UNIVERSIDAD AUTÓNOMA CHAPINGO

DEPARTAMENTO DE ENSEÑANZA, INVESTIGACIÓN Y SERVICIO EN ZOOTECNIA

POSGRADO EN PRODUCCIÓN ANIMAL

"Enseñar la Explotación de la Tierra No la del Hombre"

ACTIVIDAD ENZIMÁTICA Y CULTIVO DE HONGOS DE LA PUDRICIÓN BLANCA POR FERMENTACIÓN EN ESTADO SÓLIDO EN RASTROJO DE MAÍZ

TESIS

Que como requisito parcial
para obtener el grado de:

DOCTOR EN CIENCIAS EN INNOVACIÓN GANADERA

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Chapingo

Mayo de 2015

Chapingo, estado de México

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DEDICATORIAS

A mi familia:

A mis padres Pedro y Aurelia

A mis hijos: José Luis y Sofía Isabel

A mi esposo José Luis

A mis hermanos Clementina, Ramón, José y Luciano

AGRADECIMIENTOS

Al CONACyT por permitirme realizar mis estudios de doctorado otorgándome una beca.

Al Posgrado en Producción Animal, del Departamento de Zootecnia de la Universidad Autónoma Chapingo, por permitirme realizar mis estudios de doctorado.

A todo el personal académico y administrativo del Departamento de Zootecnia, especialmente al Dr. Sergio Iban Mendoza Pedroza y al Ing. Miguel Marín.

A la Universidad Autónoma de Aguascalientes, por permitirme realizar mi estancia, especialmente al doctor Juan Jáuregui Rincón, por su gran disposición y su calidad humana. Al doctor Juan Antonio Álvarez por todas sus asesorías. Al doctor Fidel Guevara Lara, por su apoyo y sus recomendaciones

A mi comité asesor, al doctor Luis Alberto Miranda Romero, por dirigir esta tesis y por toda la ayuda y asesorías brindadas, además de su trato amable y cordial.

A mis Asesores, el doctor Maximino Huerta Bravo, y el doctor Octavio Loera Corral, por todas sus aportaciones y correcciones para la elaboración de esta tesis.

Al doctor Alejandro Azaola Espinosa, por su trato siempre amable y por su calidad humana.

Al doctor Agustín Ruiz Flores, por su gran ayuda al revisar los documentos en inglés que se presentan en esta tesis.

A la doctora Ema de Jesús Maldonado Simán por su gran empatía y apoyo.

A la M.C. Deli Nazmín Tirado González, por su valiosa aportación en el primer capítulo de esta tesis.

A las Ingenieras Tania Alonso Meneses y Karla Ivonne Alejandro Gómez por su gran ayuda en la parte de laboratorio, sin saberlo, llegaron en un momento crucial.

A todos los docentes y compañeros del Posgrado en Producción Animal, que con su ejemplo y dedicación participaron directa o indirectamente en la realización de esta tesis.

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RESUMEN

La biomasa lignocelulósica (paja de trigo, rastrojo de maíz, bagazo de caña, etc.) se ha utilizado como alimento para rumiantes, sin embargo, debido a un alto contenido de lignina, tiene una digestibilidad baja. Para mejorar la calidad de las pajas, se ha investigado el uso de hongos de la podredumbre blanca y sus extractos de enzimas para mejorar su valor nutritivo. Se evaluó la producción de enzimas (celulasas y xilanasas) de hongos de la pudrición blanca, cultivados por fermentación en estado sólido en sustratos de rastrojo de maíz con y sin mazorca. En el primer capítulo de esta tesis, se presenta una revisión de literatura sobre el efecto potencial de la correcta suplementación de extractos enzimáticos en dietas para rumiantes, así como su protección enzimática como una alternativa para incrementar su estabilidad y actividad dentro del rumen. En el segundo capítulo se describe el crecimiento de hongos de la podredumbre blanca, la obtención de un extracto enzimático por fermentación en estado sólido en dos sustratos de rastrojo de maíz con el hongo *Trametes trogii*, su actividad enzimática y la concentración del extracto enzimático a distintas temperaturas. No hubo efecto de sustrato para la producción de enzimas ni para concentración de la enzima. En el tercer capítulo, se describe la obtención de un extracto enzimático por fermentación en estado sólido en dos sustratos de rastrojo de maíz con el hongo *Fomes* sp EUM1, su actividad enzimática, producción de gas *in vitro* y composición química del residuo, producto de la fermentación. No se observó efecto de sustrato para ninguna de las enzimas analizadas. La mayor cantidad de volumen fraccional de gas coincidió con los mismos días de mayor actividad enzimática. Los sustratos fueron modificados en su composición química a través del tiempo de fermentación.

Palabras clave: Lignocelulosa; enzimas fúngicas; degradación.

ABSTRACT

Lignocellulosic biomass (such as rice straw, maize stover and sugar cane bagasse) has long been used as a ruminant feed, yet due to a high content of lignin, which inhibits the fermentation process by rumen microbes, has a low digestibility. To improve the quality of straws, different methods have been investigated including the use of white rot fungi and their enzyme extracts to improve the nutritive value of straws. The objective of this work was to evaluate enzyme production (cellulases and xylanases) of white rot fungi grown by solid state fermentation in corn stover substrates (with and without ear of corn). In the first chapter of this thesis, a review about the potential effect of the correct supplementation of enzyme extracts in diets formulated for ruminants, and the enzymatic protection as an alternative to enhance its stability and its hydrolytic capacity inside the rumen is presented. In the second chapter, white rot fungi growth and enzymatic extract obtaining by solid-state fermentation on two corn stover substrates by *Trametes trogii* and enzymatic extract concentration at different temperatures are described. No effects were found for both substrates and concentration temperatures. In the third chapter, the obtainment and enzymatic activity of an enzymatic extract by solid-state fermentation on two-corn stover substrates with the fungus *Fomes* sp EUM1 are described; besides, *in vitro* gas production and chemical composition of the residue were determined. There was no effect of substrate for any of the analyzed enzymes. Most fractional gas volume coincided with higher enzymatic activity. Furthermore, the substrates were modified in chemical composition over time of fermentation.

Keywords: Lignocellulose; Fungic enzymes; degradation.

1. INTRODUCCIÓN GENERAL

Debido a la creciente demanda de carne, leche y sus derivados, se espera que en los siguientes años esta tendencia siga en aumento, incrementando también la demanda de alimentos para animales (Boland *et al*., 2013). Por tal motivo, es necesario que los sistemas de producción animal funcionen de manera eficiente para abastecer el mercado, debiendo tener en cuenta que la dieta animal no represente una competencia para la nutrición humana en el uso de ingredientes (Kuijk *et al*., 2015).

La biomasa lignocelulósica (como las pajas, rastrojo de maíz, bagazo de caña de azúcar, entre otros) es uno de los desperdicios más abundantes en el mundo, compuesta de paredes celulares de plantas que contienen lignina, celulosa, y hemicelulosa, es una potencial fuente de energía. Sin embargo, la lignina en la lignocelulosa, representa el mayor obstáculo para la efectiva utilización de la celulosa y la hemicelulosa en el rumen (Chaturvedi y Verma, 2013). Además, debido a la estructura cristalina que adquiere el complejo de la ligninocelulosa (Singh y Chen, 2008), la digestibilidad es baja, siendo digestible menos del 58% de la celulosa de los forrajes. La digestibilidad individual de estos componentes celulares es muy variable (Giraldo *et al*., 2007) y su proporción en los alimentos modifica la eficiencia de su utilización por el consorcio microbiano fibrolítico dentro del rumen. Por ejemplo, el contenido de Fibra Detergente Neutro (FDN), que corresponde a paredes celulares, de forrajes fibrosos va de 60.2% a 77.6% (Murphy *et al*., 2012) y su digestibilidad varía de 30% a 55% (Facchini *et al*., 2011).

Se han utilizado diversos métodos para mejorar la calidad de la lignocelulosa, incluyendo métodos físicos, químicos y biológicos. Los métodos físicos como el molido, el tratamiento con vapor y el peletizado, han probado tener poco efecto

en la digestibilidad de la lignocelulosa (Liu *et al*., 1999). Los tratamientos químicos por otro lado, han mostrado que mejoran la digestibilidad cuando se utiliza NaOH y NH_3 (Sundstol y Owen, 1984). Sin embargo, la aplicación de estos químicos, especialmente el NaOH presenta un alto riesgo para el ambiente. Los métodos biológicos incluyen el uso de hongos de la pudrición blanca y sus extractos enzimáticos para mejorar el valor nutritivo de la lignocelulosa, los cuales son económicos y amigables con el ambiente (Rodrigues *et al*., 2008; Shrivastava *et al*., 2011; Arora *et al*., 2011).

Se han utilizado diversas cepas de hongos como *Trichoderma reesei*, *Phanerochaete chrysosporium*, *Aspergillus*, *Bacillus*, *Trametes versicolor*, *Bjerkandera adusta*, *Pleurotus ostreatus* y *Fomes fomentarius* (Pérez *et al*., 2002; Ovando-Chacón y Waliszewski, 2005; Rodrigues *et al*., 2008) para la obtención de extractos enzimáticos en la degradación de materiales fibrosos. Las enzimas contenidas en estos extractos son capaces de degradar las paredes celulares y liberar su contenido en forma de azúcares disponibles. La importancia de las enzimas fibrolíticas radica en su capacidad de degradar fracciones de la pared celular, las cuales varían en función de la especie y estado fenológico del forraje (Singh y Chen, 2008). Existen evidencias de que estas enzimas son capaces de mejorar las variables de calidad para producción de carne y leche (Beauchemin y Holtshausen, 2010). Por ejemplo, la adición de enzimas fibrolíticas de diferentes hongos de la pudrición blanca a paja de trigo, ha resultado en un incremento en la degradación *in vitro* de la FDN de hasta 13% (Rodrigues *et al*., 2008). Por otro lado, debido a la variabilidad del inóculo ruminal, de los alimentos y los preparados enzimáticos (Beauchemin *et al*., 2003), el método de almacenamiento de las enzimas, el lote de fabricación y otros factores ambientales, los resultados de experimentos con preparados de celulasas y xilanasas en rumiantes han sido muy variables (Tirado-Estrada *et al*., 2011). Debido a esto, el desarrollo y la aplicación de tecnologías que puedan ayudar a mantener e incrementar la actividad enzimática, será un tema preponderante en un futuro, aunado a la suplementación de productos que permitan una mayor eficiencia en la alimentación animal (probióticos, antioxidantes, ácidos grasos,

minerales, vitaminas, entre otros). De esta manera, la aplicación de tecnologías relacionadas con la utilización del complejo de la lignocelulosa, los hongos ligninolíticos y las enzimas que estos producen en el sector agropecuario e industrial para la obtención de biocombustibles, será un tema de interés en el futuro cercano.

Parte de la investigación que se realizará en un futuro en el área podría consistir en identificar las condiciones y dosis óptimas de las enzimas fibrolíticas exógenas (Eun y Beauchemin, 2007; 2008), para utilizar productos enzimáticos mucho más estables y de mayor actividad en las condiciones particulares en el interior del rumen (Eun y Beauchemin, 2007). Los cambios posibles relacionados con la aplicación de las enzimas fibrolíticas, están relacionados con el uso de nuevas tecnologías que permitan obtener enzimas más estables y con una alta actividad.

1.1. Hipótesis

Las enzimas producidas por los hongos ligninolíticos pueden variar en su cantidad y proporción, dependiendo del tipo de sustrato en el que se cultivan.

La adición de mazorca al rastrojo de maíz puede tener un efecto positivo en el crecimiento de hongos y la producción de enzimas fibrolíticas.

1.2. Objetivos

- Evaluar la producción de enzimas de hongos de la pudrición blanca por fermentación en estado sólido en dos sustratos de rastrojo de maíz (con y sin mazorca).

- Evaluar la producción de gas y composición química de los residuos obtenidos a partir de una fermentación sólida en dos sustratos con un hongo ligninolítico

1.3. Estructura de la tesis

En el primer capítulo de esta tesis, se presenta una revisión de literatura sobre el efecto potencial de la correcta suplementación de extractos de enzimas fúngicas en dietas para rumiantes, así como su protección enzimática como una alternativa para incrementar su estabilidad y capacidad hidrolítica dentro del rumen.

En el segundo capítulo se describe el crecimiento de hongos de la podredumbre blanca, la obtención de un extracto enzimático por fermentación en estado sólido en rastrojo de maíz del hongo *Trametes trogii*, su actividad enzimática y la concentración del extracto enzimático.

