**Centro de Investigación en Alimentación y Desarrollo, A.C.**

# BÚSQUEDA, PURIFICACIÓN Y CARACTERIZACIÓN DE TRANSESTERASAS DE COMPUESTOS FENÓLICOS

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Por:

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TESIS APROBADA POR LA

COORDINACIÓN DE CIENCIA DE LOS ALIMENTOS

Como requisito parcial para obtener el grado de

DOCTOR EN CIENCIAS

Hermosillo, Sonora Agosto de 2015

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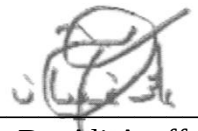
Los miembros del comité designado para la revisión de la tesis de Francisco Javier Bacame Valenzuela, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias



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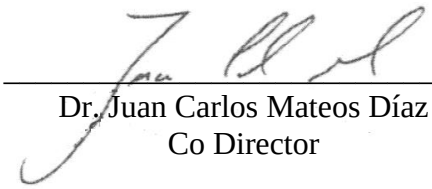

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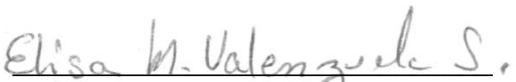

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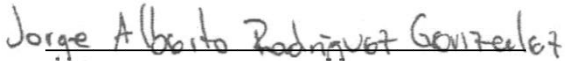

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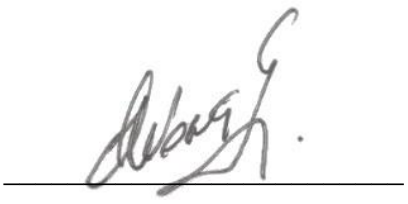

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# AGRADECIMIENTOS

Al **Consejo Nacional de Ciencia y Tecnología (CONACYT)**, por la beca N° **224317**, otorgada para realizar mi tesis de doctorado.

Al **Centro de Investigación en Alimentación y Desarrollo A. C.**, por brindarme la oportunidad de lograr mis metas.

Al **Centro de Investigación y Asistencia en Tecnológica y Diseño del Estado de Jalisco**, por apoyarme con sus instalaciones para cumplir con los objetivos de mi tesis de doctorado.

A la **Dra. Elisa Miriam Valenzuela Soto**, por creer en mí, por sus consejos y por su entera confianza para trabajar en su laboratorio.

Al **Dr. Ali Asaff**, por impulsarme para ser mejor, por su apoyo para lograr mis metas, como licenciado, maestro y doctor.

Al **Dr. Juan Carlos Mateos Díaz**, por su confianza, por transmitirme su conocimiento y por su disposición para que cumpliera con mis objetivos, estaré por siempre agradecido.

Al **Dr. Jorge Alberto Rodríguez González**, por sus aportaciones para poder cumplir con los objetivos de mi tesis.

Al **Dr. Martin Esqueda Valle**, por su participación en mi comité de tesis.

A la **Dra. María Yolanda Reyes Vidal**, por sus consejos, apoyo y motivación para continuar con mis metas.

A mis **compañeros de laboratorio** en **CIAD** y **CIATEJ** por su apoyo técnico y esos momentos que hicieron más amena mi estancia en estos laboratorios.

## DEDICATORIA

*A mi **madre**, por su apoyo fundamental durante toda mi educación.*

*A mi **esposa**, por comprender la importancia de llegar a esta meta, Te Amo.*

*A la familia **Vacame Barrios**, **Ramírez Vacame**, **Quiroz Vacame** y **Coronado Bacame** por formar esta gran familia de la cual me siento muy orgulloso.*

*A la familia **Huez Acuña** y **Martínez Aguiñaga** por aceptarme como un integrante más de la familia.*

*A mis amigos **Martha Caremi**, **Carlos Diego**, **Norberto**, **Ana**, **Edith**, **Danet** porque nuestra amistad crece con el pasar de los años.*

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## RESUMEN

Las feruloil esterasas (FAE, 3.1.1.73) son enzimas que participan en la degradación de la pared celular de plantas, rompiendo los enlaces éster entre los ácidos hidroxicinámicos y los carbohidratos. Sin embargo, en medios orgánicos, algunas FAE´s y lipasas catalizan reacciones de transesterificación, para la preparación de ésteres de ácidos hidroxicinámicos y alcoholes. Este tipo de ésteres son de interés principalmente para la industria cosmética, existiendo reportes de que estos derivados de ácidos hidroxicinámicos presentan propiedades biológicas mejoradas sobre sus precursores.

Así, la síntesis enzimática de derivados de ácidos hidroxicinámicos es una vía atractiva desde una perspectiva ambiental, económica y técnica. Actualmente el descubrimiento de nuevas fuentes de transesterasas con propiedades catalíticas mejoradas es un área de gran interés en investigación. En el presente trabajo se realizó el tamizaje de 205 cepas de hongos termotolerantes nativos del Estado de Sonora, propiedad del LBI-CIAD, para seleccionar las mejores cepas productoras de hidrolasas/transesterasas de ácidos hidroxicinámicos. La cepa B102 fue seleccionada y posteriormente identificada como *Aspergillus terreus*. Un análisis del secretoma demostró la producción de dos feruloil esterasas y una lipasa. Estas tres enzimas mostraron actividad transesterasa para la síntesis de butil esteres. Sin embargo, la capacidad de síntesis de la lipasa de *Aspergillus terreus* fue reportada previamente, seleccionando a las FAE´s para posteriores análisis. Se encontró que la actividad FAE de *Aspergillus terreus* B102 es debida a dos FAE´s; una de 50 kDa (AtFAE I) y una de 30 kDa (AtFAE II). Mediante la determinación de N-terminal se logró la identificación de la AtFAE I resultando ser una FAE tipo B. Al ser la FAE identificada se seleccionó para un mayor análisis como transesterasa. La AtFAE

I, presentó además de la actividad FAE, actividad clorogenato esterasa y transesterasa. El perfil de hidrólisis con metil ésteres mostró el siguiente orden: metil p-cumarato>metil cafeato> metil ferulato, no observando actividad sobre metil sinapinato. De acuerdo a este perfil y la determinación de amino terminal, AtFAE I corresponde a una FAE tipo B (AtFAE B), según la clasificación de Crepin et al. 2004. La especificidad por sustrato de AtFAE B contra ésteres de *p*-nitrofenilo, mostrando actividad con sustratos altamente hidrofóbicos como el *p*-nitrofenil decanoato (C10). AtFAE B logró la transesterificación de butil ferulato, butil *p*-cumarato, butil cafeato en un sistema ternario isoctano/butanol/agua. Actualmente en nuestro grupo de trabajo se continúa con el análisis bioquímico y cinético de AtFAE B y AtFAE II, así como el análisis a fondo de la versatilidad de estas enzimas como transesterasas.

**Palabras clave:** feruloil esterasa, ácidos hidroxicinámicos, transesterificación, metil éster, butil éster

## ABSTRACT

Feruloyl esterases (FAE, 3.1.1.73) are enzymes involved in the degradation process of the plant cell wall, including breaking the ester bond between the hydroxycinnamic acids and carbohydrates. However, some FAE's and lipases catalyze transesterification reactions, in organic media, for the esters of hydroxycinnamic acids and alcohols preparation. This type of esters is of major interest for the cosmetics industry, also these derivatives of hydroxycinnamic acids present enhanced biological properties on its predecessors.

Thus, the enzymatic catalysis for the preparation of hydroxycinnamic acids esters and alcohols is one of the most attractive routes, the discovery of new sources of transesterases with enhanced catalytic properties. In the present work, was carried out a screening of 205 strains of thermotolerant fungi native of the State of Sonora, LBI-CIAD collection was make in order to select the strains producing hydrolases/transesterasas of hydroxycinnamic acids. The strain B102 was selected subsequently identified as *Aspergillus terreus*. An analysis of his secretome showed the production of two feruloyl esterases and a lipase. These three enzymes showed transesterase activity in organic solvents for synthesis of butyl esters. Although, the lipase of *Aspergillus terreus* was identified and characterized in previous reports, thus it was decided to select the FAE's for further analysis. It was found that FAE activity of *Aspergillus terreus* B102 is due to two FAE's; one of 50 kDa (AtFAE I) and one 30 kDa (AtFAE II). Besides, the determination of N-terminal for AtFAE I turning out to be a FAE type B. Further analysis of its transesterase activity the enzyme AtFAE I, showed FAE activity, transesterase and chlorogenate esterase activity. The hydrolysis profile

with methyl was as a follows: methyl *p*-coumarate> methyl caffeate> methyl ferulate, not observing activity on methyl sinapinate. According to this profile and the amino terminal determination, the enzyme corresponds to a type B FAE (AtFAE B) according to the classification system proposed by Crepin et al. 2004. The substrate specificity of AtFAE B was assessed using *p*-nitrophenyl esters, finding that this enzyme can hydrolyze highly hydrophobic substrates such as it is *p*-nitrophenyl decanoate (C10). In the case of synthesis profile, transesterification of three butyl esters (butyl ferulate, butyl p-coumarate, butyl caffeate) in isooctane/butanol/water ternary system was achieved. Currently in our working group continue the biochemical and kinetic analysis of AtFAE B and AtFAE II, as well as the in-depth analysis of the versatility of these enzymes as transesterases.

**Keywords:** feruloyl esterase, hydroxycinnamic acids, transesterification, methyl ester, butyl ester

## SINOPSIS

Las feruloil esterasas (FAE's) también conocidas como ácido ferúlico esterasas, ácido cinámico esterasas o cinamoil esterasas (EC 3.1.1.73) pertenecen a una subclase de las ácido carboxílico esterasas (EC 3.1.1.1). Estas enzimas representan un grupo diverso de hidrolasas que tienen la capacidad de liberar los ácidos hidroxicinámicos y dímeros del ácido ferúlico que se encuentra en la pared de las células vegetales.

Las FAE's son producidas por microorganismos y plantas, en el caso de las FAE's microbianas son extracelulares y producidas por aquellos microorganismos que utilizan los compuestos fenólicos como fuente de carbono. Para que un microorganismo pueda degradar la pared celular requiere de complejos enzimáticos formados principalmente por hemicelulasas, celulasas, acetilxilan esteresas y FAE's que son las que liberan en última instancia los ácidos hidroxicinámicos, llamados también ácidos fenólicos, de la arabinosa. Los compuestos fenólicos liberados de los residuos agroalimentarios son empleados o tienen el potencial para distintas aplicaciones en diferentes industrias como la alimenticia, pulpa y papel, cosmetológica y farmacéutica.

De los ácidos hidroxicinámicos, el ácido ferúlico (ácido 3-metoxi-4-hidroxi-cinámico) es el más abundante en la naturaleza encontrándose hasta en un 3 % en salvado de maíz. En el área de alimentos es utilizado principalmente como precursor para la preparación de saborizantes, como la vainillina. También tiene uso en el campo de la industria cosmética por sus propiedades antienvejecimiento, contra la caída de cabello y protector

de rayos UV. En la industria médica el ácido ferúlico tiene aplicaciones gracias a su efecto para controlar la hipertensión, así como para controlar los niveles de glucosa triglicéridos y colesterol, con un efecto anti Alzheimer y anticancerígeno. Otro de los ácidos hidroxicinámicos abundantes es el ácido cafeico (ácido 3,4-dihidroxicinámico) que destaca por sus propiedades antimicrobianas contra diversos microorganismos como *Escherichia. coli, Pseudomonas aeruginosa, Bacillus cereus, Kokuria rhizophila, Staphylococcus aureus, Listeria monocytogenes, Candida albicans*. El ácido cafeico resulta también relevante por su capacidad antioxidante debido a un segundo grupo –OH en la posición orto, estabilizando la molécula por resonancia y formación de *o*-quinona. Actualmente el ácido cafeico se utiliza como precursor en la síntesis del éster fenetílico del ácido cafeico (CAPE), el cual es de gran relevancia en investigación por su actividad antitumoral y antiviral en particular anti VIH, entre otras actividades biológicas.

El ácido *p*-cumárico (ácido 4-hidroxicinámico) es reportado con un efecto benéfico en el área de salud humana ya que regula los niveles de lipoproteínas de baja densidad. Además, al ácido *p*-cumárico se le atribuyen una actividad biológica como antimutagénico, ansiolítico, sedante, analgésico y una actividad antimicrobiana contra *Staphylococcus aureus* y *Bacillus subtilis*. El mecanismo antimicrobiano del ácido *p*-cumarico no está claramente definido, pero se reporta que en el caso de bacterias patógenas de plantas inhibe la expresión de genes que codifican algunos factores de virulencia, como en el caso de *Dickeya dadantii*.

El ácido sinapínico (4-hidroxi-3,5-dimetoxi-cinámico) también es de gran potencial para la industria farmacológica por su capacidad antioxidante y antimicrobiana. En el área de salud humana el ácido sinapínico es de gran interés ya que se relaciona con actividades bilógicas como antinflamatorio, anticancerígeno y ansiolítico. El ácido sinapínico se

considera como un inhibidor de la acetilcolinesterasa por lo que podría tener aplicaciones terapéuticas en la enfermedad de Alzheimer, demencia senil, ataxia, miastenia grave, y el tratamiento de la enfermedad de Parkinson.

