMAS ESCOLARES

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