Por último, en el tercer capítulo, se describe la obtención de un extracto enzimático por fermentación en estado sólido en rastrojo de maíz del hongo *Fomes* sp EUM1, su actividad enzimática, además de las características de producción de gas *in vitro* y composición química del residuo producto de la fermentación.

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FIBROLYTIC ACTIVITY OF EXOGENOUS ENZYMES IN RUMINANTS AND THEIR PROTECTION: A REVIEW

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Submitted to *Journal of Microbiology and Biotechnology*

Fibrolytic activity of exogenous enzymes in ruminants and their protection: A review

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1.1. Abstract

The aim of this review was to analyze the potential effect of the correct supplementation of fungal enzymes extracts in diets formulated for ruminants, and the enzymatic protection as an alternative to enhance its stability and its hydrolytic capacity inside the rumen. The cell wall is composed of lignocellulose, it is quantified as the fraction corresponding to the Neutral Detergent Fiber (NDF). Depending of the media used and the conditions of the culture, white rot fungi produce different proportions of enzyme isoforms that allow them to use the energy of NDF. The use of white rot fungal enzymes in ruminant diets could increase the degradability of NDF. This effect is related to a better passage rate, dry matter intake (DMI), and productive animal behavior. The proper dosage of enzyme preparations to improve degradability of dry matter (DM) and NDF depends on the activity of cellulase and xylanase enzymes included, because they act synergistically. However, enzymatic activity depends on the structure and stability of the products inside the rumen. Immobilization can protect enzymes from inactivation by factors that can inhibit enzyme activity. In industrial processes, enzyme immobilization includes the use of proteins, silica nanoparticles and organic polymers. The only enzymatic protection studied in ruminant nutrition is the use of surfactants, which protects proteins from proteolysis. This area represents a scope about the opportunities to improve the activity of enzymes through developing their stability in the rumen.

Key Words: cell wall, fungal enzymes, ruminants, NDF degradability, enzymatic protection.

1.2. Introduction

The plant cell wall is composed of cellulose, hemicellulose, pectin, lignin and minerals, their proportions vary depending on the species and growth stage of forages. The individual digestibility of these cellular components is very variable and their proportions alter the efficiency of its use by the fibrolytic rumen microbial consortium. For example, NDF content of temperate forages, such as alfalfa, varies from 60.2% to 77.6% and its digestibility ranges from 30% to 55% [32]. It seems that the low digestibility rate is caused by the hydrolysis-resistance of the crystalline structure of the lignocellulose complex, which inhibits the availability of the energy contained in the cell wall [19].

However, in desert areas, the proportion of green temperate forages used as a source of fiber, ranges from 10% to 20%, in these regions the straws are the most common ingredient in ruminant diets. These straws have poor digestibility, because of the high amount of lignified components [19]. Due to poor digestibility of the straws, cellulase and xylanase enzymes, produced by ligninolytic fungi, have been used to improve the availability of sugars from the cell wall [10]. This process, based on enzymatic pre-digestion of the cell wall, has been widely used in the food, pulp and paper, textile, and biofuels industries [55].

In these cases, the removal of lignin and/or hemicellulose is the first step to expose the cellulose to the attack by cellulolytic enzymes to release glucose, due to the hydrolysis of the polysaccharide fraction NDF to fermentable sugars. In ruminants, the use of exogenous fibrolytic enzymes could improve growth and milk production [11]. However, the results of experiments with preparations of cellulases and xylanases in ruminants are highly variable [10], due to the ruminal inoculum, diets and enzyme preparations, the method of storing enzymes, batch, and other environmental factors. Part of the research conducted has focused in determine the conditions and optimal doses of exogenous fibrolytic enzymes, by the use of stable, highly active enzyme products in particular conditions inside the rumen [26], and the application of technologies that could help to maintain and increase enzyme activity [25]. This review examines the factors affecting the activity of exogenous enzymes within the rumen. It is also identified the enzymatic

protection as an alternative to enhance their effect on the use of forage NDF in ruminant nutrition.

1.3. Cell walls

The plant cell components have two fractions, the cellular content and the cell walls. The cellular content is the non-lignified material soluble in neutral detergent, composed of lipids, sugars, organic acids, non-protein nitrogen, soluble protein and pectin, with high energetic availability. The insoluble fraction or the neutral detergent fiber (NDF), corresponds to the cell wall of plants, consisting of lignocellulose, a rigid structure formed by polymers of cellulose, hemicellulose and lignin, which constitutes 30% to 70% of the dry matter of the forages, they also are the major reservoirs of carbon fixed in nature. Cell walls have three distinct morphological layers: 1) primary wall, composed by 9% to 25% cellulose, 20% to 50% hemicellulose, 10% to 35% of pectic substances, and 10% of proteins; 2) pectic substances which stick adjacent cells to form a middle layer; and 3) secondary wall, consisting of mature xylem tissues that support the plant, composed by 41% to 45% cellulose, 30% hemicellulose, and 22% to 28% lignin.

Cellulose makes up to about 50% of the planet's biomass, its molecule is composed of glucose monomers (50 to 15,000) linked by β-glucosidic bonds; that give rise to parallel linear chains linked by electrostatic attraction and Van der Waals forces, which give stability and resistance to chemical attack. The cellulose chains are arranged in a highly dense fiber package. It is set in parallel bundles, which surround the cell constantly making cross layers; which group themselves around a polymer matrix of hemicellulose, pectin and extensin.

The hemicellulose is an amorphous heteropolymer structure easy to hydrolyze, it represents 35% of the total dry weight of some plants. It consists of short branched chains of pentoses (D-xylose and L-arabinose), hexoses (D-galactose, D-mannose and D-glucose) and uronic acid. The two most abundant groups of the hemicellulose are the xylans and glucomannans. Besides, the hemicellulose is associated with cellulose microfibers [49] and joins the lignin. To quantify the hemicellulose is necessary to solubilize it from the NDF fraction. This is done using an acid detergent.

Lignin is the third major fraction of lignocellulosic biomass, it is a recalcitrant hydrophobic amorphous polymer embedded in cellulose and hemicellulose. This hinders the enzymatic access and the hydrolysis to these polymers, thus limiting the use of plant biomass, and its hydrolysis is very complex [36]. The lignin determination is done with permanganate (permanganate lignin fraction) or 72% H_2SO_4 (acid detergent lignin fraction, ADL). The lignin structure consists of cinnamyl alcohols derived: p-coumaryl (p-hydroxyphenyl or H units), coniferyl (guaiacyl or G units) and sinapyl (sirilgilo units or S). In the polymerization, the precursors are oxidized by peroxidases to phenoxy radicals, and they react randomly and cause multiple lignin structures. Unions between lignin components could be of condensed type (CC bonds) or not condensed (aryl alkyl ether linkage) of aromatic rings and propylic chains. CC bonds determine the high condensation of lignin and they are only formed between H or G units. The S units, by having two methoxy groups at positions 3 and 5, do not establish CC bonds, which is the reason of the binding of xylans to lignin during quick polymerization of monolignols [35]. Energy available of cell walls, defined as neutral detergent fiber digestibility (NDFD) is mainly related to the structural changes that occur in the cell wall polymers according to the type and stage of maturity of the plants [65], and in a lesser extents with its concentration [53]. The structure and content of lignin indirectly affect the degradability of the cell walls. Jung and Casler [41] describe that in grasses and legumes, lignin formation comes from the rate of change of syringyl to guaiacil, however, the cell walls of legumes are rich in cellulose and xylans, while grasses have few pectins and big ferulate content. The ferulate content is related primarily to cell wall degradability because of its ester bonds at joints among xylan and lignin. The cell walls of the grasses are more digestible than legumes [35], an example is maize fodder where this ferulate:cell wall degradability relation is negative during elongation and positive as it passes maturation time, until senescence initiates, when again it becomes negative. During the elongation of plants, diferulate´s ester bonds crossed with the arabinoxylan join the middle lamella hemicellulose with the secondary wall; meanwhile in the maturation stage, the secondary wall is thickened by an increased content of coumarate and the ester bonds of ferulate. After aging, the proportion of the secondary wall ferulate decreases by increasing other

phenolic compounds. This results in a lower proportion of ferulate to the rest of the other phenols present, which makes the dry matter less digestible [41].

1.4. Fungal enzymes production

The white rot fungi could adapt and grow in substrates with high content of lignocellulose, because of their abilities to produce enzymes degrading cellulose and hemicellulose. For example, *Trichoderma viride*, *Paecilomyces farinosus*, *Wardomyces inflatus* and *Pleurotus ostreatus*, could produce fibrolytic enzymes when they are cultivated in microcrystalline cellulose [71]. Obodai *et al*. [54] found that *Pleurotus ostreatus* growth was positively related to initial cellulose content ($r^2 = 0.6$), hemicellulose ($r^2 = 0.62$), lignin (ADL, $r^2 = 0.7$) and fiber ($r^2 = 0.7$).

Many white rot fungi produce enzymes laccase, manganese peroxidase and lignin peroxidase (Lac, MnP, and LiP) classified as phenoloxidases [70], allowing them to depolymerize, repolymerize, dimethylate and oxidize phenolic compounds, non-phenolic, aniline and xenobiotics [34]. The ligninolytic enzymes recognize catalytic domains where they bind to the cell wall to degrade the lignin [8]. First, the fiber degradation occurs by the action of low molecular weight mediators such as O_2, H_2O_2 and Mn^{2+} [63], then the penetration of the enzymes occurs [66]. According to Dashtban *et al*. [22], the laccase mediators system acts in the lignin degradation coupling the reduction of a dioxygen electron to two water molecules. The oxidation reactions catalyzed by laccases lead to the formation of free radicals, which act as intermediaries between substrates and enzymes. Since catalytic redox center of laccases are four copper ions, mediators leave sites with high redox potential and create non-enzymatic oxidative polymerization or depolymerization routes. Lignin peroxidase oxidizes substrates through several steps of electron transference and creates intermediary radicals as phenoxy. This leads to a large amount of non-enzymatic polymerization reactions and rearrangements of dimethylation and intramolecular addition, allowing oxidation of many non-phenolic aromatic substrates that do not require mediators due to their redox potential. Finally, manganese peroxidase, that contains a heme molecule as iron protoporphyrin, catalyzes the peroxide dependent oxidation of Mn^{2+} to Mn^{3+}, which then is released on the surface of the enzyme in complex with oxalate and other chelates, wherein the Mn^{3+} acts as a redox mediator whose potential

is limited only by the phenolic lignin structures. Organic acids such as oxalate and malonate, are primary compounds which act as second mediators in the production of reactive free radicals. Examples of this are: radicals with carbon center (acetic acid), peroxy radical superoxide (O_2), and radical (CO_2). In the absence of H_2O_2, these radicals can be used by MnP peroxide source and increase efficiency of lignin degradation.

The white rot fungi produce a complex of exogenous fibrolytic enzymes, cellulolytic and xylanolytic polysaccharidases. The cellulolytic complex constitutes the endo-β-glucanases, exo-β-glucanases, cellobiohydrolases, and β-glucosidases [39]. These enzymes as follows: first, the endo-β-glucanase randomly cuts β-glucosidic internal links in amorphous regions of the cellulose molecules, promoting a rapid decrease in chain length, a slow increase of free reducing groups, and thus allows formation of attack sites for exoglucanases. Then, the exo-β-glucanase gradually attacks non-reducing ends of cellulose molecules, releasing cellobiose subunits, which results in a rapid increase in sugars or reducing groups, but a little change in the size of the polymer. Finally, the β-glucosidase hydrolyzes both cellobiose, produced in the previous steps, and low molecular weight cellodextrins.

Enzymes that comprise the hemicellulolytic complex hydrolyze arabinose side chains (arabinofurosidase), release acetate groups (acetyl xylan esterase), and remove the side chains of glucuronic acid from xylose units (glucuronidase). Additionally, they act on short oligosaccharides to produce xylose (β-1,4 bonds hydrolyzing aryl xylopyranoside; β-xylosidase), on xylans and xylo-oligosaccharides to produce a mixture of xylooligosaccharides (endo-β-xylanase) [48].

1.5. Use of exogenous enzymes in production processes

The use of ligninolytic enzymes started early in the 70´s, after the first synthetic lignin models emerged. The first studies characterized the production and activities of *Phanerochaete chrysosporium* ligninases, and were done to reduce production costs and the impact of the paper industry on the environment [64]. These enzymes without having specific domains, act in extracellular environments. They can be catalytically active on a large number of substrates such as polycyclic aromatic hydrocarbons, chlorinated

pesticides, organophosphate pesticides, xenobiotics with similar lignin structure (aromatic and nitroaromatic compounds, polycyclic aromatic chemicals, herbicides, pesticides, and chlorophenol detergents), and colorants, which can be mineralized to CO_2 by several species of fungi [66].