Por tal motivo, en los últimos años se ha incrementado el interés por el estudio y descubrimiento de nuevas FAE's microbianas, al ser enzimas clave para la hidrólisis de la pared de las células vegetales y liberación de los ácidos fenólicos que se encuentran en los residuos agroindustriales. Las FAE's microbianas en un principio fueron clasificadas como tipo A o B en base a su especificidad por sustratos sintéticos como los metil ésteres de ácidos hidroxicinámicos (metil ferulato, metil cafeato, metil *p*- cumarato y metil sinapinato). Posteriormente se amplió la clasificación a cuatro tipos (A, B, C y D) tomando en cuenta para esta clasificación, además de la especificidad por sustratos sintéticos, la secuencia primaria de aminoácidos y la capacidad para liberar dímeros del ácido ferúlico. Recientemente se publicó una clasificación de las FAE's microbianas en 7 subtipos o subfamilias (SF1-7) tomando en cuenta la agrupación filogenética de sus secuencias. Sin embargo, actualmente la clasificación más utilizada para las FAE's es la que divide a estas enzimas en cuatro tipos (A, B, C y D).

Las FAE's tipo A muestran preferencia por los sustratos con sustituyentes metoxilados como el ácido ferúlico y el ácido sinapínico aunque también son capaces de hidrolizar el metil *p*-cumarato. Su secuencia primaria es similar a las lipasas pero no muestran actividad con triacilglicéridos. Este tipo de FAE's muestra la capacidad de liberar dímeros del ácido ferúlico, además de ser preferentemente inducidas utilizando salvado de trigo en el medio de cultivo. Las FAE's tipo B muestran preferencia por los sustratos que contienen uno o dos grupos hidroxilo como sustituyentes, este es el caso del ácido *p*-cumárico y el ácido cafeico, logrando hidrolizar también el metil ferulato. Las FAE B

presentan similitud en su secuencia con acetil xilano exterasas y pueden ser preferentemente inducidas cuando se agrega pulpa de remolacha al medio de cultivo. Estas enzimas no muestran la capacidad de liberar a los dímeros del ácido ferúlico.

Para el caso de las FAE's tipo C y D, ambas hidrolizan indistintamente compuestos fenólicos metoxilados o hidroxilados, tal es el caso de los ácidos ferúlico, sinapínico, *p*-cumárico y cafeico y se diferencian entre sí por su capacidad de liberar los dímeros del ácido ferúlico, siendo las FAE's tipo D quienes catalizan dicha reacción. Otra de las diferencias es que las FAE's tipo C tienen similitud en su secuencia primaria con clorogenato esterasas, mientras que las FAE's tipo D tienen similitud con xilanasas.

En la actualidad se ha reportado a un gran número de microorganismos como productores de FAE's, tal el caso de *Aspergillus awamori* IFO4033 (AwFAE A), *Aspergillus nidulans* FGSC (AnFae B), *Aspergillus niger* (AnFae A y AnFae B), *Neurospora crassa* (NcFae D), *Aspergillus flavus* (AfFae A), *Penicillium funiculosum* (PfFae B), *Sorangium cellulosum* (ScFAE D), *Aspergillus orizae* (AoFae B y AoFae C), *Fusarium oxysporum* (FoFae A, FoFae B y FoFae C), *Sporotrichum thermophile* (StFAE A y StFAE C) *Talaromyces stipitatus* (TsFae A, TsFae B y TsFae C) entre otros.

Algunas FAE's, a pesar de estar clasificadas dentro del grupo de las hidrolasas, tienen la capacidad de catalizar la reacción de transesterificación, de tal forma que también pueden ser consideradas como enzimas de síntesis, al igual que las lipasas que han sido reportadas con dicha propiedad. Las reacciones de transesterificación consisten en el intercambio de un grupo alcoxi de un ácido hidroxicinámico por otro alcohol de cadena larga o una molécula más compleja. Los ésteres resultantes tienen una mayor lipofilicidad en comparación con sus moléculas precursoras y por tanto son ideales para su formulación en vehículos oleosos, comúnmente empleados en algunas industrias,

como la cosmética. También se ha reportado que las actividades biológicas de estos derivados pueden verse incrementadas.

Aunque las reacciones de transesterificación pueden llevarse a cabo mediante vía química, resulta de mayor interés la vía enzimática al ser una tecnología más limpia y selectiva, con lo cual se facilitan los procesos de purificación y en muchos casos se disminuyen los tiempos de reacción. Por ejemplo, se ha reportado a la enzima StFAE C de *Sporotrichum thermophile* con la habilidad de catalizar la transesterificación del metil ferulato con L-arabinosa y D-arabinosa, mientras que la AnFaeA de *Aspergillus niger* tiene la capacidad para sintetizar gliceril ferulato, permitiendo en ambos casos, la obtención de productos más hidrofílicos que sus precursores. Por su parte, la enzima FoFAE-I producida por *Fusarium oxysporum* es reportada como otra enzima de síntesis al catalizar la reacción de transesterificación de derivados fenólicos, al igual que la FAE de *Humicola insolens*, reportada con capacidad para la transesterificación de alcoholes secundarios. Sin embargo, la enzima mayormente utilizada para catalizar las reacciones de transesterificación es la lipasa B de *Candida antárctica* (CAL B) al haber demostrado su alta eficiencia para este tipo de reacciones.

En la literatura se encuentran reportadas varias FAE's que ya han sido caracterizadas exhaustivamente, como es el caso de la AnFaeA de *Aspergillus niger*, la cual se reporta como una α/β hidrolasa con una triada catalítica compuesta por serina, histidina y ácido aspártico (Ser133, Asp194 y His247). Presenta > 93 % de similitud con AwFAE A de *Aspergillus awamori* y un 32 % de similitud con la lipasa de *Rhizomucor miehei.* La enzima AnFaeA también comparte características estructurales con la lipasa de *Thermomyces lanuginosa* (TIL), ambas contienen un lid que permite a TIL tener dos estados conformacionales, uno activo cuando el sitio activo está expuesto y uno inactivo

cuando el sitio catalítico está cubierto por el lid. En el caso del lid de TIL, éste corresponde a los residuos amino ácidos del 82 al 96, mientras que para AnFaeA a los residuos 71 al 77. El hecho de que AnFaeA contenga un lid no indica que esta enzima presente dos estados conformacionales ya que para esta enzima el lid siempre se encuentra en posición abierta. La enzima AnFaeA también es considerada de síntesis ya que se ha comprobado que puede sintetizar derivados fenólicos mediante reacciones de transesterificación, como es el caso de la síntesis del pentilferulato con un rendimiento del 50 al 60%.

El interés por encontrar nuevas transesterasas, tiene por objeto el descubrimiento de enzimas substitutas para la enzima CAL B (lipasa B *de Candida antárctica*), la única transesterasa comercialmente disponible, procurando evitar así la dependencia de un solo proveedor. Igualmente se buscan enzimas con capacidades mejoradas en relación a CAL B para una posible explotación comercial de los productos resultantes de los procesos de síntesis enzimática.

CAL B es considerado un catalizador robusto debido a su alta estabilidad en solventes orgánicos y a una serie de características que la diferencian de otros catalizadores. La presencia del lid cubriendo el sitio activo en la estructura de las lipasas, es una características que las distingue del resto de las esterasas, con lo cual se produce un fenómeno llamado activación interfacial. En presencia de una interface líquido-agua, el lid sufre un rearreglo para dejar expuesto el sitio activo pasando de una forma cerrada a una forma abierta. Sin embargo, en el caso de CAL B se ha demostrado la presencia del lid pero no se ha observado la activación interfacial, por lo que el sitio activo permanece abierto en todo momento. La inmovilización de CAL B y sus características particulares permiten incrementar su termoestabilidad y uso de forma continua por un mayor número

de ciclos sin una pérdida significativa de la actividad. Dichas características de CAL B pueden deberse a un proceso evolutivo para adaptarse a un clima extremo ya que *Candida antártica* fue aislada de un sedimento del lago Vanda ubicado en la Antártida. Bajas temperaturas, altas temperaturas y altas concentraciones de sal, son las condiciones ambientales que pueden producir un estrés hídrico. Las enzimas que son producidas por microorganismos que sobreviven a un estrés hídrico muestran capacidad para catalizar reacciones con un bajo contenido de agua, debido a que presentan la capacidad de ser flexibles bajo estas condiciones. Esta característica puede ser aprovechada para catalizar reacciones en solventes orgánicos, como lo es la reacción de transesterificación. Una zona con temperaturas elevadas es el desierto de Sonora donde se han reportado temperaturas que rebasan los 50 °C en temporada de verano y una precipitación pluvial promedio de 125 mm al año, lo cual ayuda a generar un ambiente de estrés hídrico. Sin embargo, pese a las altas temperaturas alcanzadas en esta región, cuenta con una gran diversidad de flora y fauna adaptada a las condiciones de estrés hídrico. Tomando en cuenta las características ambientales de esta región, en el laboratorio de biotecnología industrial del CIAD Hermosillo se estableció una colección de hongos nativos de diferentes regiones del Estado de Sonora, México, identificadas como las zonas donde se registraron las temperaturas más elevadas en el año 2009.

De acuerdo a lo anteriormente expuesto se planteó para el presente trabajo la siguiente hipótesis: “los microorganismos aislados del Estado de Sonora adaptados a sobrevivir en estrés hídrico, producen feruloil esterasas capaces de catalizar la reacción de transesterificación para la producción de derivados fenólicos”.

La colección de hongos CIAD está conformada por 205 cepas, cultivadas a 40 °C, que fueron aisladas a partir de muestras de material vegetal en descomposición (sustrato) y

muestras de suelo. Mediante métodos conocidos como "*métodos rápidos"* se seleccionaron las cepas productoras de FAE's, empleando como medio de cultivo, agar adicionado con etil ferulato como fuente de carbono, suplementado con minerales y una fuente inorgánica de nitrógeno. En un tamizaje preliminar, el 90% de las cepas presentó crecimiento en el medio cultivo utilizado, de las cuales se seleccionaron aquellas cepas que produjeron una relación halo/colonia >2, resultando un total de 20 cepas seleccionadas bajo este criterio. Este ensayo fue realizado a 20°C y 50°C obteniendo crecimiento de las veinte cepas seleccionadas a ambas temperaturas lo cual las clasifica como cepas termotolerantes.

Mediante técnicas de biología molecular, once cepas fueron identificadas como *Aspergillus terreus* (SB1-2, CG5-2, B94, B9 B17, B102, B10, B102, CG1-2, A70 y A144), dos como *Aspergillus fumigatus* (B80 y B38), dos como *Aspergillus tamarii* (CG16 y CG4-1), dos como *Aspergillus flavus* (B65 y SB9), dos como *Phialophora alba* (CG4 y B13) y una como *Neurospora tetrasperma* (A62). En un análisis filogenético las cepas de *Aspergillus terreus* fueron separadas en dos grupos: SB1-2, CG5-2, B94, B9, B17, B102, B10 y B23, similares a *Aspergillus terreus* ATCC1012. El segundo grupo quedó conformado por CG1-2, A144, A70 similares a *Aspergillus terreus* ATCC20526.

Las veinte cepas seleccionadas fueron sometidas a un segundo tamizaje utilizando tres concentraciones diferentes de etil ferulato. Para este ensayo los resultados fueron contrastados con la cepa de *Aspergillus terreus* ATCC1012, considerada como cepa de referencia. Cuando el ensayo se llevó a cabo a 25 °C, las cepas de la colección CIAD produjeron en su mayoría una actividad similar o ligeramente menor, comparada con la cepa de referencia, solo *Aspergillus tamarii* presentó actividades superiores (80-180 %). Contrario a los resultados a 25 °C, cuando el ensayo se realizó a 40 °C, las cepas de la

colección CIAD presentaron actividades 70 a 800 % mayores que la cepa de referencia, incluso comparadas con la mayor actividad observada a su temperatura óptima de crecimiento (25°C). Este hecho sugiere una relación entre las cepas adaptadas a sobrevivir en estrés hídrico y la producción de FAE´s de alta eficiencia.

Los secretomas de estas cepas, obtenidos de su cultivo en cascarilla de maíz y bagazo de caña en una proporción 2:1, fueron utilizados para un ensayo de especificidad por sustrato utilizando metil ferulato, metil cafeato, metil *p*-cumarato y metil sinapinato como sustratos. Mediante este ensayo se logró inferir que los secretomas pertenecientes a las cepas de *Aspergillus terreus* B102, B23, B10 y CG5-2 producen FAE´s tipo A ya que no presentaron actividad sobre metil cafeato como sustrato. La cepa de *Aspergillus tamarii* CG16 no mostró actividad con metil sinapinato sugiriendo que esta cepa es productora de FAE´s tipo B. El resto de las cepas mostraron actividad con los cuatro sustratos lo cual sugiere FAE´s con actividad tipo C o D y/o la producción de dos o más tipos de FAE's por una misma cepa.

Posteriormente se hizo un tamizaje de las veinte cepas con la finalidad de seleccionar aquellas que presentaran una mayor versatilidad en las reacciones de síntesis. Para este ensayo se probó la síntesis de butil ésteres (butil ferulato (BF), butil cafeato (BC), butil *p*-cumarato (BpC) y butil sinapinato (BS)) mediante una reacción de transesterificación, la cual se evaluó de forma cualitativa mediante cromatografía de capa fina (TLC). Como resultado se encontró que solo cuatro cepas de *Aspergillus terreus* (CG1-2, CG5-2, B102, B17) y una cepa de *Aspergillus tamarii* (CG4-1) lograron sintetizar los cuatro butil ésteres. Sin embargo, el secretoma de la cepa de *Aspergillus terreus* B102 presentó el mejor comportamiento en la síntesis de butil ésteres, tomando en cuenta la intensidad de las bandas del producto en la TLC, siendo la cepa seleccionada para estudios

posteriores. Mediante zimogramas en un gel nativo-PAGE se determinó la actividad FAE, utilizando los cuatro metil ésteres como sustratos por separado demostrando que *Aspergillus terreus* B102 produce dos FAE's. Mediante una purificación parcial ambas FAE's fueron separadas, observando que una de ellas presenta actividad sobre metil ferulato, metil cafeato y metil *p*-cumarato lo que la clasifica como una FAE tipo B (AtFAE I).