The study of cellulase and xylanase with biotechnology techniques began in the early 80´s, analyzing the effect of *Aspergillus* sp. and *Trichoderma* sp. enzymes in the quality of animal feedstuff [12]. During the last two decades, the use of cellulases, hemicellulases, and pectinases mainly in textile, food, wine, paper pulp, and ethanol industries has increased considerably [12].

In ruminant nutrition, the goals with the use of fibrolytic enzymes are: 1) to increase degradability of cell walls; 2) to increase digestible energy intake; 3) to reduce the amount of feed per unit of milk produced or body weight; and 4) to decrease the production costs [11]. Some models in dairy cows fed diets based on corn silage, have shown that the increase of *in vitro* NDF digestibility (IVNDFD) of this forage, is associated with better fat corrected milk and intake of dry matter [53]. Neylon and Kung [52] reported positive relationships between increased IVNDFD of hybrid sorghum and maize and milk production. Oba and Allen [53] found that cows fed exclusively with 56% DIVNDF forage, produced equal amount of milk with those fed a diet that included 30% grain and forage with lower digestibility (IVNDFD = 46.5%).

The *in vitro* digestibility of forage legumes and grasses treated with enzyme preparations evaluated in the presence of ruminal fluid, show that the use of cellulases [23], xylanases [21], ferulic acid esterases [47], and combinations of cellulase and xylanase [26] may improve the *in vitro* dry mater digestibility (IVDMD) and IVNDFD. These results depend on the enzyme preparation dose, and the type of fodder [23].

The increase of IVNDFD in plants by the use of dose and suitable types of these enzymes [26], is not necessarily related to the *in vivo* NDF digestibility evaluated in the rumen or in the total tract [53]. Since the procedures for *in vitro* digestibility measurement can be easily standardized, it has been easy to find consistent data that allow relating IVNDFD with the passage rate, the feeling of fullness (satiety) in the rumen, DM intake, and milk

production [65]. For example, Pinos-Rodríguez *et al*. [59] tried out two sheep feedlot diets (D1: alfalfa hay; D2: ryegrass) with a commercial preparation with predominant activity of xylanases. They found out that IVDMD and IVNDFD at 24 h improved by the use of enzymes (IVDMD: 4.1% and 6.1%, and IVNDFD: 16.8% and 14%, for alfalfa hay and ryegrass), as well as DM intake (2.9%, alfalfa hay; 21%, ryegrass). However, *in vivo* digestibility of DM and NDF ryegrass were better without the inclusion of enzymes (DMD: -5% and NDFD: -1.5%).

Some inconsistencies and difficulties to relate the effects of the use of enzymes in growth and milk production in ruminants, to the changes in digestibility and *in vivo* fermentation patterns, could be partly due to the measurement methods applied in some of the variables evaluated. However, it could be expected that enzyme extracts have a greater impact *in vivo*, ruminal fermentation of forage digestibility, and productive variables of dairy and beef cattle [17].

In dairy cattle, the treatment of diets with exogenous fibrolytic enzymes improve DM intake [19], gross energy intake, production and milk composition [5], and the average daily gain (ADG) in various stages of lactation [30]. Regarding beef cattle, Beauchemin *et al*. [11] found that the application of an enzyme product to a diet containing 95% barley grain; feed efficiency improved from 6% to 12% depending on the dose of enzyme used. These positive results could be related to increased digestibility of NDF and acid detergent fiber (ADF) of the diet [15]. Krause *et al*. [45] observed a 28% increase in ADF digestibility by the inclusion of exogenous enzymes in the diet. However, not in all cases the use of enzymes causes positive changes in DMD and NDFD [6], intake [5], milk production [10] or ADG. Such discrepancies may be due to the characteristics of the type of enzyme used and its interaction with the ruminal ecosystem [2].

1.6. Enzymatic synergism

The gene expression of enzymes is inducted by the presence of the substrate and the lack of glucose and cellobiose in the media. Additionally, the repression of genes that code for several types of enzymes occurs by the presence of sugars and their derivatives present in the media [31]. Gene expression can also be modulated by the amount of nitrogen

compounds [67], ions [76], and the state media (solid or liquid) [20]. Depending on the media, enzyme concentrations can vary, and could produce isoenzymes with different stability characteristics at certain temperatures, pH, affinity to active sites, isoelectric points, molecular weights, specificity, spectral characteristics and contents of sugars [28, 31, 33]. Due to the wide variety and isoforms of cellulase and xylanase produced by microorganisms, it is possible to explore conditions that permit the production of enzymes that best fit certain biotechnological processes.

Since in nature, cellulose degradation in the rumen requires the coordinated action of ligninases, celullases and xylanases, these three groups of enzymes may act synergistically [26, 28]. However, in many cases, the products evaluated in ruminants do not contain an appropriate combination of types of enzymes, which may reduce their effect on fiber digestibility. The total amount of enzyme activity of cellulase and xylanase to be supplied depends on the proportion cellulases:xylanases on the extract used. It seems that at higher rates of this proportion, a lower dose of those enzymes is required to improve the degradability of the NDF. Fortes *et al*. [28] tested *Trichoderma reseei*, *Aspergillus awamori* enzyme extracts and their combination (25:75, 50:50, and 75:25) on the degradation of the lignocellulosic complex sugarcane. Cellulases:xylanases ratios in the five products were 1.6:1, 0.64:1, 0.72:1, 0.65:1, and 0.69:1, respectively. Although the enzymatic activity of *T. reesei* enzyme extract was 15 times lower than the cellulose:xylanses *A. awamori* combination, glucose production after 24 h of incubation (without rumen fluid) and amount of reduced sugars were better using the enzymatic extract of *T. reesei* compared to the *A. awamori* (40% vs 20% vs 2.8 and 5.4 g / L). Whilst, the release of xylose was better with the enzymatic extract of *A. awamori* (73% vs 56%). The combination of different enzymatic activities could have positive effects in fiber degradation, but different fungi combined extracts may have synergistic or negative interactions [28].

Sufficient supplementation of cellulase activity in relation to xylanases, is key to determine the optimum dose of enzyme extract for fiber degradability. Although, it is difficult to predict the potential of enzymatic extract in the rumen as a reference of its enzymatic activity, there could be more consistency between evaluations without rumen

fluid of the same extract, considering the relation cellulases:xylanases, enzyme stability and structure, depending on the fungi from which the extract was obtained [28].

Several studies have shown that cellulase:xylanase enzymatic extract ratios from 0.01 to 1.5:1 [27], improved degradability of NDF from alfalfa and corn silage (evaluated *in vitro* with rumen fluid). They were linearly associated to the total dose of cellulase supplemented (R^2 = 0.58 to 0.75). Depending on the type of forage, it could have greater relation between improvement of the activity NDFD and individual certain types of cellulases, such as endoglucanases, exoglucanases, β-glucosidases, or combination of cellulase and xylanase activity. Eun and Beuchemin [26] explain that the use of xylanases in proper combination with cellulases improve glucose release, by increasing xylose removal. It is possible that by hemicellulose removal, some hemicellulose-cellulose bonds are broken, increasing accessibility to cellulose. Consequently, pre-treatments such as washing, use of ligninolytic enzymes, polyethylene glycol (PEG), and surfactants, could improve the effect of cellulase and xylanase enzymes on NDF degradability [40].

In recent years, research has been conducted regarding the use of cellulosomes for more efficient use of lignocellulose. Resch *et al*. [62] examined a cocktail of three enzymes of the fungus *Hypocorea jecorina* and cellulosome of *Clostridium thermocellum*. These two combined systems have a better effect on the degradation of microcrystalline cellulose, which can help to design products that could degrade certain kinds of recalcitrant polysaccharides.

1.7. Predominant conditions of the rumen

1.7.1. pH

The pH in the rumen varies from 6 to 7. Food fermentation in the rumen produces volatile fatty acids (VFA) and lactic acid occasionally. Changes in ruminal pH are due to species, physiological animal stage, diet, fermentation patterns and environmental temperature. pH values below 6.0 are reported when ruminants consume high concentrate amounts. In cattle, it has been reported the lowest pH (5.6) at 6 h postprandial [58], although may be values below 5.0 if diets are high in starch [49]. Low ruminal pH has a negative effect on

ruminal fermentation and microbial growth [17]. A pH below 6.0 inhibits the growth of microbes and fiber digestion rates are depressed. Also, structural carbohydrates probably will not be degraded after a pH of 5.8 [60]. Low pH for extended periods of time may reduce fiber digestion and availability of energy [16]. Degradation of cellulose increases at pH of 6.0 or higher, whereas at pH ≤5.5 there is no degradation and sometimes the inhibitory effect of a low pH in the rumen microbes is not completely remedied, even when the pH of media was adjusted to a neutral range [38]. The advantage of exogenous fibrolytic enzymes is that they act at pH 6.0 or lower. For example, it has been observed that endoglucanase, exoglucanase and β-glucosidase of ligninolytic fungi have optimal pH of 4.5, 5.0 and 5.5, respectively [50].

1.7.2. Temperature

Average temperature interval in the rumen ranges between 38 and 42 °C [77]. The peak of microbial fermentation occurs after feed intake and rumen temperature reaches 41 °C [24]. Experiments carried out with ruminants, have shown a negative correlation between ruminal pH and temperature of the rumen [75]. These experiments are directly related to the proportion of grains in the diet, as this directly influences the ruminal pH. For example, Alzahal *et al*. [3] evaluated Holstein dairy cows fed with a diet consisting of a mixture of chopped hay, and gradually changed to a diet high in grains for two weeks. They observed that the values of ruminal pH were not lower than 5.6 during feeding of both diets. They found an association of low ruminal pH with high ruminal temperature, although the maximum ruminal temperature ranged between 39.0 and 40.0 °C. Environment

temperature also affects body and rumen temperature. Beatty *et al*. [9] observed a sustained difference between body and ruminal temperature of 1 °C. Body and ruminal daily temperatures observed were 40.9 and 42.5 °C, respectively, in the warmest day (25.2 °C). Fibrolytic enzymes fungi such as *Trichoderma reseei* have an optimum temperature for activity of 50 °C [50], indicating that the enzymes may be highly stable in the rumen.

1.7.3. Osmolarity

Normal osmolarity in the rumen ranges from 240 to 360 mosmol/L [56]. Variations in osmolarity, are due to intake level; since there is a cation and anion release (such as phosphates, sulfur and chlorine), sugars and starch in the rumen; from digested food. Consequently, there is an increase in osmolarity, with values ranging from 290 to 400 mosmol/L, depending on the diet [61]. Diets high in grain enhance the occurrence of acute acidosis in ruminants ($pH \leq 5$), resulting in a high osmolarity in the rumen [57]. Acidosis in the bloodstream occurs as response to the highly acidic rumen environment; due to high AGV concentration not absorbed at the rate they are produced. High osmotic pressure in the rumen causes water to be quickly pulled inward blood through the rumen wall, to neutralize the osmotic pressure and to increase the ruminal pH by the removal of hydrogen ions [57]. Although rumen osmolality is maintained slightly hypotonic respect to blood serum (290 mosmol/L), its values vary for short periods during the day. For example, Malafaia *et al*. [51] experimented with calves grazing on Tanzania grass and supplemented with 600 g of concentrated (67% ground corn, 33% soybean meal) and high amounts of sodium (90 g/d) chloride. They observed that levels in the rumen osmolarity remained within normal range (235-281 mosmol/L) throughout the day along the experiment. Fungal enzymes could be highly stable in ruminal environment and its osmolarity. Sun *et al*. [69] reported activity of cellulases produced by *Trametes ressei* using rice straw as a treated substrate, in alkaline conditions for biofuel production. Research has also been conducted with some cellulases called halophylic, which work well in environments with high osmotic pressure [79]. It is feasible that the osmotic pressure within the rumen allows the activity of enzymes that degrade the fibers of the lignocellulosic materials.

1.7.4. Proteolytic Activity

Some research on the hydrolysis of protein extracted from the rumen fluid, indicate elevated levels of protein degradation at initial incubation times. For example, Kohn and Allen [44] reported a 47.3% degradation of CP in soy flour at 16 h of incubation. Even a pre-incubation *in vitro* of rumen fluid in an enriched culture has yielded enzyme extracts with higher proteolytic activity, 78.5% of CP degraded in BSA at 12 h of incubation [74].

Some factors that influence the hydrolysis of the protein in the rumen are the food type and the proportion of concentrate in the diet, because they change the microbial population [37]. The microbial and food contained proteases could have an additive or synergistic effect on the rate of protein degradation [37]. Some plants used as fodder, have proteases that enhance the splitting of nutrients inside the rumen. Consequently, the work of rumen microorganisms is more efficient [43]. However, high protein hydrolysis in the rumen, sometimes leads to inefficient utilization of N for the synthesis of microbial protein causing loss of nitrogen. This is because the rate of protein degradation by rumen microorganisms is much faster than the rate by which ammonia might be incorporated into microbial protein [72]. Furthermore, there is an effect of glycosylation on the stability of an exogenous xylanase exposed to commercial proteases or *in vitro* ruminal fluid. Glycosylation increases stability by protection against proteolytic inactivation under similar rumen conditions [73]. This indicates that it is possible to increase the stability of enzymes by protection methods.

1.8. Enzyme protection

Enzyme immobilization methods have been widely used in the last four decades because they prevent enzymes from denaturation and proteolysis, maintaining its full performance status for successful use in various industrial processes. This improves the properties of enzymes such as the activity, stability and substrate specificity [68].

The first studies in immobilization of enzymes began in the 60´s; since then there has observed an increase in the use of these technologies for application in the biomedical, textile, paper, hydrolysis of lignocellulose, and bioremediation industries. In these areas, most enzymes have been used in free form so that new ways have been implemented in

order to protect them from inactivation and even increase their activity and optimization. Recent studies in this regard, have investigated the effect of β-glucosidase on the degradation of lignocellulose [39].

Research has focused in obtaining enzymes from spores to which food is provided to produce such enzymes. An example is the work done by Attitalla and Salleh [7], whom

immobilized spores of two strains of *Trichoderma harzianum* with the adjuvants xylan and carboxymethylcellulose (CMC) on alginate spheres. These researchers obtained *in vitro* production of carboxymethylcellulase and xylanase in free suspensions of fungal spores. Spores trapped in the alginate with and without adjuvant showed high enzyme production. Besides, alginate encapsulation not only prolonged the metabolic activity of the fungus *Trichoderma,* but also promoted a slow release of microbial spores in the medium for successful production of the enzyme.

The immobilization process may lead to changes in optimal pH and temperature of enzymes. Therefore, methodologies to use these catalysts under desirable conditions could be designed. Dragomirescu *et al*. [38] lyophilized enzyme preparations of *Trichoderma viride* with cellulase and cellobiase activities. Subsequently, they were immobilized on porous matrices using physical adsorption methods in ceramic matrices and entrapment in sol-gel glass. The highest cellulase and cellobiase activities were observed in preparations immobilized by physical adsorption on ceramic supports being 1.3 to 2 times greater than those observed by entrapment. In the case of cellobiase substrate, the immobilization method did not influence the pH optimum of the enzymes. However, in the hydrolysis of CMC the optimum pH being was less acid (5.0). Furthermore, Dragomirescu *et al*. [25] determined a 20 °C decrease on the optimum temperature of the enzyme with respect to the initial temperature, in the samples with changes in optimum pH. While Abraham *et al*. [1] reported a 20% increase on the optimum temperature optimum for immobilized compared to free enzyme, at the same optimum initial pH. Immobilization allows some changes regarding the optimum pH and temperature for an enzyme, and then these catalysts could be used in more optimal production processes.

The use of enzymes in the hydrolysis of lignocellulosic materials to obtain biofuels is a growing area of interest. However, the potential of replacing the commonly used raw materials by lignocellulosic materials to obtain biofuels such as ethanol is not yet profitable by enzymatic hydrolysis [39]. Because of these limitations, other researchers have made efforts to protect these enzymes. Eckard *et al*. [39] tested colloidal casein proteins that can form monolayers on hydrophobic surfaces to avoid enzyme deactivation. The results of this study suggest that the steric barrier provided by caseins and whey

proteins adsorbed by lignocellulosic biomass, could induce the cellulase activity by inhibition of its adsorption in non-productive sites. Furthermore, Blanchette *et al*. [14], by combining a cellulase obtained from *Trichoderma viride* with polystyrene nanospheres, both free and immobilized cellulose showed the same activity in soluble CMC. However, the enzyme of the immobilized complex showed higher affinity in its action on the microcrystalline cellulose. Furthermore, the immobilized cellulase was more efficient in the degradation of natural cellulose structures in thickened walls of cultured wood cells. These results suggest that the enzymes bound to nanoparticles can enhance the catalytic efficiency in physically insoluble substrates. Several authors [1, 39], have shown that the cellulase activity improves with immobilization, since it provides improved stability at higher temperatures in processes of lignocellulose degradation, and provide an advantage over the free enzyme through reuse and storage stability resulting of immobilization. These properties were corroborated by Abraham *et al*. [1] working with pre-treated biomass hemp. These authors found 89% and 93%, as the maximum hydrolysis at 48 h for both free and immobilized enzyme with, respectively.

Improvements obtained for enzymes with industrial application by modifying proteins have focused primarily on the activity, thermostability, stability in certain solvents, specificity, substrate and multiple tolerance reactions, also called multienzyme systems. Due to economic and environmental protection requirements, the multienzyme co-immobilizated systems have been reinforced. This has been mainly through sequential reactions enzymatic processes by the individual action of the catalysts [68]. The use of various technologies associated with proteins could increase the degradation of cellulose of raw materials for biofuel. This could be done by immobilizing different combinations of cellulase, xylanase and laccase/ligninase in nanoparticles [14]. This technology has already been applied successfully in several studies. Therefore, the co-immobilization is another viable option to degrade efficiently cellulose. Cho *et al*. [18] co-immobilized

simultaneously three cellulases labeled with cysteine, including endoglucanase, exoglucanase and β-glucosidase in gold nanoparticles and nanoparticles of magnetic silica bound in gold. Their results indicate that it is possible to use this technology for successful production of cellobiose and glucose.

The search for new methods to convert lignocellulosic biomass into fermentable sugars for the production of biofuels at an affordable cost has led to the implementation of technologies that combine protein engineering and immobilization to degrade biomass. An example of this is the creation of artificial structures that emulate cellulosome activity present in cellulolytic *Clostridium* and *Ruminococcus* species, which contain multicellulases associated to greater biomass degradation [4]. Anderson *et al*. [4] demonstrated that is possible to assemble multienzyme complexes on the cell surface; and therefore it is possible that these aggregates can be used to degrade biomass. Furthermore, complexes reconstituted from a cellulase enzyme mixture from a cellulosome [46], allow enzymes reattach cellulosomes. *In vitro* re-association of artificial enzymes cellulosomes allows to obtain 12 times more synergism in the substrate (crystalline cellulose) compared to non-complexed components, reaching up to 80% of the activity present in a natural cellulosome. Additionally, protein complex activity and increased stability were retained. By partially supplementing cellulosome components with three selected recombinant cellulose, activity in crystalline cellulose was increased, and cellulosome natural activity was reached [46]. These results show that it is possible the reconstitution of *in vitro* complex, which is a step towards the creation of cellulases highly and efficiently designed.

Although, the research on immobilization in ruminant nutrition has focused on stabilization of exogenous enzymes using surfactants [42], and on protection of amino acids from rumen fermentation [78], still there is an area of growing opportunity on the study of food exogenous enzymes protection for ruminants through immobilization.

1.9. Conclusions

Cell walls have different proportions of cellulose, hemicellulose and lignin, quantified by the determination of NDF. Some mathematical models indicate that NDF degradability is related to improved rate of passage, intake of DM and animal productive performance.

White rot fungi produce a variety of forms and isoforms of enzymes that allow them to use the energy of NDF. The inclusion of these enzymes in ruminant diets increases NDF degradability, which means improvement in feed conversion for the production of meat and milk; however, this result depends on the dose used. Although the combination of

extracts of cellulase and xylanase enzymes works synergistically in specific ranges of cellulase:xylanase proportion, sometimes combination of extracts from different fungi may have negative effects. Variability on animal response to the use of enzyme products depends on enzyme structure and their stability within the rumen. The addition of specific enzymes increases degradability of some fiber types. The expression of genes that encode enzymes depends on the medium and culture conditions in which the enzymes are produced. Although there are temperature and pH conditions within the rumen appropriate for the activity of the fungal enzymes, some factors might inhibit their activity (proteases for example). The immobilization could protect enzymes from inactivation. In bioremediation, protection of enzymes has permitted fungal enzymes reuse, and cost reduction in lignocellulose degradation. Some successful methods for enzyme protection and bioremediation of industrial processes include the use of whey protein, silica nanoparticles, and surfactants. For enzyme protection in ruminant nutrition, only surfactants, as Tween 80, have been tested. This represents an area of opportunity to improve the enzymatic activity of ligninolytic fungi to improve stability within the rumen.

The production of enzymes in suitable media could promote gene expression forms suitable for treatment of diets with different proportions and types of fibers enzymes. This process is essential to obtain specific products for the degradation of some materials in ruminant diets. Furthermore, the design of enzyme products includes the correct cellulase:xylanase ratio, and it depends on the feed to be treated. These conditions coupled with the use of techniques for protecting enzymes, could help to define dose supplementation and to reduce variability in the productive performance of ruminants.

2.0. References

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GROWTH AND FIBROLYTiC ENZYME PRODUCTION OF *TRAMETES TROGII* IN CORN STALK WITH AND WITHOUT EARCORN

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Submitted to *BioResources*

GROWTH AND FIBROLYTIC ENZYME PRODUCTION OF *TRAMETES TROGII* IN CORN STALK WITH AND WITHOUT EARCORN

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1.1. Abstract

The objective was to evaluate the growth of eight white-rot fungi and the enzymatic activity of *Trametes trogii* on corn stalk with and without earcorn. Growth area and radial growth rate (RGr) were calculated. Cellulase and xylanase production of *Trametes trogii* were determined and cellulase concentration was done at three temperatures. The growth area and the RGr were not influenced (P>0.05) by substrate, however fungal strain influenced radial growth (P<0.05). There was a significant effect of day for both enzymes and substrate by day interaction for cellulase (P<0.05). No effects were found for both substrates and concentration temperatures (P>0.05). Nevertheless, it was found that the potential that can reach the concentration at 22°C is reduced 50% when temperature was approximately 30°C. Therefore, addition of earcorn did not improve any of the studied variables, and it is better to use corn stalk without earcorn for fungi and fibrolytic enzyme production.

Keywords: Xylanases; Cellulases; Lignocellulose

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1.2. Introduction

Ruminants degrade plant cell walls due to the rumen microbes that digest most of plant carbohydrates. Thus, forages with high plant cell walls content could be used as a main feed ingredient in ruminant diets (Kuijk *et al.,* 2015). Plant cell walls are composed by 35-50% cellulose, 25-35% hemicellulose, and 10-15% lignin (Mendoza *et al.,* 2014). However, forage ruminant digestibility varies from 30% to 55% (Gallardo *et al.,* 2010), due to crystallization of these molecules and to the lignin content in forages (Singh and Chen, 2008). The effect of biological treatment with fibrolytic fungus of straws and stalks has been studied (Arce-

Cervantes *et al.,* 2013a) aiming to reduce the lignin content and to improve the nutritive value of fibrous substrates (Tuyen *et al.,* 2012). However, loss of cellulose and hemicellulose occur during the degradation of lignocellulose with white rot fungi (Arora and Sharma 2009); and the effectiveness of fungal delignification is difficult to elucidate due to lack of standardized culture conditions and information on fungal strains used (Kuijk *et al.,* 2015).

Recently, Arce-Cervantes *et al.* (2013b) used exogenous fibrolytic microbial enzymes as feed additive in ruminants and obtained variable results. The use of exogenous fibrolytic enzymes can improve meat and milk production in ruminants (Beauchemin and Holtshausen, 2010). However, due to variability of ruminal inoculum, food and enzyme preparations and environmental factors, the results of experiments with preparations of cellulases and xylanases in ruminants have been highly variable. A good deal of the research conducted has aimed to identify conditions and optimal dose of exogenous fibrolytic enzymes (Eun and Beauchemin, 2008) via stable, highly active enzyme products under certain conditions inside the rumen (Eun and Beauchemin 2007), and the use of technologies that help to maintain and to increase enzyme activity (Sheldon 2007).