La segunda FAE producida por la cepa seleccionada *Aspergillus terreus* B102 presentó actividad sobre los cuatro sustratos pudiendo ser clasificada como una FAE tipo C o tipo D (AtFAE II). En los análisis preliminares del secretoma de *Aspergillus terreus* B102 se mostraron resultados que sugerían la producción de FAE´s con actividad tipo A. Sin embargo este resultado se pudo confundir debido a la combinación de ambas ó a una baja concentración de las FAE´s en el secretoma (AtFAE I y AtFAE II). Mediante SDS-PAGE y utilizando marcadores de bajo peso molecular se demostró que AtFAE I es de aproximadamente 57 kDa y AtFAE II de 30 kDa.

Las bandas obtenidas en SDS-PAGE fueron recuperadas para la determinación de la secuencia N terminal mediante el método de MALDI/TOF. Como resultados se obtuvo un péptido QDAFEAKXHS para AtFAE I mientras que en el caso de AtFAE II no se logró obtener un péptido probablemente por la baja concentración de la enzima en la banda a secuenciar. El péptido de AtFAE I fue analizado en la base de datos NCBI BLAST resultando con un 90% de identidad con FAE B de *Aspergillus terreus* NIH 2624, razón por la cual se pasó a nombrar a AtFAE I como AtFAE B.

Con ambas enzimas separadas y con AtFAE B identificada se utilizó un sistema ternario isoctano/butanol/agua para demostrar cual FAE es la responsable de catalizar la reacción de transesterificación. Para este ensayo se probó la síntesis de butil ferulato (BF), butil

cafeato (BC), butil *p*-cumarato (BpC) y butil sinapinato (BS). Utilizando el método de HTPLC se logró obtener los rendimientos en síntesis para ambas FAE´s, resultando para AtFAE B un 5.8% de BF, 3% de BpC, 2% BC y 0% de BS, mientras que para AtFAE II 4.1% de BF, 2% de BF, 0% de BpC y 5.2% de BS. Con estos resultados se obtuvo el perfil de hidrólisis para ambas FAE's siendo para AtFAE B BF>BpC>BC y para AtFAE II BS>BF>BC>BpC. La enzima AtFAE II presentó una mayor versatilidad al resultar positiva también para la síntesis de fenetil éster de ácido caféico (CAPE), el cual fue determinado de forma cualitativa por TLC.

Con los resultados anteriores se comprobó que la biodiversidad microbiana del Estado de Sonora tiene un potencial biotecnológico importante, en particular como nuevas fuentes de FAE's. Se demostró que la cepa *Aspergillus terreus* B102 es productora de dos FAE´s (AtFAE B y AtFAE II), ambas con la capacidad de hidrólisis y de transesterificación (síntesis).

Los resultados obtenidos en este trabajo justifican la continuación de los estudios a fondo sobre la colección de hongos termotolerantes CIAD y en particular las FAE's de *Aspergillus terreus* B102. Por ello resulta de sumo interés la optimización de los rendimientos de síntesis de los butil ésteres, evaluando los efectos de las condiciones de la reacción de transesterificación, tales como temperatura, concentración de la enzima y de los sustratos, entre otros, además de un análisis en el rendimiento de los productos de dicha reacción utilizando las enzimas inmovilizadas. Por otra parte es importante conocer a fondo la versatilidad de AtFAE B y AtFAE II para sintetizar derivados fenólicos, para ello es necesario utilizar sustratos más complejos como geraniol o timol los cuales son reportados con diferentes actividades biológicas que pudieran incrementarse mediante la obtención de derivados fenólicos. Sin embargo, para ampliar

el conocimiento de las FAE's de *Aspergillus terreus* B102 es necesario la clonación y sobreexpresión de dichas enzimas, lo cual facilitaría los análisis ya que se obtendría la enzima en cantidades elevadas y se facilitaría su purificación.

La cepa seleccionada en este estudio *Aspergillus terreus* B102 también puede ser explotada no solo para la producción de FAE's, si no también mediante un análisis completo del sistema de carbohidrato esterasas para determinar la eficiencia catalítica en la hidrolisis de polisacáridos de los residuos agroindustriales.

En el tamizaje final de las cepas de la colección CIAD fueron seleccionadas cinco cepas de las cuales cuatro fueron descartadas a efectos de acotar los alcances del trabajo de tesis. Sin embargo, dichas cepas tienen potencial para producir enzimas con capacidades de síntesis que pueden resultar interesantes, por lo que su explotación como productoras de transesterasas y otras enzimas degradadoras de residuos agroalimentarios resulta relevante como tema de investigación. Esta consideración también puede ser extrapolada a toda la colección de cepas de hongos termotolerantes CIAD, como posibles cepas productoras de enzimas de interés industrial.

Capítulo I.- Francisco J Bacame-Valenzuela , Jorge A Rodriguez , Juan C Mateos-Díaz , Manuel Kirchmayr , Elisa M Valenzuela-Soto, Yolanda Reyes-Vidal , Martin Esqueda and Ali Asaff (2015) Screening of Sonoran Desert Fungal Strains for feruloyl Esterase Activity. Journal of pure and applied microbiology.9: 131-138.

# Screening of Sonoran Desert Fungal Strains for feruloyl Esterase Activity

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(Received: 18 March 2015; accepted: 20 April 2015)

**During last two decades, feruloyl esterases have been intensely studied for industrial or medical applications. Also screening of microbes from thermal and extreme environments as source of this kind of enzymes with improved properties is an active research field. In this context, screening and selection of fungal strains, for feruloyl esterase activity, were developed at Sonoran Desert, one of the hottest and driest regions of America. Sampling was carried out from soil and plant residues decomposing on soil. Samples were suspended in water, diluted, spread onto agar and cultured at 40 °C. Isolated strains were spread onto selective agar, choosing those with feruloyl esterase hydrolytic halo/colony diameters ratio>2. Selected strains were identified by molecular methods and their secretomes were assessed for methyl hydroxy cinnamates specificity. Twenty thermotolerant fungal strains, showing high feruloyl esterase activity, were found and identified as *Aspergillus flavus* (2), *Aspergillus fumigatus* (2), *Aspergillus tamarii* (2), *Aspergillus terreus* (11), *Neurospora tetrasperma* (1) and *Phialophora alba* (2). At least 10 of those shown 2.5 to 3.8 higher feruloyl esterase activities, than *Aspergillus terreus* ATCC1012, a mesophile strain. Secretomes exposed scarce specificity for substrates. Selected feruloyl esterases-producing strains show high potential to be used as biotechnological tools.**

**Key words:** Desert climate, Hydroxycinnamoyl esterases, Fungi.

Thermostable enzymes have been a topic of intense research during the last two decades, because their increased reactivity at high temperatures (70–100 °C) and long-term stability at ambient temperature make them suitable for cost effective industrial processes[1, 2, 3]. This also stimulated the screening and isolation of microbes from thermal environments as sources of new enzymes that could significantly increase the window for enzymatic bioprocess operations[4].

One of the hottest regions in North America is the Sonoran Desert, where temperatures of up to 50 °C have been recorded, although is considered as one of the wettest deserts and averages from 125 mm of rain a year[5]. Sonoran Desert has a great biological diversity and geological alternation, where many species have evolved to have specialized adaptations to the desert climate[6]. In Mexico, it covers more than half of the State of Sonora, two-thirds of the peninsula of Baja California and is present in all the islands of The Sea of Cortes. Of the seven subdivisions that comprise it, four are located in Sonora: the highlands of Arizona; the plains of Sonora; the Central Gulf Coast and the lower Colorado River Valley[6].

The feruloyl esterases (FAE) (EC 3.1.1.73) represent a diverse group of hydrolases that catalyze the cleavage and formation of ester bonds

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between polysaccharides of the cell wall of plants and phenolic acids[7]. Over the past decade, research carried out on microbial FAEs has increased intensely. The number of papers related to isolation, purification and characterization of FAEs and patents for applications in biotechnological processes, both in industrial and medical areas grew exponentially[7, 8]. Nowadays, the interest towards FAEs with new properties continues and represents an important area of research[7, 9].

According the current importance of FAEs and the particular characteristics of the Sonoran Desert, the objective of this work was to develop a screening of fungal strains for feruloyl esterase activity from samples collected from ten regions from Sonora State, Mexico.

## MATERIALS AND METHODS

### Sample collection

Climate charts of Sonora State, Mexico, were used in order to choose the hottest regions during the year 2009. Three regions, with records of temperatures between 45 to 50 °C, were selected. Sampling was carried out from soil and plant residues decomposing on soil with or without macroscopic evidence of fungal growth. In the case of soil samples, around 100 g from the depth of 0–10 cm and from the depth of 20–30 cm were randomly collected. All samples were transported to the laboratory in sterile zip lock bags on the same day.

### Isolation and cultivation of fungi

Petri dishes containing potato dextrose agar (PDA, procured from Difco) were employed to isolate strains. 10g of soil sample were diluted in 125 ml of sterile tween 80 solution (0.05% v/v) by vortexing. 1.5 g of milled and sieved sample, containing plant residues, was diluted in 13.5 ml of sterile tween 80 solution (0.05% (v/v)) by vortexing. Then, 0.5 ml of serial diluted supernatant was spread onto the surface of the medium and incubated at 40°C for 3 d. Isolated strains were preserved at 4 °C in sloped tubes containing PDA, covered with sterile mineral oil. They were used to propagate fungi on PDA Petri dishes used as inocula.

### Screening of fungal strains for FAE activity

Screening was performed using a minimal medium described by Rodríguez *et al.,* (2006) with little modifications in carbon source. Petri dishes containing selective agar (1, 2 or 3 g/l of ethyl ferulate, 4 g/l of urea, 5 g/l of $K_2HPO_4$, 1 g/l of $MgSO_4$, 15 g/l of bacteriological agar and 1% (v/v) bromocresol purple aqueous solution (0.1% (w/v), pH 6.5) were prepared. The released ferulic acid from ethyl ferulate by FAE activity decrease pH, discoloring or changing to yellow the purple color of the medium. Magnesium sulfate and potassium phosphate dibasic were procured from J.T. Baker; urea, bromocresol purple and ethyl ferulate from Sigma-Aldrich-Fluka (Mexico) and bacteriological agar from Difco.

Inoculum was picked with sterile wooden stick and transfer by touching each dish. For preliminary screening, plates were incubated at 40°C for 5 d and then, hydrolytic-halos and colony diameters were measured in order to establish halo/colony ratios. For secondary screening *Aspergillus terreus* ATCC 1012, a known FAEs-producing strain was included as positive control. Also, *Aspergillus niger* ATCC 16888 was used as a second control because it is a known FAEs-producing strain which has been extensively studied[11, 12]. Pre-selected and mesophilic control strains were incubated both at 25 and 40°C in order to compare efficiency of FAEs production.

For classifying the selected FAEs producing strains as mesophilic, thermotolerant or thermophilic according Mouchacca (1997)criterion, they were inoculated in a PDA medium using 24 wells microplates, incubated at 20°C and 50°C for 5 d.

### Molecular identification of isolates

All strains were grown on PDA at 30°C for 72 h. Subsequently, fungal mycelium was collected by scraping the surface of the solid culture and DNA was extracted with the commercial kit Dneasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR was carried out with Illustra ™ PureTaq Ready-To-Go PCR Beads (GE Healthcare, Uppsala, Sweden) on a Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, USA). Primers ITS1 and ITS4 were employed to amplify an approx. 600 bp fragment from the ribosomal gen cluster under the same reaction conditions described elsewhere[14]. PCR products were sent to Macrogen USA Corp. for sequencing of both strands. Consensus sequences were obtained with

the CLC Main Workbench 5.5 software package (CLCBio, Aarhus, Denmark) and compared to recorded sequences from GenBank database using the BLAST algorithm[15].

**Solid fermentation and secretome extraction**

Selected strains for FAE activity were culture on solid-state fermentation. 125 ml Erlenmeyer flasks containing 7 g of a milled and sieved (40 mesh) mixture of corn bran and sugar cane bagasse (2:1 w/w) were sterilized at 121°C, 15 min. Then, 4.5 ml of inoculum ($3 \times 10^7$ spores/ml) and 8.5 ml of minimal medium (4 g/l of urea, 5 g/l of $K_2HPO_4$, 1 g/l of $MgSO_4$) were added and incubated at 30°C for 48 h. Then, cultures were dried at 30°C for 24 h and milled and sieved (60 mesh). 1 g of sieved material was extracted with 5 ml MOPS buffer (2.5 mM, pH 7.2) by vortexing during 1 min and steeping for 10 minutes in ice bath. This mixture was centrifuged at 10000 ×g, 4°C, for 10 min and recovered supernatants containing secretomes were kept at 4°C for further analyses.

**FAE substrate specificity**

Ninety six well plates containing 100 µl of methyl ferulate, methyl caffeate, methyl *p*-coumarate and methyl sinapinate solutions (0.1% (w/v)) and 1% (v/v) bromocresol purple aqueous solution (0.1%(w/v)) were added with 50 µl of secretomes. Discoloration, or change to yellow, in the purple color of the medium after 5 min of secretome addition was considered positive for FAE activity. Depol™ 740L (Biocatalysts Ltd., Wales, UK), a commercial enzymatic extract with feruloyl esterase activity was used as positive control.

## RESULTS

**Preliminary screening of fungal strains for FAE activity**

Sampling localities were grouped into three regions according their proximity among them and their location in the State of Sonora, Mexico (fig 1). A total of 205 fungal strains were isolated from the collected samples. Although, more than 90% of the strains were able to growth in the culture medium containing ethyl ferulate as sole carbon source, just those with a halo/colony diameter ratio > 2, at least at one of the three concentrations of ethyl ferulate employed, were considered as good candidates for FAE activity. The halo/colony diameter ratio is a broad accepted criterion for selecting strains which produce %enzymes of

**Table 1.** Georeferenced sampling places and molecular characterization of the selected isolates.

| Region | North latitude | West longitude | Isolated | Reference strain | Identity (%) |
|---|---|---|---|---|---|
| I | 30°09'54.10" | 111°14'44.30" | B80 | *Aspergillus fumigatus(ATCC1022* | 99 |
| | 28°00'55.90" | 111°03'4.10" | B94 | *Aspergillus terreus(ATCC1012) (ATCC1012)* | 100 |
| | 28°04'05.50" | 110°45'12.90" | B102 | *Aspergillus terreus(ATCC1012)* | 100 |
| | 28°18'59.60" | 111°14'40.60" | B17 | *Aspergillus terreus(ATCC1012)* | 100 |
| | | | B13 | *Phialophora alba(ICMP17034)* | 100 |
| | 28°48'09.70" | 111°12'13.20" | A70 | *Aspergillus terreus(ATCC20526)* | 99 |
| | | | A144 | *Aspergillus terreus(ATCC20526)* | 100 |
| | | | A62 | *Neurospora tetrasperma(ATCC MYA-4615)* | 99 |
| | | | B9 | *Aspergillus terreus(ATCC1012)* | 100 |
| | 28°33'50.80" | 111°00'28.40" | B65 | *Aspergillus flavus(ATCC20043)* | 99 |
| | 29°16'20.30" | 111°05'43.60" | B23 | *Aspergillus terreus(ATCC1012)* | 99 |
| | | | B38 | *Aspergillus fumigatus(ATCC1022)* | 99 |
| II | 29°31´48´´ | 109°31´48´´ | CG1-2 | *Aspergillus terreus(ATCC20526)* | 100 |
| | | | CG5-2 | *Aspergillus terreus(ATCC1012)* | 100 |
| | | | CG16 | *Aspergillus tamarii(NRRL427)* | 100 |
| | | | CG4 | *Phialophora alba(ICMP17034)* | 100 |
| | | | CG4-1 | *Aspergillus tamarii(NRRL427)* | 100 |
| III | 31°31'55.01" | 113°25'40.05" | SB1 | *Aspergillus terreus(ATCC1012)* | 99 |
| | | | B10 | *Aspergillus terreus(ATCC1012)* | 100 |
| | | | SB9 | *Aspergillus flavus(ATCC20043)* | 99 |

interest[12, 16, 17]. According to this condition, 20 fungal strains were selected and classified as thermotolerant since they were able to growth at 20 and 50°C, following the criterion suggested by Mouchacca (1997). Growth at 20°C was higher than growth at 50°C after 5 d of incubation. Twelve strains were selected from region I, five from region II and three from region III. From all selected strains, just three were isolated from soil samples (0–10 cm depth) in the region I and the rest of strains from plant material.

It is known that FAEs are involved in the degradation of lignocellulosic materials[18], thus most selected fungal strains were isolated from this kind of material; even the strains isolated from soil which include samples collected from the surface, rich in products resulting from decomposition of dead vegetal matter. Moreover, 85% of the selected strains come from region I and region II where a great variety of vegetation is found, including trees as "mesquite" (*Prosopis* sp.), sarcocaule shrub and microphyll desert shrub.

**Molecular characterization**

Of the 20 selected FAEs-producing strains, two were identified as *Aspergillus flavus*, two as *Aspergillus fumigatus*, two as *Aspergillus tamarii*, eleven as *Aspergillus terreus*, one as *Neurospora tetrasperma* and two as *Phialophora alba* (table 1).

The presence of a large number of strains belonging to the genus *Aspergillus* coincides with the report of Bonnin *et al.,* (2008), which points out species of *Aspergillus* genus as efficient producers of enzymes degrading plant cell wall. Other species of *Aspergillus* reported as producers of FAEs are *A. niger*, *A. awamori*, *A. nidulans*, *A. oryzae*, *A. tubingensis*, *A. flavipes* and *A. foetidus*[7, 20]. The existence of *Aspergillus terreus* and *Aspergillus fumigatus* in the Sonoran Desert areas were already reported by Cruz *et al.,* (2005) and Kithsiri *et al*., (2003).

Higher ratios of native strains at 40°C suggest adaptive modification to environmental conditions that promote higher production of

**Table 2.** Selection of feruloylesterases-producing strains using ethyl ferulate as substrate.

| Isolated | [a]Ratio | | | | | |
|---|---|---|---|---|---|---|
| | 25 °C | | | 40 °C | | |
| | [b]EF(1 g/L) | EF(2 g/L) | EF(3 g/L) | EF(1 g/L) | EF(2 g/L) | EF(3 g/L) |
| *A. niger*(ATCC 16888) | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. terreus*(ATCC 1012) | 2.8 | 4 | 3.25 | 2 | 3 | 3 |
| B102 | 6.25 | 3.75 | 4 | 1.25 | 9.37 | 11.25 |
| SB1 | 4 | 2 | 1.25 | 4 | 3.5 | 3 |
| B23 | 5 | 2 | 2 | 1 | 7.25 | 6 |
| B17 | 0 | 1 | 1.25 | 6 | 5 | 2 |
| A70 | 0 | 2.5 | 1 | 5.5 | 3 | 4 |
| CG1 | 0 | 1 | 1 | 5.5 | 7.5 | 3.5 |
| A144 | 0 | 2.5 | 1 | 4 | 4 | 3 |
| B10 | 2.5 | 3.25 | 1.25 | 1.75 | 11.25 | 11.25 |
| CG5-2 | 3 | 1 | 1.75 | 1.75 | 9.37 | 8.75 |
| B94 | 2.5 | 1 | 1 | 1 | 10.25 | 5.75 |
| B9 | 2 | 3 | 4.25 | 5.25 | 5.5 | 7.25 |
| B80 | 5 | 3.5 | 4.25 | 1.25 | 11.25 | 8.75 |
| B38 | 5.5 | 4.5 | 5 | 1.25 | 11 | 8.75 |
| SB9 | 3.5 | 3.25 | 3.75 | 1.25 | 10 | 6 |
| B65 | 2 | 3 | 4.5 | 1.25 | 5 | 5.75 |
| CG16 | 7.5 | 8.25 | 8.25 | 1.75 | 10 | 8.5 |
| CG4-1 | 7.5 | 7.25 | 9.25 | 1.25 | 7.5 | 10 |
| CG4 | 3.75 | 2.5 | 2.5 | 1 | 3.5 | 2.25 |
| B13 | 3.75 | 2.5 | 2.5 | 1 | 2.5 | 2 |
| A62 | 0 | 1 | 1 | 1 | 2.5 | 1.75 |

[a]Ratiobetween hydrolytic halo and colony diameters; [b]Ethylferulate

enzymes with FAE activity and/or enzymes with higher catalytic efficiency. It is also expected a higher enzymatic thermostability according the sources, such was observed with enzymes isolated from thermophiles[23].

**Phylogenetic tree**

The phylogenetic tree shows the selected strains which were identified as feruloyl esterase producers (Fig. 2). It should be noted that the fungal strains identified as *A. terreus* are divided into two groups. The first one is composed by strains SB1-2, CG5-2, B94, B9, B17, B102, B10 and B23, being the last the most different among them. This group turned out to be similar to the strain *A. terreus* ATCC1012. In the case of the second group, which is composed by strains CG1-2, A144 and A70, it was proven to be similar to the strain of *A. terreus* ATCC20526, being A70 strain the most different among them.

**Secondary screening of fungal strains for FAE activity**

Since 90% of the selected strains during the preliminary screening correspond to *Aspergillus* genus, for secondary screening *Aspergillus terreus* ATCC 1012 and *Aspergillus niger* ATCC 16888 were included as controls. Table 2 shows FAE activity tests developed at 25 and 40°C, at three ethyl ferulate concentrations, in order to establish differences on growth, enzymatic activities and possible advantages of native strains compared with controls. At any ethyl ferulate concentration, *A. terreus* ATCC 1012 established higher ratios at 25° C than 40°C since it is considered a mesophile strain. However, *A. niger* ATCC 16888 was unable to growth, even at the lowest ethyl ferulate concentrations because this strain may not produce the amount or type of enzymes required to hydrolyze and metabolize ethyl ferulate which also is toxic for many species of fungi. The antimicrobial activity of cinnamic acid derivatives has been largely reported[24, 25, 26]. At 25°C and at any ethyl ferulate concentration, native strains of *Aspergillus* genus and *Phialophora alba* established similar or lower ratios than *A. terreus ATCC* 1012. Just *A. tamarii* strains showed higher ratios (80–180%) than control. However, at 40°C, native strains increased their ratios between 70 to 800%, while *A. terreus* ATCC 1012 decreased around 8 to 30%. Stand out some *A. terreus* native strains, like B102, B23, CG1, B10, CG5-2, B94, and *A. flavus, A. fumigatus*, and *A. tamarii* strains which halo/colony diameter ratios results around 2.5 to

**Table 3.** Feruloyl esterase activity of the selected strainson different substrates

| Isolated | Strains | [a] MF | [b] MC | [c] MpC | [d] MS |
|---|---|---|---|---|---|
| B102 | *Aspergillusterreus* | + | - | + | + |
| SB1 | *Aspergillusterreus* | + | + | + | + |
| B23 | *Aspergillusterreus* | + | - | + | - |
| B17 | *Aspergillus terreus* | + | + | + | + |
| A70 | *Aspergillus terreus* | + | + | + | + |
| CG1 | *Aspergillus terreus* | + | + | + | + |
| A144 | *Aspergillus terreus)* | + | + | + | + |
| B10 | *Aspergillus terreus* | + | - | + | + |
| CG5-2 | *Aspergillus terreus* | + | - | + | + |
| B94 | *Aspergillus terreus* | + | + | + | + |
| B9 | *Aspergillus terreus* | + | + | + | + |
| B80 | *Aspergillus fumigatus* | + | + | + | + |
| B38 | *Aspergillus fumigatus* | + | - | + | + |
| SB9 | *Aspergillus flavus* | + | + | + | + |
| B65 | *Aspergillus flavus* | + | + | + | + |
| CG16 | *Aspergillus tamarii* | + | + | + | - |
| CG4-1 | *Aspergillus tamarii* | + | - | + | - |
| CG4 | *Phialophora alba* | + | + | + | + |
| B13 | *Phialophora alba* | + | + | + | + |
| A62 | *Neurosporatetrasperma* | + | + | + | + |

[a]Methylferulate; [b]Methylcaffeate; [c]Methyl *p*-coumarate; [d]Methylsinapinate

3.8 times higher than *A. terreus* control.

**FAE substrate specificity**

Feruloyl esterase specificity was tested using four synthetic substrates as shown in table 3. Such substrates are used in the classification of Crepin *et al.,* (2004) which states that there are four FAEs types (A, B, C, D). This classification is based on the substrate specificity and primary sequence. In the case of *A. terreus*, secretomes obtained from B102, B23, B10 and CG5-2 strains were not able to hydrolyze methyl caffeate suggesting type A FAEs production which have preference for substrates whose substituents are methoxy groups. However these strains showed the largest halo/diameter colony ratios (table 3). On the contrary, most of the strains with the lowest halo/colony ratios, produced secretomes capable of hydrolyzing the four substrates showing that contain unspecific FAEs (type C or D) or FAEs mixtures. The larger halo/colony ratios may be due to a higher enzyme specificity and thus to a higher catalytic efficiency instead to an increased enzyme production.