Future research on the use of fibrolytic enzymes should focus in obtaining them via solid fermentation on lignocellulose substrates to: 1) be used as feed additives in diet formulation for ruminants (Arce-Cervantes *et al.,* 2013b), or 2) obtain the enzyme extracts and protect the enzymes (Abraham *et al.,* 2014). Among the microorganisms studied to obtain the enzyme extracts are the white-rot fungi such as *Fomes* sp EUM1 (Arce-Cervantes 2013a); *Trametes versicolor,* and *Bjerkandera adusta* (Rodrígues *et al.,* 2008). These studies indicate that enzyme extracts from white-rot fungi could be used to develop new approaches to overcome low digestibility of some plant cell walls. Disregarding of the focus on the enzymatic production of fungi, it is still required research on the growth and fibrolytic enzyme production of several fungi on different substrates. Therefore, the objective of this study was to evaluate the growth of eight white-rot fungi and the enzymatic activity of *Trametes trogii* on corn stalk with and without earcorn.

1.3. EXPERIMENTAL

1.3.1. Microorganisms and their Maintenance

Strains of *Sporotrichum pulverulentum, Phanerochaete chrysosporium, Bjerkandera adusta, Trametes zonatus, Trametes trogii, Pleurotus ostreatus, Trametes hirsuta,* and *Fomes* sp EUM 1 fungi were used. The fungi strains were donated from Universidad Autónoma Metropolitana-Ixtapalapa and the Universidad Autónoma de Aguascalientes. The fungi were spread on potato-dextrose-agar (PDA; Difco,) medium for their conservation and later they were activated in Petri dishes using the same medium and incubated at 28°C until the mycelium colonized most of the Petri dish area. Fungi were maintained for future periodical spreadings on Petri dishes with PDA, later they were refrigerated at 4°C.

1.3.2. Radial Growth Rate (RGr)

Fungi were sown in Petri dishes with PDA- 2 mm milled corn stalk (39 g L^{-1}; 2% p/v) with and without earcorn (WE and CS) media, sterilized at 121°C for 15 minutes in autoclave. The medium was inoculated with 1.0 cm^2 of media with fungus mycelium, and incubated at 28°C. The radium (r) of growth was measured daily using a Vernier. The growth area ($\pi * r^2$) was determined at four days of sowing and RGr ($cm^2\ d^{-1}$) was calculated by linear regression (Nussenbaum *et al*. 2013).

1.3.3. Cellulase and Xylanase Production by *Trametes trogii*

Methodology described by Arce-Cervantes (2013a) was used to determine cellulase and xylanase production of *Trametes trogii*. For activation of fungi, it was sown a disc of 1.0 cm diameter of media with mycelium growth of *T. trogii* in Petri dishes with media; then they were incubated at 28°C for seven days. Later, four discs were inoculated (1.0 cm media diameter each) with mycelium growth of *T. trogii*. The Petri dishes had 10.0 g of humid substrate (80%) and sterile (121°C for 20 min). The growth substrate was composed by 2 mm milled

corn stalk (80% dry basis) with or without earcorn and 2 mm milled wheat bran (20% dry basis). The inoculated dishes were incubated at 30°C.

Extracts of fermentation in solid state were obtained at 0, 3, 6, 9, 12, 15, and 18 days of incubation as follows: the inoculated substrate was completely transferred to an 250 mL Erlenmeyer flask, 90 mL distilled water were added and covered with adherent paper. The flask was put in an ice bath with orbital agitation (120 rpm) for 30 minutes. Afterward, the flask content was filtered; 40 mL of filtered media were transferred to a centrifuge tube of 50 mL capacity, they were centrifuged at 2,703 g for 12 minutes. With a micropipette, 1.8 mL of supernatant were transferred to 2 mL microcentrifuge tubes and were frozen at -20ºC.

The enzyme activity was measured via quantification of reducing sugars liberated by the activity of enzyme extracts (μmol g^{-1} DM of substrate), at incubation time mentioned previously. For determination of xylanases it was used as substrate a solution of xylan from beechwood (0.5%, p/v; Sigma-Aldrich) in citrates buffer (50 mM; pH 5.3). In test tubes (11x100 mm), 800 μL of substrate, 100 μL of citrates buffer (50 mM, pH 5.3), and 100 μL of enzyme extract were added, they were vigorously mixed. The mix was incubated for 20 minutes at 50°C, immediately after it was added 1.0 mL of reactant DNS, (Miller *et al*., 1959); then the tubes were introduced into a boiling water bath for 5 minutes. For stopped the reaction the samples were placed in an ice bath. Absorbance of each tube was determined using an UV-vis spectrophotometer at 540 nm. The reference solution consisted of enzymatic extract boiling-inactivated.

Enzymatic activity of cellulases was determined using as substrate a solution (1% p/v) of carboxymethylcellulose (Sigma-Aldrich) dissolved in citrate buffer (50 mM, pH 5.0). The procedure described for xylanase was followed. The enzymatic activity of both types of enzymes was defined as the μmoles of sugar produced per gram of substrate per minute. The calibration curves to relate the quantity (μmoles) of sugar with the absorbance were obtained using xylose (Merck) and glucose (Sigma-Aldrich) as reference for the xylanase and cellulase.

1.3.4. Concentration of Cellulolytic Activity of Extracts

The crude enzymatic extracts at nine day were concentrated using a Concentrator Vacufuge Plus (Epperdorf, Germany). 5.5 mL of crude extract were transferred into tubes for microcentrifuge, they were placed into the Vacufuge Plus in the vacuum aqueous mode (248 x g 20 mbar) for 150 minutes at 22^{o}C (room temperature), and at 30 ^{o}C. The final volume depended on the temperature (400 µL and 200 µL; at 22°C and 30°C, respectively. The cellulolytic activity was determined following the procedure described previously; a sample aliquot of 100 µL of crude enzymatic extract and concentrated enzymatic extract at 22 and 30°C was used.

1.3.5. Experimental Design and Statistical Analysis

A completely random design with three replicates per treatment was used, in an 8x2 (fungi*substrate), 7x2 (day of incubation*substrate), and 3x2 (enzymatic extract*substrate) factorial arrangement for growth, enzymatic activity, and concentration of enzymatic activity. When applicable, a multiple mean comparison following the Tukey procedure was used. The GLM procedure of SAS (SAS 2014) was used for the statistical analysis. The response variables were growth at four days (cm^2), RGr (cm^2 day^{-1}), cellulolytic and xylanase activity.

1.4. RESULTS AND DISCUSSION

1.4.1. Radial Growth Rate

Although *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum* were the fungi with the greatest growth and RGr, respectively; it has been reported a greater enzyme activity of cellulase and xylanase by the fungus *T. trogii* compared to other fungi (Tirado *et al*., 2014). Due to this, this fungus was selected to perform essays of enzymatic concentration from a crude enzymatic extract.

Table 1. Growth and radial growth rate (RGr) of eight white-rot fungi in Petri dishes with agar-corn stalk.

Fungi	Growth (cm^2 in 4 d)	RGr (cm^2 d^{-1})
Sporotrichum pulverulentum	0.13^{c}	21.21^{a}
Phanerochaete chrysosporium	63.62^{a}	16.97^{b}
Bjerkandera adusta	20.23^{b}	12.37^{c}
Trametes zonatus	13.56^{b}	11.78^{cd}
Trametes trogii	19.24^{b}	10.60^{d}
Pleurotus ostreatus	12.83^{b}	10.60^{d}
Fomes spp EUM 1	2.77^{c}	2.48^{e}
Trametes hirsute	0.13^{c}	0.01^{f}
MSD	7.41	1.735

Means without a common literal within columns are different (P<0.05).
MSD, minimum significant difference.

1.4.2. Cellulase and Xylanase Production by *Trametes trogii*

Table 2 shows the values of enzymatic activity of *T. trogii* at different days of growth by fermentation in solid state on corn stalk with and without earcorn. It was not found a significant effect (P>0.05) of substrate for any of the two enzymes, neither for the substrate by day interaction (P>0.05) for the xylanase activity. On the other hand, it was found a significant effect of day (P<0.05) for both enzymes, and for the substrate by day interaction (P<0.05) for cellulase.

The greatest activity was observed in the enzymatic extract at day 9 of incubation of *T. trogii* in corn stalk with earcorn (22.9 μmoles of glucose g^{-1} min^{-1}). In the corn stalk without earcorn, the enzymatic extract showed two peaks of cellulolytic activity, at 3 and 12 d (16.58 and 18.27 μmoles of glucose g^{-1} min^{-1}). This could be because when the corn stalk has earcorn, the fungus start to consume the reserve polysaccharides (starch) and afterward it begins the digestion of cellulose. On the contrary, when the substrate does not have reserve polysaccharides, the fungus is forced early to produce cellulases to liberate the sugar that will be used as energy source for the fungus (Okamoto *et al*., 2011).

The xylanase activity was not different (P>0.05) for the extracts obtained for the culture of *T. trogii* in corn stalk with or without earcorn; however, the highest

peak of enzyme activity was on the 18 day of incubation in corn stalk without earcorn (63.07 μmoles of glucose g^{-1} min^{-1}, Table 2).

Table 2. Enzymatic activity of *Trametes trogii* in corn stalk with and without earcorn a different days of incubation.

Days of incubation	Cellulase in corn stalk		Xylanase in corn stalk	
	without earcorn	with earcorn	without earcorn	with earcorn
	---------- μmoles g^{-1} min^{-1} ----------			
0	2.99	3.87	2.18	3.24
3	16.58	5.14	12.55	2.78
6	11.32	8.40	8.75	5.10
9	11.63	25.63	12.84	22.89
12	18.27	17.47	20.96	18.13
15	1.74	7.87	15.88	9.27
18	12.80	18.89	63.07	14.51
SEM	1.32		3.97	
Effect	Significance levels (*p*) for each kind of enzyme			
Substrate	0.37*		0.14	
Day	0.001		0.04	
Substrate*Day	0.04		0.19	

SEM = standard error of the mean

In the corn stalk with earcorn, *T. trogii* has a peak of maximum xylanase activity at 9 day (22.89 μmoles of glucose g^{-1} min^{-1}). This result is similar to the one observed with the cellulolytic activity, and it could be influenced by the presence of reserve polysaccharides. These results are different compared to the profile of xylanase activity reported with other fungi and types of substrates, where the peaks for maximum activity have been observed between days one and four (Costa *et al.,* 2010). However, the profiles of enzymatic activity vary according to the conditions of cultures because the enzyme production occurs due to the presence of specific substrates, among other factors (Obodai *et al.,* 2003). Additionally, the hydrolytic activity of specific enzymes depends on chemical composition and structure of fiber; for example, Ma *et al.* (2011) tested the degradation of fractions of cell walls of corn stalk using enzymatic extracts of *Auricualira polytricha* and *Stereum hirsutum.* They found that A. *polytricha* degraded the lignin, cellulose and hemicellulose; on the contrary, *S. hirsutum* did

not degrade lignin nor hemicellulose, even if it presented high xylanase activity. The removal of xylan from the substrates may increase the digestion of cellulose to glucose. Gao *et al.* (2012), observed a 43% reduction of corn stalk xylan by the treatment with xylanase extract from *Gloeophyllum trabeum* KU-41. This increased from 0.7 to 2.5 times the adsorption of cellulases. According to this, in the adaptation process to use the fibrous substrates, the fungi should produce a high xylanase activity previous to the cellulolytic activity; however, in this study the performance of the activity of both enzymes on the incubation time was similar.

1.4.3. Concentration of Cellulolytic Activity of Extracts

Figure 1 shows the cellulolytic activity of enzymatic extracts of *Trametes trogii*, cultured by fermentation in solid state on corn stalk with and without earcorn, the extracts were crude, as they are obtained, or concentrated at two temperatures (30 and 22°C). The statistical analysis showed no effect of both substrate and concentration temperature ($P>0.05$). It was not observed a significant effect of substrate by type of concentration interaction. When the concentration was made at 22°C is was possible to concentrate the enzymatic activity 145 % more than the crude extract. Meanwhile, at 30°C the concentration achieved was 77% higher than in the crude extract. This means that the potential that can be reached is reduced 50% when it is concentrated at 30°C compared to 22°C. This could be explained by the effect of temperature during the concentration. Obtaining enzymatic extracts at industrial scale requires of specific conditions. The temperatures reported for purification of some enzymes from white-rot fungi, generally are not higher than 4°C (Tien y Kirk 1988). Temperatures higher than 20°C could denature enzymes very quickly (Daniel, 1996). The substrates treated with white-rot fungi, depending upon the geographical zone, includes residuals of crop residues such as wheat, rice, maize, bamboo, sugarcane, aquatic lilies, among others. Several of these materials are used for bio-combustibles production, or pretreated in ruminant nutrition. The type of lignocellulosic material that has been used as substrate, vary in its composition, since the content of

cellulose, hemicellulose and lignin varies among species (Grabber, 2005). Additionally, the fungi-substrate combinations show great variation in delignification and accessibility of cellulose and hemicellulose (Kuijk *et al*., 2015) by the enzymatic ligninolytic, cellulolytic, and xylanasic action. This could explain why the substrates differed when their enzymatic activity was evaluated on the concentrated extracts.