In the case of *A. tamarii* CG16, strain secretome was not able to hydrolyze methyl sinapinate suggesting type B FAEs production, since they have a strong preference for hydroxylated substrates. Just in two cases (B23 and CG4-1), methyl sinapinate and methyl caffeate were not hydrolyzed at our analysis conditions,


**Fig. 1.** A: Map of México. B: Map of Sonora State, including the regions where FAEs-producing strains were isolated.

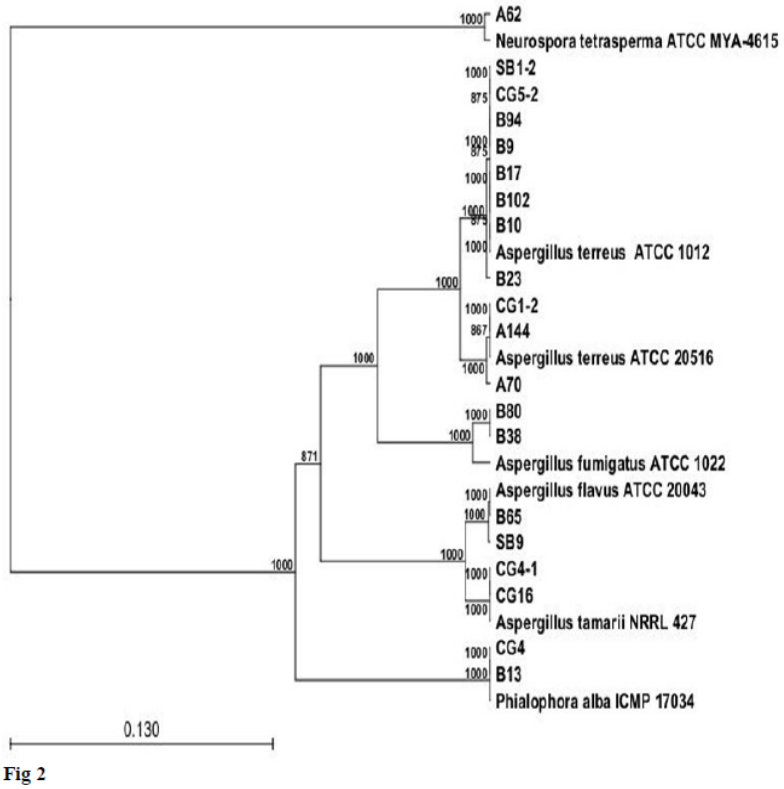


Fig 2

**Fig. 2.** Phylogenetic tree for FAEs producing strains from the Sonoran Desert collection.Numbers at the nodes are bootstrap values (of 1,000 replicate)

probably due to a low enzyme concentration produced by these strains.

Several strains of *Aspergillus terreus* have been reported as producers of different types of FAEs that can be induced by the employed substrate. *Aspergillus terreus* strain MUCL35566 was reported as a producer of type B FAE when grown on sugar beet pulp as it showed activity on methyl caffeate but not on methyl sinapinate. Also, *A. terreus* strain MUCL35503 was reported as type B FAEs producer when it was grown on oat xylan. However, when this strain was grown on sugar beet pulp, hydrolytic activity on methyl caffeate and methyl sinapinate was observed, suggesting type A and B FAEs production[28]. It was reported that *A. terreus* strain GA2 exhibits FAE activity, although classification was not included[29]. On the other hand, it is also reported that *A. terreus* MTCC11096 produces three types of FAEs, one type A and two type C[30]. Bouzid *et al*., (2006) found that *Aspergillus flavus* strain BRFM821, grown on sugar beet pulp, presented activity on methyl sinapinate and methyl caffeate as substrates, suggesting the production of type A and B FAEs. When *A. flavus* strain BRFM821 was grown on oat xylan, it produced just a type B FAE which showed activity on methyl caffeate but not on methyl sinapinate. In the case of *Aspergillus tamarii*, three strains have been reported producing type B FAEs (*A. tamarii* MUCL14048, *A. tamarii* MUCL 18828, *A. tamarii* MUCL 43440) when they were grown on sugar beet pulp and oat xylan[28]. No reports about FAEs production by *Aspergillus fumigatus*, *Neurospora tetrasperma* and *Phialophora alba* were found and this work is the first that notice these fungal species as FAEs-producing strains. Currently, secretomes are being employed to obtain zymograms in order to establish the number of FAEs and FAE's specificity.

## CONCLUSION

Results show that the ecosystem of the Sonoran Desert is a good source of FAEs-producing thermotolerant fungi, which show advantages over other FAEs-producing mesophile strains. The characterization of FAEs produced and their applications are interesting and promising topics for future studies due to characteristics of the environment in which microorganisms develop and the diversity of types of FAEs that could be induced by specific substrates and culture conditions.

## ACKNOWLEDGMENTS

The authors acknowledge technical assistance of the Q.B. Socorro Vallejo Cohen, M.C. César Oropeza and M.C. Aldo Gutiérrez. FJ Bacame-Valenzuela thanks to the Mexican Council for Science and Technology (CONACYT) for his PhD scholarship (No. 326666). The study was financially supported by FOMIX-CONACYT-Jalisco (No. 2012-07-190698).

## REFERENCES

1. Córdova J., Roussos S., Baratti J., Nungaray J., Loera O. Identification of Mexican thermophilic and thermotolerant fungal isolates. *Micologia aplicada international,* 2003; **15**(2): 37–44.
2. Hilden K., Hakala T.K., Lundell T. Thermotolerant and thermostable laccases. *Biotechnology Letters*, 2009;**31**(8): 1117–1128.
3. Morgenstern I., Powlowski J., Ishmael N., Darmond C., Marqueteau S., Moisan M.C., Quenneville G., Tsang A. A molecular phylogeny of thermophilic fungi. *Fungal Biology*, 2012; **116**(4): 489–502.
4. Pernilla T., Mamo G., Nordberg E. Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microbial Cell Factories*, 2007;**6**(9): 1–23.
5. Balling R.C, Klopatek J.M., Hildebrandt M.L., Moritz C., Watts C.J. Impacts of land degradation on historical temperature records from the Sonoran Desert. *Climatic Change*, 1998; **40**(3–4): 669–681.
6. Sánchez J.J. Plantas nativas de Sonora: las plantas del desierto sonorense. *Revista Universidad de Sonora,* 2007; **19**: 20–22.
7. Fazary A.E., Ju Y.H. Feruloyl esterases as biotechnological tools: current and future perspectives. *Acta Biochimica et Biophysica Sinica*, 2007; **39**(11): 811–828.
8. Fazary A.E., Ju Y.H. The large-scale use of feruloyl esterases in industry. *Biotechnology and Molecular Biology Reviews,* 2008; **3**:95–110.
9. Shin H.D., Chen R.R. A type B feruloyl esterase from *Aspergillus nidulans* with broad pH applicability. *Applied Microbiology and Biotechnology*, 2007; **73**(6): 1323–1330.
10. Rodríguez J.A., Mateos Díaz J.C., Nungaray J., González V., Bhagnagar T., Roussos S., Cordova

J., Baratti J. Improving lipase production by nutrient source modification using *Rhizopus homothallicus* cultured in solid-state fermentation. *Process Biochemistry*, 2006; **41**(11): 2264–2269.
11. Faulds C., Kroon P., Saulnier L., Thibault J., Williamson G. Release of ferulic acid from maize bran and derived oligosaccharides by *Aspergillus niger* esterases. *Carbohydrate Polymers*, 1995; **27**(3): 187–190.
12. Henríquez M., Vergara K., Norambuena J., Beiza A., Maza F., Ubilla P., Araya I., Chávez R., San-Martín A., Darias J., Darias M.J., Vaca I. Diversity of cultivable fungi associated with antarctic marine sponges and screening for their antimicrobial, antitumoral and antioxidant potential. *World Journal of Microbiology and Biotechnology*, 2014; **30**(1): 65–76.
13. Mouchacca J. Thermophilic fungi: biodiversity and taxonomic status. *Cryptogamie Mycologie*, 1997; **18**(1): 19–69.
14. Segura G.L., Kirchmayr M.R., Flores B.E., Gschaedler M.A. PCR-RFLP of the ITS-5.8S regions as an identification tool for yeasts: advantages and disadvantages. *e-Gnosis*, 2010: **8**, 2.
15. Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. Basic local alignment search tool. *Journal of Molecular Biology, 1990;* **215**(3): 403–410.
16. Fernandes G.E., Valério H.G., Feltrin T., Van Der Sand S.T. Variability in the production of extracellular enzymes by entomopathogenic fungi grown on different substrates. *Brazilian Journal of Microbiology*, 2012; **43**(2): 827–833.
17. Florencio C., Couri S., Sanchez F.C. Correlation between agar plate screening and Solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains. *Enzyme Research;*2012: doi:10.1155/2012/793708.
18. Zhang S.B., Pei X.Q., Wu Z. Multiple amino acid substitutions significantly improve the thermostability of feruloyl esterase A from *Aspergillus niger*. *Bioresource Technology*, 2012; **117**: 140–147.
19. Bonnin E., Saulnier L., Brunel M., Marot C., Lesage-Meessen L., Asther M., Thibault J.F. Release of ferulic acid from agroindustrial by-products by the cell wall-degrading enzymes produced by *Aspergillus niger* I-1472. *Enzyme and Microbial Technology,* 2008; **31**(7): 1000–1005.
20. Koseki T., Fushinobu S., Shirakawan H., Komai M. Ocurrence, properties, and applications of feruloyl esterases. *Applied Microbiology and Biotechnology*, 2009; **84**(5), 803–810.
21. Cruz-Hernández M., Contreras-Esquivel J.C., Lara F., Rodríguez R., Aguilar C. Isolation and evaluation of tannin-degrading fungal strains from the Mexican Desert. *Zeitschrift für Naturforschung,* 2005; **60**(11–12): 844–848.
22. Kithsiri W.E., Turbyville J.T., Zhongge Z., Bigelow D., Pierson S., VanEtten H.D., Whitesell L., Louise M., Canfield L.M., Gunatilaka A.L. Cytotoxic constituents of *Aspergillus terreus* from the rhizosphere of *Opuntia versicolor* of the Sonoran Desert. *Journal of Natural Products*, 2003; **66**(12): 1567–1573.
23. Maheshwari R., Bharadwaj G., Bhat M. Thermophilic fungi: Their physiology and enzymes. *Microbiology and Molecular Biology Reviews*, 2000; **64**(3): 461–488.
24. Bisogno F., Mascoti L., Sanchez C., Garibotto F., Giannini F., Kurina-Sanz M., Enriz R. Structure-antifungal activity relationship of cinnamic acid derivatives. *J. Agricultural and Food Chemistry,* 2007: **55**(26): 10635–10640.
25. Ergün B.C., Çoban T., Onurdag F.K., Banoglu E. Synthesis, antioxidant and antimicrobial evaluation of simple aromatic esters of ferulic acid. *Archives of Pharmacal Research,* 2011; **34**(8): 1251–1261.
26. Khatkar A., Nanda A., Kumar P., Narasimhan B. Synthesis and antimicrobial evaluation of ferulic acid derivatives. *Research on Chemical Intermediates*, 2015; **41**(1): 299–309.
27. Crepin V.F., Faulds C.B., Connerton I.F. Functional classification of the microbial feruloyl esterase. *Applied Microbiology and Biotechnology,* 2004; **63**(6): 647–652.
28. Bouzid O., Record E., Asther M., Haon M., Navarro D., Asther M., Lesage-Meessen L. Exploration of members of *Aspergillus* sections Nigri, Flavi, and Terrei for feruloyl esterase production. *Canadian Journal of Microbiology,* 2006; **52**(9): 886–892.
29. Kumar C.G., Kamle A., Mongolla P., Joseph J. Parametric optimization of feruloylesterase production from *Aspergillus terreus* strain GA2 isolated from tropical agro-ecosystems cultivating sweet sorghum. *Journal of Microbiology and Biotechnology,* 2011; **21**(9): 947–953.
30. Kumar C.G., Kamle A., Kamal A. Purification and biochemical characterization of feruloyl esterases from *Aspergillus terreus* MTCC 11096. *Biotechnology Progress*, 2013; **29**(4): 924-932.

Capítulo II. Francisco Javier Bacame-Valenzuela, Ali Asaff¨, Elisa Miriam Valenzuela-Soto, Martin Esqueda, Jorge Alberto Rodriguez, and Juan Carlos Mateos-Diaz (2015) New feruloyl esterase type B of Aspergillus terreus B102 (AtFAE B) exhibits ability to synthesize hydroxycinnamic acid derivatives by transesterification reaction. Enviado a Enzyme and Microbial Technology.

# New feruloyl esterase type B of Aspergillus terreus B102 (AtFAE B) exhibits ability to synthesize hydroxycinnamic acid derivatives by transesterification reaction

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Highlights:

Type B feruloyl esterase (AtFAE B) from *Aspergillus terreus* B102 was first identified.

The feruloyl esterase AtFAE B exhibited the ability to catalyze the transesterification reaction for the production of phenol derivatives.

The enzyme AtFAE B showed potential as biotechnological tool due to its feruloyl esterase, chlorogenate esterase and transesterasa activity.

**Abstract**

Feruloyl esterases (FAE's, EC 3.1.1.73) hydrolyze ester bonds between phenolic acids and carbohydrates present in the plant cell wall. However, in the absence of water, FAE's are able to catalyze esterification and transesterification reactions. An enzyme with FAE activity (AtFAE B) was purified from secretome of *Aspergillus terreus* B102, a native strain isolated from Sonoran Desert cultivated by solid state fermentation on sugar cane bagasse and lime cooking liquor of corn. Based on a N-terminal amino acid sequence, substrate specificity and electrophoretic analyses, AtFAE I was identified as a feruloyl esterase type B (AtFAE B) of 57 kDa. AtFAE B present a profile of hydrolysis of methyl ferulate > methyl *p*-coumarate > methyl caffeate, and didn't show activity towards methyl sinapinate. Moreover AtFAE B showed chlorogenate esterase activity. The activity of AtFAE B was probed using a series of substrate *p*-nitrophenyl (C2 to C10), showing activity C2-C10. The ability to synthesize AtFAE B was verified by the production of phenol derivatives by a transesterification reaction using a ternary system isooctane /1-butanol / water. A yield of 5.8 % for butyl ferulate, 3 % butyl *p*-coumarate, 2 % butyl caffeate was obtained. Thus, AtFAE B can be used to catalyze the hydrolysis reaction as feruloyl esterase, chlorogenate esterase and catalysis of the transesterification reaction. Showing that *Aspergillus terreus* B102 is producing AtFAE B with capacity for synthesis, this result is extremely important since this type of FAE usually have no ability to synthesis.

**Key words**: feruloyl esterase, hydroxycinnamic acids, transesterification, methyl ester, butyl ester

## Introduction

Feruloyl esterases (FAE's) (EC 3.1.1.73) are critical enzymes involved in the complete degradation of lignocellulose, breaking the ester bonds between hydroxycinnamic acids and arabinoxylans or certain pectins present in plant cell walls [10, 45]. The first FAE was identified in *Streptomyces viridosporus*, later these enzymes were demonstrated to be produced by a wide range of microorganisms when cultivated on complex substrates such as xylan, pectin, wheat bran, or sugar beet pulp [7, 8, 21]. FAEs have been purified and characterized from several mesophiles *Streptomyces olivochromogenes*, *Pseudomonas fluorescens* subsp. cellulosa, an anaerobic fungus *Neocallimastix* strain MC-2, *Penicillium pinophilum*, and from several species of *Aspergillus* [3, 14, 15] and thermophiles such as *Schizophyllum commune*, *Clostridium stercorarium*, *Clostridium thermocellum*, and *Humicola insolens* [2, 9, 11, 25]. The microbial FAEs have been classified in four types (A, B, C and D) based on their specificity for methyl esters of hydroxycinnamic acids and their ability of releasing diferulic acid from complex substrates, this classification also considers the primary sequence of the enzymes [5]. Hydroxycinnamic acids *p*-coumaric ferulic, caffeic and sinapinic are of industrial interest mainly due to antioxidant properties. However these hydroxycinnamic acids have low solubility in hydrophobic phases limiting industrial application. A solution to this problem is the modification of these compounds by reaction of transesterification starting from a methyl ester and aliphatic alcohols resulting in a more lipophilic molecule. *Fusarium oxysporum* feruloyl esterase (FoFAE-I) catalyzes efficiently the transesterification of hydroxylated derivatives of cinnamic acid, such as *p*-coumaric and caffeic acid esters while the conversion rate is reduced in the case of methoxy

derivatives such as ferulic and especially, sinapinic acid esters in a ternary system hexane/1-butanol/water [39]. In particular the feruloil esterase A from *Aspergillus niger* (AnfaeA), a higher yield and initial rate was achieved in the case of methyl sinapinate, followed by methyl ferulate. The yield for the methyl *p*-coumarate was very low and there was no enzyme synthetic activity detected for MCA in *n*-hexane/1-butanol/water [41]. *Aspergillus terreus* strain MTCC11096 is reported as produced three feruloyl esterases named AtFAE-1, AtFAE-2 and AtFAE-3 with molecular weights of 74, 23 and 36 kDa respectively [22], AtFAE-1 belonged to type A while AtFAE-2 and AtFAE-3 were type C FAE. The ability of synthesis of these FAEs has not been reported. FAE's with the ability to catalyze the synthesis reactions are few, even more those that are classified as type B. Thus, the aim of this study was to demonstrate the production of a new type B feruloyl esterase from *Aspergillus terreus* B102 catalyzing the synthesis of hydroxycinnamic acid derivatives by a transesterification reaction in a ternary system isooctane/1-butanol/water.

## Methods

### Microorganism

The laboratory strain B102 of *Aspergillus terreus*, isolated from Sonoran Desert was used in the present investigation. The stock culture was maintained on potato dextrose agar at 4°C.

### AtFAE B production by solid State Fermentation

Production of *Aspergillus terreus* B102 Ferulic Acid Esterase type B (AtFAE B) by Solid State Fermentation (SSF) was performed using a solid medium containing liquor cooking corn (nejayote) and bagasse 2:1, and 65 % of formulated basal salt solution (4 g/L of urea, 5 g/L of $K_2HPO_4$, 1 g/L of $MgSO_4$) sterilized at 121 °C, 15 min. The culture media was inoculated with $1X10^7$ spore/ mL of spore suspension of 3 days grown old culture and incubated at 40 °C for 4 days. Fermented cultures were extracted with 4 mL MOPS 2.5 mM, pH 7.2 buffer for 1 g material by vortexing during 1 min and steeping for 10 minutes in ice bath. This mixture was centrifuged at 10000 x g, 4 °C, for 10 min and recovered supernatants containing secretomes were kept at 4 °C for further analyses.

### Purification

The enzymes were concentrated by precipitation with ethanol 90%, the supernatant was discarded and the resulting precipitate was centrifuged at 10000 xg for 5 min. The pellet was resuspended in MOPS 2.5 mM pH 5 buffer and separating the enzymes was with

10% resin diethylaminoethyl (DEAE) batch, equilibrated with MOPS 2.5 mM pH 5 buffer. The crude extract was pleasing to the DEAE resin, stirred and incubated on ice for 1 hour, FAE protein activity in the supernatant was recovered.

Electrophoresis and zymogram

Molecular mass was determined by SDS-PAGE 12% resolving gel and 4% stacking gel according to the Laemmli [29] method using low molecular weight standards (BIO RAD). Zymograms were performed in a native-PAGE, after the protein migrated is made two washes with distilled water for 30 minutes under stirring and then the pH of the gel buffer is adjusted with 2.5 mM MOPS buffer pH 7.2 with stirring. The bands revealed in 10 mL MOPS 2.5 mM pH 7.2 buffer containing 50 mM of methyl ferulate and 50 mg phenol red and incubated at 40 ° C for 5 minutes until the appearance of yellow bands.

Protein Sequencing

To determine the N-terminal sequence, the protein was first separated by means of the SDS-PAGE procedure using a 12% acrylamide gel and then electroblotted onto polyvinylidenedifluoride membrane (PVDF, Bio-Rad) with a Tris-borate buffer (Tris 50 mM, 50 mM borate, pH 8.3). Immediately, the membrane was colored with Ponceau

red; the band observed at 57 kDa was cut and subjected to analysis on an Applied Biosystem Model 476 A gas-phase sequencer.

## Methyl ester synthesis

The methyl esters (methyl ferulate, caffeate, p-coumarate and sinapinate) as FAE's substrates for hydrolysis and synthesis were chemically synthesized. 1g of ferulic acid, caffeic acid, *p*-coumaric acid or sinapinic acid was dissolved in 10 mL of anhydrous methanol. Then 5% hydrochloric acid was added to the reaction mixture and maintained under reflux for 24 hours at 65 °C. Synthesis was qualitatively monitored by TLC, using hexane: ethyl acetate 2: 1 (v/v). Each methyl ester was purified on a silica column packed with 60 Å Sigma Aldrich. Elution was with a gradient ethyl ether of 0-100 with petroleum ether. 5 mL fractions were collected and the purity was determined by TLC under the same conditions mentioned above.

## Substrate specificity

AtFAE B activity was measured by a spectrophotometric method using methyl ferulate (MFA), methyl *p*-coumarate (MpCA), methyl caffeate (MCA), chlorogenic acid (ACL) and different *p*-nitrophenyl esters (*p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl valerate (C5), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl decanoate (C10)). One volume of each substrate stock (50 mM) was dissolved in *tert*-butanol containing 5 mM of *p*-nitrophenyl (a pH indicator) and mixed with nine volumes of 2.5

mM MOPS (pH 7.2). 20 µl of each enzyme solution at an appropriate dilution in MOPS 2.5 mM (pH 7.2) were placed in each microplate well, and 100 µl of substrate was quickly added using an eight-channel pipette. Subsequently, the plate was positioned in the microplate reader and shaken for 5 s after each reading. The decrease in absorbance at 415 nm was recorded every 15 min at 30 °C [30]. The one unit corresponds to 1 umol of product released per minute in the assay conditions

Enzymatic synthesis of butyl esters

The enzymatic synthesis of butyl esters was carried out in a ternary system isooctane/1-butanol/water. The reaction mixture contained 50 mM of methyl ester, 75 mM 1-butanol and 20 µl of enzyme. The reaction was placed at 35 ° C for 72 hours and1000 rpm. A procedure for quantitative analysis of the butyl ester by high performance thin layer chromatography (HPTLC) is presented. 10 µl of sample and 0.1 micrograms per lane standard plating thin layer chromatography (TLC) was injected. The mobile phase used was hexane: ethyl acetate in a ratio of 2:1(v/v) to migrate TLC. The plates were analyzed by densitometry at 294 nm. The standards used were synthesized by chemically by modifying the protocol for methyl esters, changing the ethanol by butanol.

## Results

### Production of AtFAE B

The fungal *Aspergillus terreus* is part of the list of producers of FAE microorganisms as reported above for the strain *Aspergillus terreus* AG2 [23] and *Aspergillus terreus* strain B102 [1]. This last was used for this work and the secretome produced by *Aspergillus terreus* B102 after four days of fermentation were extracted with 2.5 mM MOPS buffer pH 7.2 in a ratio 1:4 (ferment: buffer w/v). After the fermentation, it was obtained with a total crude extract activity of 54.7 mU.

### Purification of AtFAE B

The crude extract was used as starting material in a two-step purification protocol. The supernatant presented a FAE activity of 9.2 mU/mg which was recovered and named AtFAE I. In the first step it was made a protein precipitation with 90% ethanol, obtaining a specific activity of 0.7219 mU/mg with methyl *p*-coumarate as substrate. In the second purification step the recovered pellet was resuspended in 2.5 mM MOPS pH 7.2 buffer to which subsequently 10 mL of DEAE resin was added for each 100 extract. The supernatant with FAE activity was recovered named as AtFAE I. A yield of 30% (Table 1) was obtained for AtFAE I.

Table 1. Summary of partial purification of FAE's from *Aspergillus terreus* B102.

| Purification Step | Total Protein (mg) | Total Activity (mU) | Specific Activity (mU/mg) | Recovery (%) | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 7370 | 54.7 | 0.0074 | 100 | 1 |
| Ethanol precipitation | 46.4 | 33.5 | 0.7219 | 61 | 97 |
| DEAE | 1.8 | 16.66 | 9.2 | 30 | 1247 |

Electrophoresis and zymogram

The purified AtFAE B was used to perform a zymogram using methyl ferulate as substrate (Figure 1). A single yellow band indicated the presence of only one FAES after purification. Through SDS-PAGE gel and using low molecular weight markers from Bio Rad was estimated a molecular weight of 57 kDa to AtFAE I approximately (Figure 1). These results differ with reported by Kumar et al. [22] which mentions that *Aspergillus terreus* MTCC 11096 produces three FAE's with molecular weights of 74, 36 and 23 kDa when grown maize bran for 7 days. Microbial FAE's show great variability in molecular weight, also have been reported both in monomeric and dimeric form as in the case of *Aspergillus niger* [21] Species of *Aspergillus* have been reported as producers of FAE's as, *A. niger* CBS 120.49 (AnFae A) 36 kDa, *A. niger* CBS 120.49 (FAE-I B) 63 kDa, A. *clavatus* (AcFAE), *A. falvus* (AfFae A), *A. awamori* (AwFae A) y *A. oryzae* (AoFae), *A. tubingensis* (AtFae A) [6, 12, 13, 19, 31, 40, 43, 48]. In the case of FAE's type B molecular weights of *Aspergillus nidulans* FGSC A4 (AnFae B) reported 56 kDa; *Aspergillus oryzae* (AoFAE B) 55 kDa; *Penicillium funiculosum* (PfFAE B) 53 kDa, similar AtFAE I like the results [18, 20, 31].

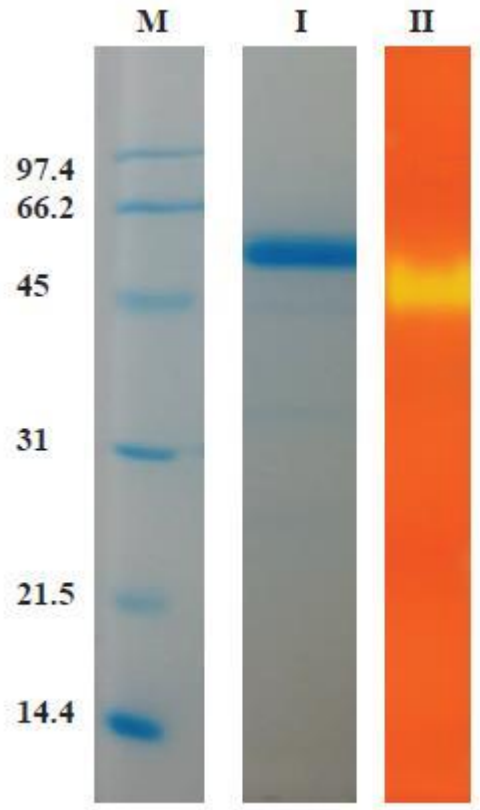


Figure 1. SDS-PAGE of partial purified FAEs from *A. terreus*. (M) molecular weight standards, (I) Atfae I, (II) native-PAGE zymogram of partial purified FAE from *A. terreus* using methyl ferulate.

## Substrate specificity

The substrate specificity of the AtFAE B was performed using methyl ferulate, methyl caffeate, methyl *p*-coumarate and methyl sinapinate. The hydrolysis profile of these substrates allowed us to confirm the AtFAE as type B according to Crepin et al. [5]. Type B FAEs show preference for hydroxycinnamic acids containing one or two hydroxyl substitutions, as found in *p*-coumaric and caffeic acids, respectively. AtFAE B presented activity with methyl ferulate > methyl *p*-coumarate > methyl caffeate, but not with methyl sinapinate (Figure 2). Kumar et al. [22] reports that *Aspergillus terreus* 11096 MTCC produces a type FAE type A and two FAE's type C of 74, 23 and 36 kDa respectively, Zhang et al [47] report an FAE type A from *Aspergillus terreus* CBE332.5 of 35 kDa, which differs from that found in this study to AtFAE B. This can be attributed to genetic diversity that exists in this microorganism as *Aspergillus terreus* 11096 MTCC was isolated agro-industrial waste from one region of India. Moreover *Aspergillus terreus* B102 for our study was isolated from the central region of the state of Sonora Mexico, a region that part of the Sonora desert area.