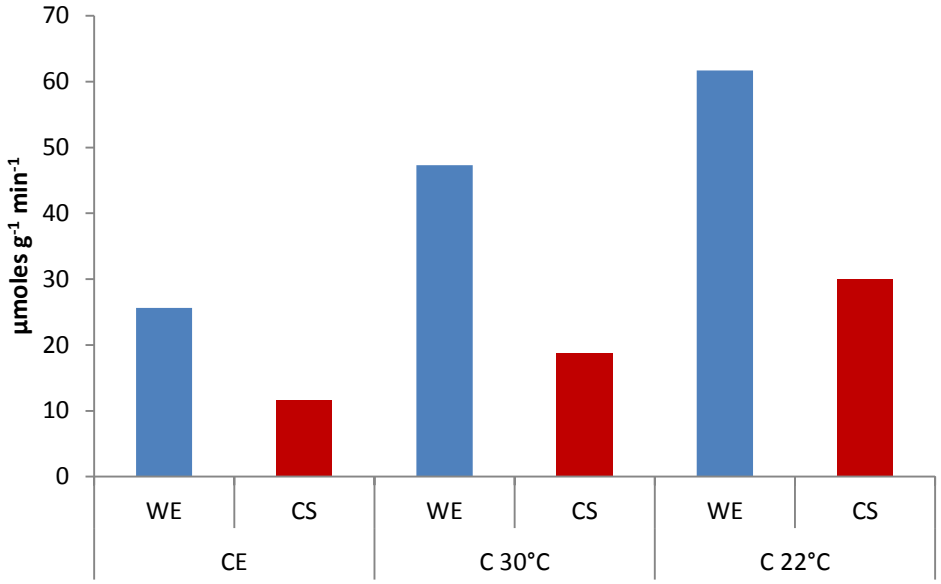

Fig 1. Cellulolytic activity of crude extracts (CE) and concentrated at 30°C and 22°C of *Trametes trogii* cultured by fermentation in solid state on corn stalk with (WE) and without earcorn (CS).

In ruminant nutrition, the direct treatment of fibrous materials with white-rot fungi has shown promising results (Labarere y Bois, 2001). In some studies, it has been demonstrated that the addition of exogenous fibrolytic enzymes into the ruminant diets, may increase the *in vitro*, *in situ* and *in vivo* digestibility of dry matter, and the neutral detergent fiber in fibrous feeds (Colombatto *et al.,* 2002). The use of lignocellulosic materials to obtain enzymatic extracts that could be applied to feeds of low digestibility for ruminants is an opportunity to increase the cellulase activity in the rumen. Arce-Cervantes *et al*. (2013b) observed weight gain in sheep by the addition of a ligninolytic extract into the diet. Increases in both weight gain and digestibility were observed.

1.5. CONCLUSIONS

1. Addition of earcorn to the substrates of corn stalk did not improve fungi growth, neither cellulase and xylanase production, therefore is better to use corn stalk without earcorn for fungi and fibrolytic enzyme production.
2. The white rot fungi *Trametes trogii* have a potential to degrade lignocellulosic material, as corn stalk, for enzyme production.

1.6. ACKNOWLEDGEMENTS

This project was supported by Universidad Autónoma Chapingo (DGIP-135501012). The authors also thank CONACyT for the financial support to the first author during her Doctoral studies.

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EFECTO DE *Fomes* sp. SOBRE SUSTRATOS DE MAÍZ PARA LAS CARACTERÍSTICAS DE ACTIVIDAD ENZIMÁTICA, COMPOSICIÓN QUÍMICA Y PRODUCCIÓN DE GAS *in vitro*

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Enviado a *Revista Mexicana de Micología*

Efecto de *Fomes* sp sobre sustratos de maíz para las características de actividad enzimática, composición química y producción de gas *in vitro*

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1.1. Resumen

Los hongos de la pudrición blanca

han sido utilizados ampliamente para la degradación de materiales lignocelulósicos, ya que son capaces de hidrolizar compuestos de la pared celular (celulosa, hemicelulosa y lignina). Los compuestos lignocelulósicos pueden ser aprovechados en diversos campos por el pretratamiendo biológico de hongos. El objetivo de este trabajo fue evaluar el efecto de *Fomes sp* EUM1 cultivado por fermentación en estado sólido sobre rastrojo de maíz con y sin mazorca (CM y SM) para las características de actividad enzimática (celulasas y xilanasas), composición química y producción de gas *in vitro* para volumen fraccional, velocidad máxima, fase de retardo y fase lag (Vf, Vm, S, L). No hubo efecto de sustrato ($P>0.05$) para ninguna de las enzimas analizadas. Las celulasas mostraron un pico de máxima actividad a los tres días de incubación; la mayor actividad de xilanasas se encontró a los seis días. La mayor cantidad de Vf coincidió con los mismos días de mayor actividad enzimática. El Vm se redujo linealmente en ambos sustratos. Además, los sustratos fueron modificados en su composición química a través del tiempo de fermentación.

Palabras Clave: xilanasas; celulasas; fermentación en estado sólido; lignocelulosa

Effect of *Fomes* sp on corn substrates for enzymatic activity characteristics chemical composition and *in vitro* gas production

1.2. Abstract

White rot fungi have been widely used for the degradation of lignocellulosic materials, due to its capabilities of hydrolyzing the cell wall plants components (cellulose, hemicellulose and lignin). Lignocellulosic compounds can be utilized in several fields, after biological pretreatment by fungi. The aim of this study was to evaluate the effect of *Fomes* sp EUM1 grown by solid state fermentation of corn stover with and without cob (CM and SM) for the characteristics of enzyme activity (cellulase and xylanase), chemical composition and *in vitro* gas production (Vf, Vm, S, L). There was no effect of substrate (P> 0.05) for any of the analyzed enzymes. The cellulases showed a peak of maximum activity after three days of incubation; the highest xylanase activity was found at six days. Most Vf coincided with higher enzymatic activity. The Vm decreased linearly in both substrates. Furthermore, the substrates were modified in chemical composition over time of fermentation.

Keywords: xylanases; cellulases; solid state fermentation; lignocellulose

1.3. Introducción

Los subproductos agrícolas, como la paja de arroz, rastrojo de maíz, hojas de palma de aceite y bagazo de caña de azúcar, son abundantes en muchos países (Sarnklong *et al.*, 2010; Devendra, 2009; Wan Zahari *et al.*, 2003); sin embargo, debido a su alto contenido de fibra detergente neutro (NDF) y de lignina y a su bajo contenido de proteínas, presentan valores de digestibilidad bajos en la alimentación para rumiantes (Karunanandaa y Varga, 1996; Karunanandaa *et al*., 1995) Diversas tecnologías han sido investigadas para mejorar el valor nutritivo de subproductos agrícolas. Tratamientos físicos, como cocción al vapor, molienda y granulación se han reportado para aumentar el consumo y la digestibilidad de hojas de palma de aceite y por lo tanto, el rendimiento del ganado (Wan Zahari *et al*., 2003). Los métodos biológicos, como el uso de hongos de la podredumbre blanca y sus extractos enzimáticos han sido considerados para mejorar el valor nutritivo de alimentos con baja calidad por ser procedimientos amigables con el medio ambiente y por sus costos. (Arora y Sharma, 2011). Existen reportes en donde se han utilizado los hongos para mejorar la calidad nutricional de ingredientes como la paja de trigo utilizando hongos de podredumbre blanca (Arora y Sharma, 2011; Arora *et al*., 2011) donde se ha observado que los efectos de la interacción de los hongos con los sustratos dependen de la características bioquímicas del sustrato, la cepa fúngica utilizada y la duración de la fermentación (Karunanandaa y Varga, 1996; Karunanandaa *et al*., 1995; Okano *et al*, 2006, Zadrazil y Puniya, 1995). No hay muchos datos disponibles sobre el valor nutritivo de rastrojo de maíz tratado con hongos, aunque ha sido reportada una alta pérdida de componentes de lignina y otra fibras en el rastrojo de maíz tratado con *Ceriporiopsis subvermispora* (Wan y Li, 2010). Existen evidencias de que el efecto de los hongos en la deslignificación y posteriormente en la degradación en el rumen de un sustrato es

altamente dependiente sobre el sustrato y cepa fúngica (Tuyen *et al*., 2012). La cepa del hongo termotolerante *Fomes* sp. EUM1 es capaz de crecer a temperaturas superiores a 45 °C en medio basado en rastrojo de maíz y producir enzimas lignocelulolíticas como xilanasas, celulasas y lacasas en fermentación en estado sólido (Arce-Cervantes *et al.*, 2013; Membrillo *et al.*, 2008). El objetivo de este trabajo fue evaluar el efecto del hongo *Fomes* sp EUM1 cultivado por fermentación en estado sólido en rastrojo de maíz con y sin mazorca para la producción enzimática de celulasas y xilanasas; composición química y producción de gas.

1.4.Materiales y Métodos

1.4.1. Material biológico: se utilizó una cepa del hongo *Fomes* sp. EUM1 donado por La Universidad Autónoma Metropolitana unidad Iztapalapa. La cepa de *Fomes* sp EUM1 fue activada y conservada en agar (15 g L^{-1}), extracto de malta (40 g L^{-1}) y extracto de levadura (3 g L^{-1}), para lo cual se resembró un disco (1cm ϕ) de medio con crecimiento micelial de *Fomes sp* EUM1, en cajas Petri conteniendo el medio de cultivo referido, y se incubaron a 30 ºC durante 15 días. También se usó rastrojo de maíz (RM) variedad AS1503 (ASPROS) con y sin mazorca (CM y SM), el cual fue proporcionado por el Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias de Pabellón de Arteaga, Aguascalientes. El rastrojo se cortó en trozos de aproximadamente 5 cm y se secó en una estufa de aire forzado a 60 ºC por 3 días. Posteriormente se molió en un molino Thomas Wiley con criba de 2 mm y se almacenó en bolsas de plástico.

1.4.2. Cultivo de *Fomes* sp EUM1 por fermentación en estado sólido: Diez gramos de sustrato húmedo (70%) y compuesto de RM (80%) con o sin mazorca, y salvado de trigo

(20%), fueron colocados en matraces de 125 mL de capacidad y esterilizados en autoclave a 121 °C por 20 minutos. Los matraces se inocularon con cuatro discos (1cm ϕ) de agar extracto de malta con crecimiento micelial de *Fomes* sp EUM1 y se incubaron a 30ºC. La fermentación en estado sólido fue detenida a 0, 3, 6, 9,12, 15, 18, 21 y 24 días de incubación para determinar la actividad enzimática, para lo cual se tomaron al azar tres matraces por tratamiento y por tiempo de incubación. A los matraces se les agregó 90 mL de agua destilada y se colocaron en baño de hielo con agitación rotacional a 120 rpm por 30 minutos. Posteriormente, el contenido del matraz se decantó y se filtró a través de tela. El filtrado fue transferido a tubos de 50 mL y se centrifugó a 2,7039 g durante 10 minutos. Una muestra de 2 mL del sobrenadante se transfirió a tubos de microcentrífuga y se almacenó en congelación (-20 °C) hasta ser usado para la determinación de actividad enzimática.

1.4.3. Medición de actividad enzimática: se determinaron las actividades celulolítica y xilanolítica. Para la actividad celulolítica se utilizó como sustrato enzimático carboximetilcelulosa (CMC) al 1% (p/v) disuelta en buffer de citrato de sodio (pH 5.0). A 800 µL del sustrato CMC se adicionaron 100 µL de buffer de citrato de sodio y 100 µL de la muestra de sobrenadante de cada día de incubación. La mezcla se agitó en un vórtex y se incubó a 50 ºC por 20 min. La reacción se detuvo mediante la adición de un mililitro del reactivo ácido 3,5-dinitrosalicílico (DNS), se mezcló y colocó en un baño maría a ebullición por 5 minutos. Posteriormente, los tubos con la mezcla se colocaron en un baño de agua con hielo. Se usó un blanco con extracto enzimático inactivado por ebullición, antes de la adición del DNS. La absorbancia se midió en un espectrofotómetro UV-VIS (Lambda 35, Perkin Elmer) a 540 nm (Miller, 1959). La actividad de xilanasas se midió

usando como sustrato una solución de xilano de madera (SIGMA) al 0.5 % (p/v) disuelto en buffer citrato de sodio (pH 5.3). La mezcla de reacción consistió de 800 µL de xilano, 100 µL de buffer de citrato de sodio y 100 µL del sobrenadante de cada tiempo, se agitó e incubó por 12 minutos a 50 ºC en baño maría, y se agregó un mililitro del reactivo DNS y se colocaron los tubos en un baño maría a ebullición por cinco minutos. Inmediatamente después, se colocaron los tubos en un baño de agua con hielo para detener la reacción y se midió la absorbancia de la misma forma descrita que para las celulasas (Miller, 1959). La actividad enzimática se definió como los µmoles de azúcar producido por gramo de sustrato por minuto. Para las curvas de calibración se utilizó glucosa y xilosa como referencia de los azúcares liberados por la actividad celulolítica y xilanolítica y obteniendo las ecuaciones de regresión para la absorbancia en función de la cantidad (µmoles) de azúcar.