## Protein Sequencing

Identification of AtFAE I was performed using the method of EDMA, for which the corresponding band was transferred PVDF membrane. The EDMA analysis of the band corresponding to native PAGE AtFAE I show homology with feruloyl esterase of *Aspergillus terreus* NIH2624. The N-terminal amino acid sequence (QDAFEAKXHS) of this enzyme corresponds to FAE B-2 of *Aspergillus terreus* NIH2624 (Table 3), for that

reason if we consider the specificity for substrate AtFAE I and its primary sequence we can conclude that AtFAE B corresponds to a type B feruloyl esterase. Has been reported that some FAE's type B present a high sequence identity FAE's type C because conserved 13 amino acids in the vicinity of the active site such as FAE B of *Aspergillus nidulans* and *Aspergillus oryzae* [18, 31].

AtFAE B presented chlorogenate esterase activity of 3.3 U/mg (Figure 2), which allows to hydrolyze chlorogenic acid (an ester molecule composed for caffeic acid and quinic acid). Previously chlorogenate esterase activity for the enzymes produced by strains of *Aspergillus terreus* was not reported, and this report is presented for the first time chlorogenate esterase activity produced by *Aspergillus terreus* FAE's. Moreover it is extremely interesting one FAE with chlorogenate esterase activity, since AtFAE B can be attributed three different activities, feruloyl esterase, chlorogenate esterase and enzymatic transesterification as demonstrated in the following section.

To characterize thorough the hydrolytic activity of AtFAE B *Aspergillus terreus* B102 esters of *p*-nitrophenol were used as substrates. AtFAE B showed activity from C2 to C10, (Figure 3). Topakas et al. [37] reports that FoFae B and FoFae A *Fusarium oxysporum*, and StFae B and StFae C *Sporotrichum thermophile* showed activity with *p*-nitrophenyl esters, the maximum activity was shown by *p*-nitrophenyl propionate, except FoFae A which presented its maximum activity with C2, in contrast AtFAE B showed maximum activity with C10. These four enzymes showed a minimal preference for C4 and AtFAE B with C2. Other types of FAE's B (FoFaeB and StFaeB) showed no activity with *p*-nitrophenyl decanoate (C10) is highly hydrophobic which can cause an

expulsion of this compound from the vicinity of the active site, with the consequence that the catalyst is not produced [37]. On the other hand, the ratio between the hydrolysis of methyl ferulate and C2, the FAE type B showed a similar ratio FoFaeB 1.86 and StFaeB 1.64, in the case of the AtFAE B ratio is 1.28. Contrary to these results FAE type A showed a higher preference for *p*-nitrophenyl acetate, FoFaeA showed a ratio 0.12, indicative that it is more of a general plant cell wall-acting esterase [37]. Thus, we show that AtFAE B showed a greater ability to hydrolyze hydrophobic compounds. Showing a different hydrolysis profile with *p*-nitrophenyl esters as substrate compared with FAE's previously reported.

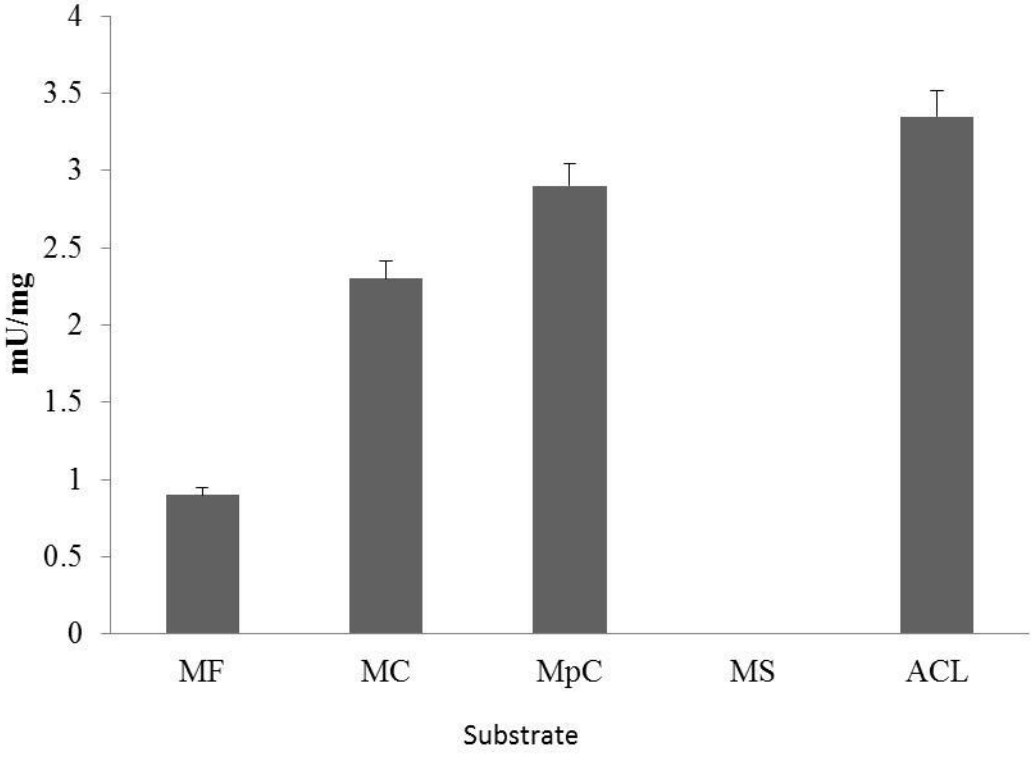


Figure 2. Feruloyl esterase activity with synthetic substrates for AtFAE I. (MF) methyl ferulate (MC), methyl caffeate (MpC), methyl *p*-coumarate (MS), methyl sinapate and (ACL) Chlorogenic esterase activity.

Table 2. Identification of AtFAE I from *Aspergillus terreus* B102.

| Enzyme | Peptide (N terminal) | Homologues | Primary sequence of FAE B from *Aspergillus terreus* NIH 2624 | kDa | Ident. (%) |
|---|---|---|---|---|---|
| AtFAE I | QDAFEAKXHS | FAE B | MAPIHYLLPIITLGSAALARQDAFEAKCHSFANKI<br>HLPNVHVNFASYVPGGTNLTLADNPSSCGATSQS<br>VSADVCRVAMAVATSNSSEITLEAWFPRNYTGRF<br>LSTGNGGLSGCIQYYDMAYTTGFGFATVGANNG<br>HNGTSGEPFYHHPEVLEDFAYRSIHTGVVIGKKLT<br>KMFYEEGFNKSYYLGCSTGGRQGFKSVQKYPND<br>FDGVVAGAPAFNFANLISWSAHFYPITGPPGSDTY<br>LSPAMWKVAHDEIIRQCDQIDGAKDGIIEDPSLCN<br>PIMETIICKPGASSDNCLSAAQAKTVREVLYPLYG<br>VNGTLLYPRMQPGSEVLAAPIMYNGQPFAYSTD<br>WYRYVVYNDPNWNGTTFDVQDAAAALAQNPYN<br>IQTWDADLTPFRKSGGKVLTYHGLQDQLISSENSK<br>LYYARVAETMGMPPEELDEFYRFFQISGMGHCGG<br>GDGAYGIGNGLATYSGKDPENNVLMAMVQWVE<br>KGIAPETVRGAKFANGPGSTVEYSRKHCRYPRRN<br>VFKGPGNYTDENAWECVV | 57.5 | 90 |

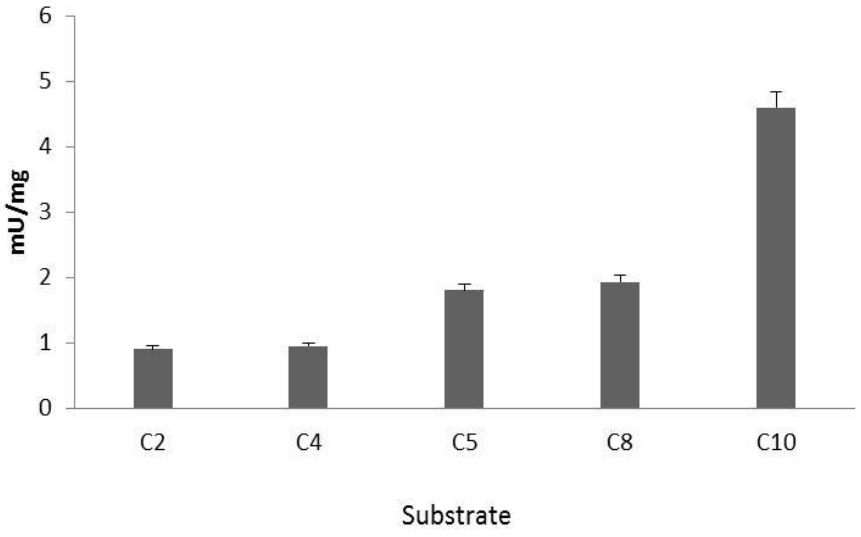


Figure 3. Specificity activity of AtFAE B towards *p*-nitrophenyl esters. *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl valerate (C5), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl decanoate (C10).

Butyl ester synthesis

The results of the synthesis of phenolic derivatives (figure 4) show how the reaction was affected by the nature of the methyl ester used. The percentage of conversion was achieved for the synthesis of butyl ferulate followed by butyl *p*-coumarate, butyl caffeate and butyl sinapinate (BF> BpC> BC). The hydrolysis profile shown by AtFAE B is similar to profile of synthesis for FAE type B, which coincides with that reported by other authors where it is mentioned that the hydrolysis profile is similar to profile synthesis. Using ternary systems in synthesis has been reported for other FAE's as the FAE type B of *Sporotrichum thermophile* showed a higher yield for the synthesis of BpC by a transesterification reaction in a ternary system composed of hexane/1-butanol/water [38]. This enzyme shows similarity between the profile hydrolysis (MpC > MC > MF) and profile synthesis (MpC >MC >MS) because does not show synthesis activity for sinapinic acid derivatives. The synthesis profile AtFAE B shows its versatility to catalyze transesterification reactions.

This is probably due to the origin of *Aspergillus terreus* B102 which was isolated Sonoran Desert adapted to survive in hydric stress due to the higher temperatures zone to 50 °C. This could be related to its versatility to catalyze transesterification reactions. This characteristic is shared with the lipase B from *Candida antarctica* (CAL B), this yeast was isolated from the Antarctic which is also adapted to survive in hydric stress caused on this occasion by the low temperatures in the area. This enzyme is produced by a microorganism psychrophilic, which gives it a high extreme flexibility on conditions of low water activity favoring the catalytic activity. In summary we can conclude that

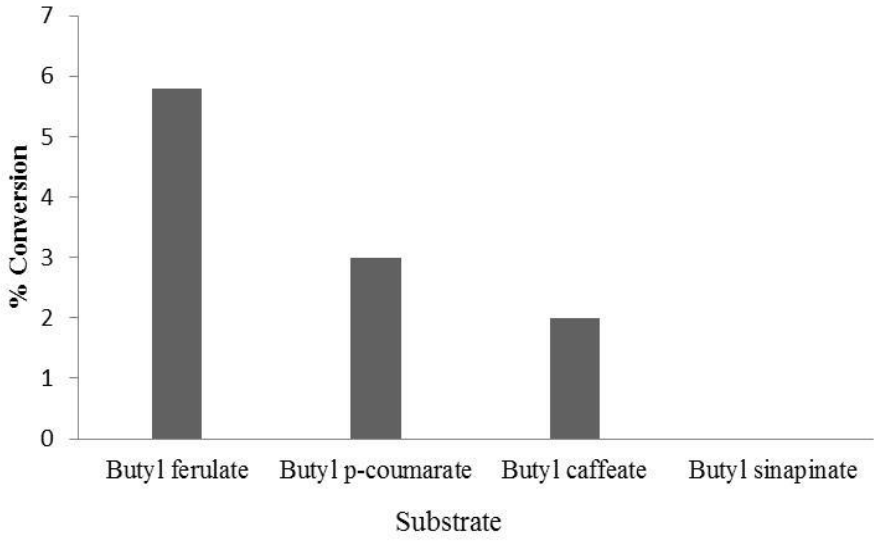


Figure 4. Enzymatic synthesis of butyl esters with AtFAE B by transesterification reaction in ternary system isooctane/1- butanol /water.

the enzymes isolated from microorganisms adapted to survive a water stress, may be used for the synthesis of compounds, especially the production of phenol derivatives by a transesterification reaction. The Cal B has been used to synthesize many compounds due to its ability to catalyze transesterification reactions, under different conditions in organic solvents as a medium of reaction [4, 24, 26, 27, 32, 33, 34, 35 36, 44, 46].

Yields above were reached after 48 hours for AtFAE B of reaction for the synthesis of 5.8 % BF, 3 % for BpC, 2 % for BC; in the case of the FAE type B *Sporotrichum thermophile* yields close to 10% were obtained in a time of 140 hours [38]. Moreover of FoFAE I of *Fusarium oxysporum* for BF showed a yield of about 12% at a time 140 hours in ternary system hexane/butanol/water [39].

Other FAE's have been reported as synthetic enzymes for phenol derivatives as the case of *Aspergillus niger* AnFae A, which was used for the production of pentyl ferulate glyceryl ferulate and butyl hidroxicinnates [27,2 8, 17, 41]. In specific is reported to AnFae A yield of 78% for MS followed BF with 42%, 2% for BpC and no activity was detected in BC after 120 hours of reaction [41]. For StFaeC of *Sporotrichum thermophile* is reported capacity for synthesis of phenol derivatives such as D-arabinose feruloyl [42]. Considering the results presented for AtFAE B show that has the ability to synthesize phenol derivatives, with conversion yields for the synthesis of derivatives of the hydroxycinnamic acids are significantly higher than those reported for commercial lipases and other FAE's with ability to synthesis.

Moreover FAE's type B reported as AnFAE B of *Aspergillus nidulans*, NcFAE B of *Neurospora crassa*, Aofae B of *Aspergillus oryzae* and FAE B *Sporotricum thermophile* and *Penicillium funiculosum* have not been reported with synthesis capacity in the production of phenolic derivatives. Therefore the importance of reporting AtFAE B with the ability to synthesize phenol derivatives of industrial interest. Thus, in our working group is continuing with the optimization in the transesterification reaction in ternary systems AtFAE B.

## Conclusions

In conclusion, in this study the production of feruloyl esterase from *Aspergillus terreus* B102 (AtFAE I) was demonstrated, which present a profile of FAE type B (AtFAE B). For this reason this report is the first to show the production of a FAE type B by a strain of *Aspergillus terreus*. This FAE could be an alternative for use as a biotechnological tool feruloyl esterase, esterase chlorogenate and enzymatic transesterification activity. The hydrolysis profile AtFAE B using *p*-nitrophenyl esters as substrate showed that this enzyme has the ability to hydrolyze highly hydrophobic substrates such as C10. This characteristic shows that AtFAE B is a new FAE from *Aspergillus terreus*. Currently in the research group continues to work with AtFAE B to find their biochemical properties and kinetic parameters. In addition, studies are continued to further demonstrate the versatility of the enzyme by synthetic reactions with more complex substrates in order to expand the synthetic ability of AtFAE B.

**References**

[1] Bacame-Valenzuela FJ, Rodriguez JA, Mateos-Díaz JC, Kirchmayr M, Valenzuela-Soto EM, Reyes-Vidal MY, Esqueda M, Asaff A, Screening of Sonoran Desert Fungal Strains for feruloyl Esterase Activity. J Pure App Microbial. 9 (2015) 131-138.

[2] Blum DL, Kataeva IA, Li XL, Ljungdahl LG, Feruloyl esterase activity of the Clostridium thermocellum cellulosome can be attributed to previously unknown domains of XynY and XynZ. J Bacteriol. 182 (2000) 1346-1351.

[3] Borneman WS, Ljungdahl LG, Hartley RD, Akin DE, Purification and partial characterization of two feruloyl esterases from the anaerobic fungus Neocallimastix strain MC-2. Appl Environ Microbiol. 58 (1992) 3762-3766.

[4] Choo WS, Birch EJ, Stewart I, Radical scavenging activity of lipophilized products from transesterification of flaxseed oil with cinnamic acid or ferulic acid. Lipids. 44 (2009) 807-815.

[5] Crepin VF, Faulds CB, Connerton IF, Functional classification of the microbial feruloyl esterases. Appl. Microbiol. Biotechnol. 63 (2004) 647-652.

[6] Damásio RL, Pinto CM, Brenelli LB, Citadini AP, Mandelli F, Cota J, Ferreira AF, Salvador VH, Alavaredo DA, Segato F, Zerlotti MA, Oliveira NM, Dantas SW, Squina

FM (2012) Biomass-to-bio-products application of feruloyl esterase from Aspergillus clavatus. Appl Microbiol Biotechnol. (2012) DOI 10.1007/s00253-012-4548-4.

[7] Deobald LA, Crawford DL, Activities of cellulase and other extracellular enzymes during lignin solubilisation by Streptomyces viridosporus. Appl Microbiol Biotechnol. 26 (1987) 158-163.

[8] Donaghy J, McKay AM, Purification and characterization of a feruloyl esterase from the fungus, Penicillium expansum. J Appl Microbiol. 83 (1997) 718-726.

[9] Donaghy JA, Bronnenmeier K, Soto-Kelly PF, McKay AM, Purification and characterization of an extracellular feruloyl esterase from the thermophilic anaerobe Clostridium stercorarium. J Appl Microbiol. 88 (2000) 458-466.

[10] Faulds CB, What can feruloyl esterases do for us?. Phytochem. Rev. 9 (2010) 121-132.

[11] Faulds CB, Sancho AI, Bartolome B, Mono- and dimeric ferulic acid release from brewers' spent grain by fungal feruloyl esterases. Appl Microbiol Biotechnol. 60 (2002) 489-493.

[12] Faulds CB, De Vries RP, Kroon PA, Visser J, Williamson G. Influence of ferulic acid on the production of feruloyl esterases by Aspergillus niger. FEMS Microbiol Lett. 157 (1997) 239-244.

[13] Faulds CB, Williamson G, Ferulic acid esterase from Aspergillus niger: purification and partial characterization of two forms from a commercial source of pectinase. Biotechnol Appl Biochem. 17 (1993) 349-359.

[14] Faulds CB, Williamson G, The purification and characterization of ferulic acid esterase from Streptomyces olivochromogenes. J Gen Microbiol. 137 (1991) 2339-2345.

[15] Ferreira MA, Wood TM, Williamson G, Faulds CB, Hazlewood GP, Gilbert HJ, A modular esterase from Pseudomonas fluorescens subsp. cellulosa contains a non-catalytic cellulose binding domain. Biochem J. 294 (1993) 349-355.

[16] Ghatora SK, Chadha BS, Saini HS, Mahalingeshwara K. Bhat b, Craig B. Faulds Diversity of plant cell wall esterases in thermophilic and thermotolerant fungi. J Biotechnol. 125 (2006) 434-445

[17] Giuliani S, Piana C, Setti L, Hochkoeppler A, Pifferi PG, Williamson G, Faulds CB, Synthesis of pentylferulate by a feruloyl esterase from Aspergillus niger using water-in-oil microemulsions. Biotechnol Lett. 23 (2001) 325-330.

[18] Koseki T, Hori A, Seki S, Murayama T, Shiono Y, Characterization of two distinct feruloyl esterases, AoFaeB and AoFaeC, from Aspergillus oryzae. Appl Microbiol Biotechnol. 83(2009) 689-696.

[19] Koseki T, Takahashi K, Fushinobu S, Iefuji H, Iwano K, Hashizume K, Matsuzawa H, Mutational analysis of a feruloyl esterase from Aspergillus awamori involved in substrate discrimination and pH dependence. Biochim Biophys Acta. 1722 (2005) 200-208.

[20] Kroon PA, Williamson G, Fish NM, Archer DB, Belshaw NJ, A modular esterase from Penicillium funiculosum which releases ferulic acid from plant cell walls and binds crystalline cellulose contains a carbohydrate binding module Eur. J. Biochem. 267 (2000) 6740-6752.

[21] Kroon PA, Faulds CB, Williamson G, Purification and characterization of a novel esterase induced by growth of Aspergillus niger on sugar-beet pulp. Biotechnol Appl Biochem. 23 (1996) 255-262.

[22] Kumar CG, Kamle A, Kamal A, Purification and biochemical characterization of feruloyl esterases from Aspergillus terreus MTCC 11096. Biotechnol. Progr. 29 (2013) 924-932.

[23] Kumar CG, Avijeet K, Poornima M, Joveeta J, Parametric Optimization of Feruloyl Esterase Production from Aspergillus terreus Strain GA2 Isolated from Tropical Agro-Ecosystems Cultivating Sweet Sorghum. J. Microbiol. Biotechnol. 21(2011) 947-953.

[24] Lee GH, Widjaja A, Ju YH, Enzymatic synthesis of acid derivatives. Biotechnol. Lett. 28 (2006) 581-585

[25] MacKenzie CR, Bilous D, Ferulic acid esterase activity from Schizophyllum commune. Appl Environ Microbiol. 54 (1998) 1170-1173.

[26] Malhotra D, Mukherjee J, Gupa MN, Lipase catalyzed transesterification of castor oil by straight chain higher alcohol. J Biosci Bioeng. 119 (2015) 280-283.

[27] Matsuo T, Kobayashi T, Kimura Y, Hosoda A, Taniguchi H, Adachi S, Continuous synthesis of glyceyl ferulate using immobilized Candida Antarctica lipase. J Oleo Sci. 7(2008) 375-380.

[28] Matsuo T, Kobayashi T, Kimura Y, Tsuchiyama M, Oh T, Sakamoto T, Adachi S, Synthesis of glyceryl ferulate by immobilized ferulic acid esterase. Biotechnol Lett. 30 (2008b) 2151-2156.

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

[30] Ramirez L, Arrizon J, Sandoval G, Cardador A, Bello-Mendoza R, Lappe P, Mateos-Diaz JC, A new microplate screening method for the simultaneous activity quantification of feruloil esterases, tananases, and chlorogenate esterases. Appl Biochem Biotechnol. 151 (2008) 711-723.

[31] Shin HD, Chen RR, A type B feruloyl esterase from Aspergillus nidulans with broad pH applicability. Appl Microbiol Biotechnol. 73 (2007) 1323-1330.

[32] Sun S, Zhou W, Enhanced enzymatic preparation of lipophilic feruloylated lipids using distearin as feruloyl acceptors: optimization by response surface methodology. J Oleo Sci. 63 (2014) 1011-1018.

[33] Sun S, Yang G, Bi Y, Xiao F, Chemoenzymatic synthesis of feruloylated monoacyl- and diacyl-glycerols in ionic liquids. Biotechnol. Lett. 31(2009) 1885-1889.

[34] Sun S, Shan L, Liu Y, Jin Q, Wang X, Wang Z, A novel, two consecutive enzyme synthesis of feruloylated monoacyl- and diacyl-glycerolsin a solvent-free system. Biotechnol. Lett. 29 (2007) 1947-1950.

[35] Sun S, Shan L, Jin Q, Liu Y, Wang X, Solvent-free synthesis of glyceryl ferulate using a commercial microbial lipase. Biotechnol. Lett. 29 (2007b) 945-949.

[36] Tomke PD, Rathod VK, Ultrasound assisted lipase catalyzed synthesis of cinnamyl acetate via transesterification reaction in a solvent free medium. Ultrasonics Sonochem. 27 (2015) 241-246.

[37] Topakas E, Christakopoulos P, Faulds C.B (2005) Comparison of mesophilic and thermophilic feruloyl esterases: characterization of their substrate specificity for methyl phenylalkanoates. J Biotechnol. 115 (2005) 355-366.

[38] Topakas E, Stamatis H, Biely P, Christakopoulos P, Purification and characterization of a type B feruloyl esterase (StFAE A) from the thermophilic fungus Sporotrichum thermophile. App Microbiol Biotechnol. 63 (2004) 686-690.

[39] Topakas E, Stamatis H, Biely P, Kekos D, Macris BJ, Christakopoulos P, Purification and characterization of a feruloyl esterase from Fusarium oxysporum catalyzing esterification of phenolic acids in ternary water-organic solvent mixtures. J Biotechnol. 102 (2003) 33-44.

[40] Udatha D, Mapelli V, Panagiotou G, Olsson L, Common and Distant Structural Characteristics of Feruloyl Esterase Families from Aspergillus oryzae. PLOS ONE 7 (2012) e39473. doi:10.1371/journal.pone.0039473.

[41] Vafiadi C, Topakas E, Apostolos A, Faulds CB, Christakopoulos P, Enzymatic synthesis of butyl hydroxycinnamates and their inhibitory effects on LDL-oxidation. J Biotechnol. 133 (2008) 497-504.

[42] Vafiadi C, Topakas E, Alderwick LJ, Besra GS, Christakopoulos P, Chemoenzymatic synthesis of feruloyl D-arabinose as a potential anti-mycobacterial agent. Biotechnol Lett. 29 (2007) 1771-1774.

[43] de Vries RP, Michelsen B, Poulsen CH, The faea genes from *Aspergillus niger* and *Aspergillus tubingensis* encode ferulic acid esterases involved in the degradation of complex cell wall polysaccharides. Appl Environ Microbiol. 63 (1997) 4638-4644.

[44] Wang Y, Cao X, Enzymatic synthesis of fatty acid ethyl esters by utilizing camellia oil soapstocks and diethyl carbonate. Bioresource technol. 102 (2011) 10173-10179.

[45] Wong DW, Feruloyl esterase. Appl. Biochem. Biotechnol. 133 (2006) 87-112.

[46] Yoshida Y, Kimura Y, Kadota M, Tsuno T, Adachi S, Continuos synthesis of alkyl ferulate by immobilized Candida antarctica lipase at high temperature. Biotechnol Lett. 28 (2006) 1471-1474.

[47] Zhang SB, Wang L, Liu Y, Zhai HC, Cai JP, Hu YS, Expression of feruloyl esterase A and its application biomass degradation. Protein Expres Purif. (2015) Doi.org/10.1016/j.pep.2015.08.015.

[48] Zhang SB, Pei XQ, Wu ZL, Multiple amino acid substitutions significantly improve the thermostability of feruloyl esterase A from Aspergillus niger. Bioresour Technol. 117 (2012) 140-147.