1.4.4. Análisis químico: se determinaron las concentraciones (%) de fibra detergente neutro (FDN), fibra detergente ácido (FDA), lignina detergente ácido (LDA y materia orgánica mediante la calcinación de la materia seca a 550 °C por 3 h en una mufla (Van Soest, 1991).

1.4.5. Producción de gas ***in vitro*** **de sustratos tratados con** ***Fomes*** **sp EUM1:** con el fin de determinar cambios en la composición de los sustratos por efecto del cultivo de *Fomes* sp EUM1 en función del tiempo de incubación, se utilizó la técnica de producción de gas *in vitro* (Theodorou *et al*., 1994). En frascos de vidrio color ámbar de 60 mL de capacidad, se colocaron 200 mg de sustrato tratado con *Fomes* sp EUM1, y se les adicionó 45 mL de inóculo ruminal y un flujo continuo de CO_2. Los frascos se cerraron herméticamente con un tapón de goma y aro de aluminio. Los frascos se colocaron en un baño maría a 39 °C

y se midió el volumen de gas a las 0, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, 42, 48, 60 y 72 h de incubación.

El inóculo ruminal se obtuvo de dos borregos criollos (53 kg ± 6.8 peso vivo) con cánula ruminal, alimentados con heno de alfalfa (10%), rastrojo de maíz (70%) y concentrado (20%). Al momento de la extracción del líquido ruminal los borregos se encontraban en ayuno de 12 h. El líquido se filtró a través de cuatro capas de gasa y se mezcló en una proporción de 1:9 (v:v) con una solución mineral reducida compuesta de 4 g de Ca_2CO_3, 0.45 g de K_2HPO_4, 0.45 g de KH_2PO_4, 0.45 g de $(NH_4)_2SO_4$, 0.90 g de NaCl, 0.18 g de $MgSO_4$, 0.07 g de $CaCl_2$, 0.5 mg de Na_2S y 0.5 mg de L-Cisteína por litro de solución. Se adicionaron unas gotas de rezasurina (0.1%) como indicador de las condiciones reductoras.

Con los valores de volumen acumulado de gas a cada tiempo de incubación, se estimaron los parámetros de la cinética de producción de gas: volumen máximo (Vm; mL g^{-1}), tasa de producción de gas (S, h^{-1}) y la fase lag o de retardo (L, h) de la producción de gas, del modelo (volumen acumulado) $Va = Vm /(1+e^{2-4*s+(t-L)})$ (Schofield y Pell, 1995). Además se calcularon los volúmenes fraccionales de gas (mL g^{-1}) para los intervalos de tiempo de 0 a 8 h (Vf_{0-8}), 8 a 24 h (Vf_{8-24}) y de 24 a 72 h (Vf_{24-72}) de incubación.

1.4.6. Diseño experimental y análisis estadístico: se usó un diseño completamente al azar con tres repeticiones por tratamiento. El arreglo de tratamientos fue factorial 9x2, correspondientes a nueve días de incubación y dos tipos de sustrato (CM y SM). Las variables fueron: actividad de celulasas, actividad de xilanasas, parámetros de la producción de gas (Vm, S y L), volumen fraccional de gas (Vf_{0-8}, $Vf8_{-24}$ y Vf_{24-72}) y

contenido de FDN, FDA, LDA y MO. Se utilizó el procedimiento GLM y la prueba de comparación múltiple de medias de Tukey (SAS 9.3 SAS Institute Inc., Cary, NC, USA).

1.5. Resultados y Discusión

El análisis estadístico mostró que no hubo efecto de sustrato ($P>0.05$) ni interacción sustrato por día para ninguna de las enzimas analizadas (Tabla 1). Por otro lado, la actividad de celulasas y xilanasas varío en función de los días de incubación ($P>0.01$; Tabla 1). Las celulasas mostraron un pico de máxima actividad a los tres días de incubación, mientras que la mayor actividad de xilanasas se encontró a los seis días de incubación (Tabla 1). Se observó un segundo pico de actividad de celulasas y xilanasas a 12 y 18 días de incubación, pero éstos fueron menores al primer pico de actividad. Esto indicó una actividad enzimática secuencial donde el primer grupo de enzimas que digieren el sustrato (RM+salvado de trigo) son las celulasas y posteriormente las xilanasas. Por otro lado se observó un pico de mayor actividad de xilanasas en comparación a la actividad de celulasas. Arce-Cervantes *et al*., (2013) encontraron un comportamiento de la actividad de celulasas y xilanasas de *Fomes* sp EUM1 similar a la presente investigación, observando la máxima actividad de xilanasas y celulasas en el día 5 de fermentación en estado sólido. Márquez-Araque *et al*., (2007) encontraron que el pico de máxima actividad de xilanasas en bagazo de caña de azúcar fue obtenido a los 14 días de incubación, lo cual indica que la producción de la enzima está relacionada con el sustrato usado para el cultivo del hongo.

Tabla 1. Actividad de celulasas (CEL) y xilanasas (XIL) de *Fomes spp EUM 1* cutivado por fermentación en estado sólido sobre rastrojo de maíz con y sin mazorca (CM y SM)

Sustrato	Día	CEL (μmoles g^{-1} min^{-1})	XIL (μmoles $g^{-1}$$min^{-1}$)
CM	**0**	415.37	451.75
	3	1731.20	1573.42
	6	949.53	3295.64
	9	179.25	972.59
	12	1583.98	1344.53
	15	287.87	678.98
	18	668.06	583.42
	21	210.92	573.42
	24	407.31	369.81
SM	**0**	563.98	568.42
	3	1948.14	2518.42
	6	1704.25	3592.03
	9	171.75	526.20
	12	783.42	783.42
	15	245.37	245.37
	18	221.75	186.20
	21	208.98	172.87
	24	221.48	333.70
EEM		424.35	434.21
Efecto	Niveles de significancia (*p*) para cada tipo de media		
Sustrato		0.8408	0.6217
Día		<0.0001	0.0017
Sustrato*Día		0.7411	0.8328

EEM= Error estándar de la Media

Estas diferencias pueden atribuirse a factores como el tipo de sustrato, el tamaño de partícula, la estructura de la fibra, la humedad del cultivo, entre otros (Membrillo *et al*., 2011) Eun y Beauchemin (2008) mencionan que una relación xilanasa:celulasa con mayor cantidad de celulasas favorecerá la degradación de la FDN. En la presente investigación la relación con mayor celulasas se encontró a los seis y nueve días de incubación (0.39:1 y 0.23:1), pero de igual forma, el pico de mayor actividad de xilanasas se dio a los seis

días, por consiguiente, se consideró que el día seis de incubación con *Fomes* sp EUM1 es el apropiado para la obtención de extracto enzimático con mejor actividad.

Respecto a la producción de gas por la fermentación de los residuos de sustrato según los días de incubación, el Vf_{0-8} y Vm fueron afectados por la interacción sustrato*días de tratamiento ($P>0.05$; Tabla 2). El Vf_{0-8} incrementó entre los tres y nueve días de tratamiento, respecto al sustrato sin tratamiento, siendo mayor y a un tiempo más corto cuando el RM tuvo mazorca (Figura 1). Puesto que el Vf_{0-8} corresponde principalmente a gas proveniente de la fermentación de azúcares solubles, el incremento de esta variable nos indica que la actividad de celulasa y xilanasas de *Fomes sp* EUM1 induce la solubilización de carbohidratos solubles fermentables entre los 3 y 9 días de tratamiento con el hongo. Posteriormente, el Vf_{0-8} se redujo posiblemente debido a que *Fomes* sp EUM1 metaboliza tales carbohidratos. El incremento de Vf_{0-8} en los primero días coincidió con el tiempo de máxima actividad de xilanasas (Tabla 1), lo cual explicó el incremento de los azúcares a partir de la digestión de la xilosa, principalmente, aunque en parte puede ser atribuido a la actividad de celulasas. Dicho incremento en la cantidad de azúcares solubles corresponde al 98% en sustrato CM a los 3 días de tratamiento, y 70.6 % en el sustrato SM a las 6 días de tratamiento, con respecto al sustrato sin tratamiento con *Fomes* sp EUM1. El Vm se redujo linealmente en ambos sustratos (CM o SM) a una tasa de reducción de -3.92 y -3.06 mL g^{-1} d^{-1} (R^2; 0.6285 y 0.7855; Figura 1). Estos resultados concuerdan con los reportados por Arce-Cervantes *et al*., (2013), quienes observaron que el tratamiento del heno de alfalfa y el rastrojo de maíz, tratados con un extracto enzimático de *Fomes* sp. EUM1, el Vm de fermentación por microorganismos ruminales fue menor ($P< 0.05$). Puesto que el Vm representa la fermentación potencial del sustrato, lo anterior indica que el tratamiento con *Fomes* sp EUM1 reduce la fermentación

ruminal potencial del sustrato a una tasa constante, debido posiblemente a la asimilación de los carbohidratos por parte del hongo, lo cual disminuye la cantidad de carbohidratos fermentables disponibles para los microorganismos ruminales. Es posible que la reducción constante de Vm se deba a que el hongo continúa produciendo celulasas y xilanasas aunque su actividad sea menor (Tabla 1).

Tabla 2. Producción de gas para las variables de gas de 0 a 72 h (Vf_{0-8}, Vf_{8-24} y Vf_{24-72}), Vm, S y L), de rastrojo de maíz con y sin mazorca (CM y SM) cultivados con *Fomes* spp EUM1

Sustrato	Día	Vf_8	Vf_{24}	Vf_{72}	Vm	S	L
CM	0	32.803	140.877	136.840	287.500	0.034	6.920
	1	51.053	121.230	100.700	249.400	0.034	3.091
	3	65.087	117.720	102.103	262.400	0.032	1.936
	6	44.033	90.703	98.593	217.933	0.029	4.002
	9	46.843	87.197	87.717	205.867	0.030	2.474
	12	30.353	77.720	103.510	196.367	0.029	6.392
	15	30.703	73.160	109.120	200.100	0.028	6.179
	18	15.967	63.333	109.123	174.167	0.031	10.277
	21	19.827	81.230	112.983	196.967	0.032	9.097
	24	15.613	72.107	106.670	177.800	0.033	10.098
SM	0	26.843	158.420	155.440	312.933	0.036	7.954
	1	45.087	105.790	126.317	257.767	0.027	3.571
	3	36.667	82.633	127.020	231.567	0.026	5.009
	6	45.790	105.090	122.107	254.533	0.028	3.834
	9	41.227	83.683	114.037	226.533	0.027	3.951
	12	38.773	111.403	122.107	251.000	0.031	4.812
	15	34.210	99.473	114.037	229.100	0.031	5.643
	18	29.297	93.157	112.633	217.567	0.031	6.836
	21	16.667	83.337	114.737	197.367	0.031	9.152
	24	20.877	91.753	114.387	208.900	0.032	8.301
EEM		5.205	12.045	6.959	17.290	0.002	0.959
Efecto		Niveles de significancia (*p*) para cada tipo de media					
Sustrato		0.474	0.105	<0.001	0.007	0.662	0.745
Día		<0.001	<0.001	<0.001	<0.001	0.008	<0.001
Sust*Día		0.026	0.135	0.435	0.046	0.112	0.089

EEM= Error Estándar de la Media

Un comportamiento similar fue observado en el hongo *V. volvácea*, que al ser cultivado en paja de trigo, no produjo cambios significativos en la composición química del sustrato después de 49 días de incubación, presentando un decremento en la producción de gas total comparado con el control. Esto puede explicarse si se considera que el hongo puede secretar compuestos que inhiben el proceso de fermentación de los microorganismos del rumen. (Tuyen *et al*., 2012).

Los días de tratamiento del sustrato con *Fomes sp* EUM1 también afectaron (P>0.01) al Vf_{8-24} y Vf_{24-72} (Cuadro 2). Ambas variables disminuyeron con respecto al testigo, pero la reducción fue mayor para el Vf_{8-24} (Figura 2). Puesto que el Vf_{8-24} y Vf_{24-72} puede asociarse a la fermentación de almidón-hemicelulosa y con la de celulosa, lo anterior sugiere una mayor y más rápida digestión del almidón y xilano que de celulosa, lo que se puede atribuir a la mayor actividad xilanasa.

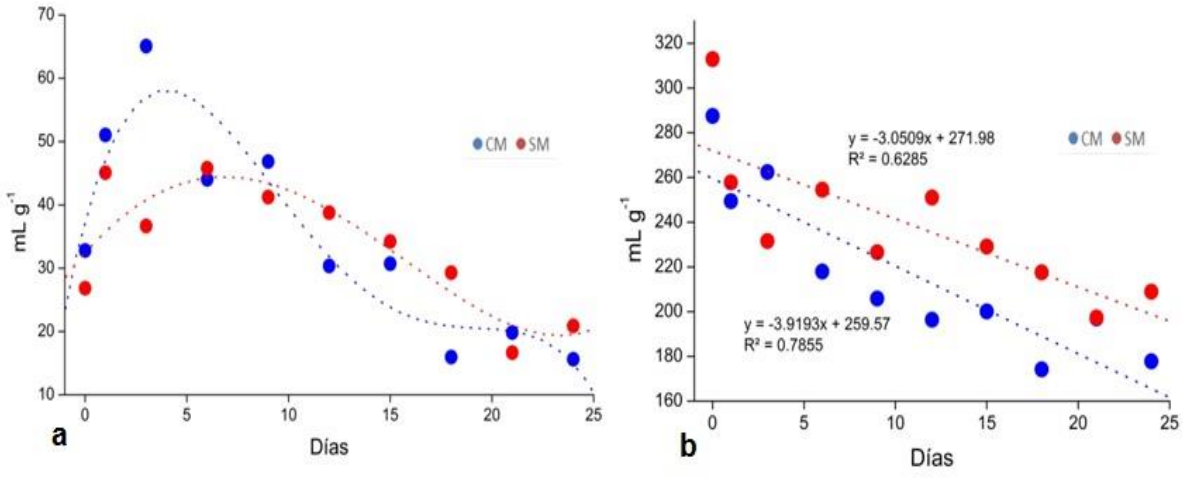

Figura 1. Producción de gas de 0 a 8 horas (a) y volumen máximo (b) de fermentación *in vitro* por un inóculo ruminal, del rastrojo de maíz con (CM) y sin mazorca (SM) tratado con *Fomes sp* EUM1 a distintos días de incubación.

La principal reducción del gas de fermentación se mostró en los primeros seis días y en los primeros nueve días de tratamiento con *Fomes* sp UAM1, para Vf_{8-24} y Vf_{24-72},

respectivamente. Esto coincide con el tiempo de mayor actividad celulolítica y xilanolítica.

El Vf_{24-72} fue mayor (P<0.01) en el RM-SM (122.28) que en el RM-CM (106.74), pero la reducción porcentual media del Vf_{24-72} respecto al testigo fue 22.0 *vs*. 26.4%, lo que indicaría indirectamente la cantidad de celulosa fermentable que digirió y metabolizó *Fomes* sp UAM1, antes de someterla a la fermentación *in vitro* por los microorganismos ruminales.

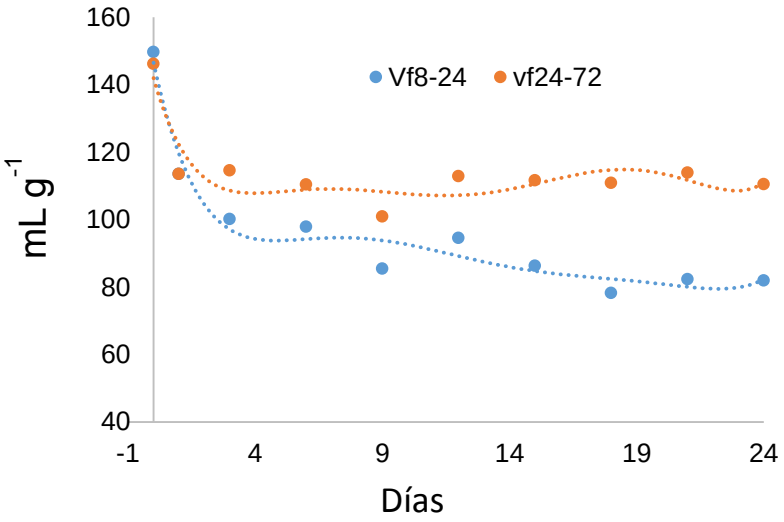

Figura 2. Producción de gas de 8 a 24 h (Vf_{8-24}) y 24 a 72 h (Vf_{24-72}) de fermentación ruminal *in vitro* de rastrojo de maíz tratado con *Fomes* sp EUM 1 a distintos días de incubación.

Tuyen *et al*. (2012) al probar paja de trigo, con 11 hongos de la podredumbre blanca para realizar una fermentación en estado sólido durante 49 días, observó que *Ceriporiopsis subvermispora*, *Lentinula edodes* y *Pleurotus eryngii* produjeron mayor cantidad de gas que el tratamiento testigo, pero otros hongos no incrementaron la disponibilidad de compuestos fermentables de la paja, para los microorganismos ruminales. Shrivastava *et al*. (2011) encontraron que la producción de gas incrementó respecto al testigo, cuando la paja de trigo fue tratada con *P. ostreatus* y T. versicolor por 20 y 10 días; sin embargo, la producción de gas fue menor a períodos más largos de tratamiento.

La fase Lag (L) fue modificada (P<0.01) por los días de tratamiento del sustrato con *Fomes* sp EUM1 (Tabla 2). Éste parámetro (L) disminuyó de 7.5 h en el testigo a 3.5 h a los tres días de incubación, y se mantuvo así hasta los nueve días (Figura 3) de tratamiento. Lo anterior mostró que el tratamiento con *Fomes* sp EUM1 reduce el tiempo de retardo para la fermentación del sustrato a base de RM, lo cual puede explicarse por el incremento de azúcares solubles fermentables (Figura 1) y al incremento de la actividad celulasa y xilanasa de *Fomes* sp EUM1. Arce-Cervantes *et al*. (2013) también encontraron que el tiempo lag (L) para la producción de gas de fermentación *in vitro* del bagazo de caña, se redujo con el tratamiento por medio del extracto enzimático de *Fomes* sp EUM1.

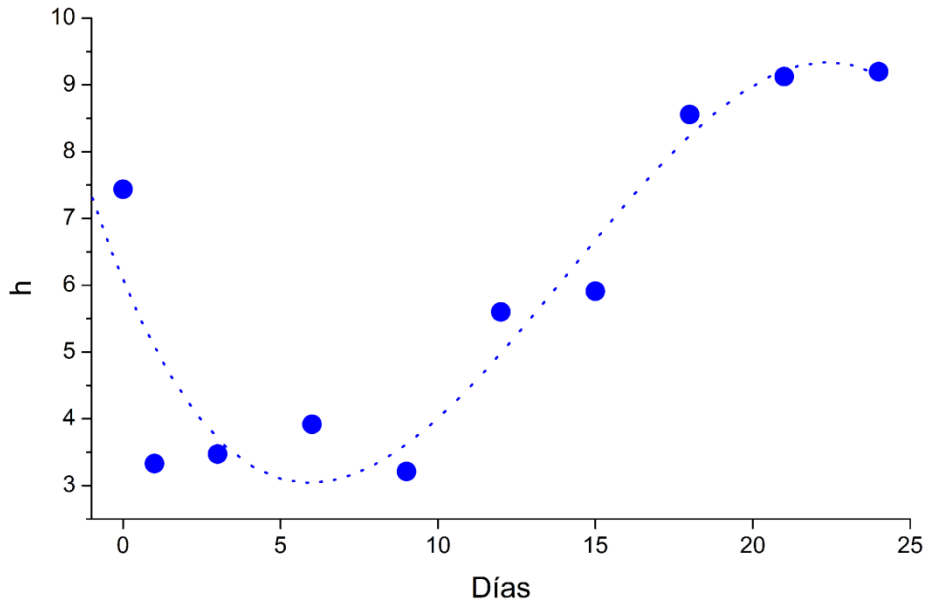

Figura 3. Tiempo Lag (L) de la producción de gas de fermentación del rastrojo de maíz tratado con *Fomes* sp EUM1, a distintos días de incubación en fermentación solida

La segunda fase del gráfico (Figura 3) muestra un incremento del tiempo Lag (L), lo cual fue atribuido a la fermentación de los azúcares solubles libres (Figura 2) por *Fomes* sp EUM1, y al incremento de la FDA y LDA entre los 9 y 24 días de tratamiento. A los 24

días de tratamiento el valor de L fue mayor que el del tratamiento testigo, lo que fue resultado de la utilización de los carbohidratos de más fácil fermentación, por parte de *Fomes* sp EUM1 de manera que los residuos de 24 días contenían carbohidratos fibrosos menos fermentables.

Respecto a la composición de los residuos de los sustratos, se encontró interacción (P<0.01) sustrato*día para FDN, FDA y contenido de celulosa (Tabla 3). También se encontró efecto de días de tratamiento (P<0.01) para FDA, LDA, hemicelulosa y celulosa.

Tabla 3. Composición química (%) de un control y rastrojo de maíz con y sin mazorca incubado con *Fomes sp* EUM1 durante 24 días.

Sustrato	Día	FDN	FDA	LDA	HC	CEL	CEN	MO
CM	**Control**	65.58	30.67	4.19	34.91	26.48	1.20	99.36
	0	68.22	40.50	7.92	27.73	32.58	2.09	98.80
	3	65.75	41.28	7.59	24.48	33.69	1.61	99.33
	6	76.72	50.44	8.95	26.28	41.50	2.80	98.57
	9	67.04	40.87	7.38	26.17	33.49	0.79	99.09
	12	74.88	42.12	7.67	32.76	34.45	2.20	97.91
	15	68.26	40.57	6.93	27.69	33.64	0.67	98.39
	18	69.34	40.72	7.22	28.63	33.50	1.43	97.20
	21	72.98	43.86	7.76	29.13	36.10	0.91	99.21
	24	76.40	49.22	7.27	27.17	41.95	0.64	97.80
	Control	70.41	35.70	4.41	34.71	31.29	2.40	98.85
	0	74.76	45.82	9.06	28.94	36.76	2.50	97.60
	3	72.85	45.37	8.83	27.48	36.54	1.41	98.87
	6	70.01	45.03	10.43	24.99	34.60	2.41	97.49
	9	70.05	43.92	8.72	26.13	35.21	0.97	98.79
SM	**12**	70.14	42.83	8.29	27.31	34.54	1.66	97.50
	15	72.04	42.60	8.28	29.45	34.31	1.13	98.59
	18	70.56	42.94	8.10	27.61	34.84	2.51	97.59
	21	68.25	43.43	8.11	24.82	35.32	1.21	99.03
	24	67.53	42.33	7.69	25.20	34.63	1.15	98.34
EE		1.72	1.68	0.39	1.52	1.68	0.37	0.37
Efecto		Niveles de significancia (*p*) para cada tipo de media						
Sustrato		0.8560	0.0262	<0.0001	0.2297	0.9293	0.0750	0.0750
Día		0.5860	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	<0.0001
Sust*Día		<0.0001	0.0065	0.6809	0.1901	0.0068	0.3320	0.3320

Estos resultados indican que los sustratos fueron modificados en su composición a medida que el hongo fermentó dichos materiales a través del tiempo. Además se observaron diferencias entre los dos sustratos utilizados para las variables de FDA y LDA. El procedo de esterilización resultó en un aumento en FDN, FDA, LDA y CEL, estos resultados concuerdan con los reportados por (Tuyen *et al*., 2012) donde observaron que el proceso de esterilización por autoclave resultó en pajas con contenidos de FDN, FDA, LDA y celulosa mayores a los presentes en la paja intacta (sin esterilizar en autoclave) Esto puede deberse a que los vapores pueden solubilizar algunos compuestos nitrogenados y otros elementos, lo cual puede resultar en pérdida de N y cenizas, incrementando las proporciones de FDA FDA y LDA (Tuyen *et al*., 2013). Aunque no se encontraron diferencias entre días para la variable de FDN, se observa un incremento a través del tiempo, esto probablemente a la concentración de sustrato debido a la acción del hongo. (Olivera *et al*., 2014). La variable FDA presentó diferencias entre sustratos siendo mejor el RM SM. El día 6 presentó el valor más alto de FDA y LDA con 47.74 y 9.69, respectivamente (Figura 4).

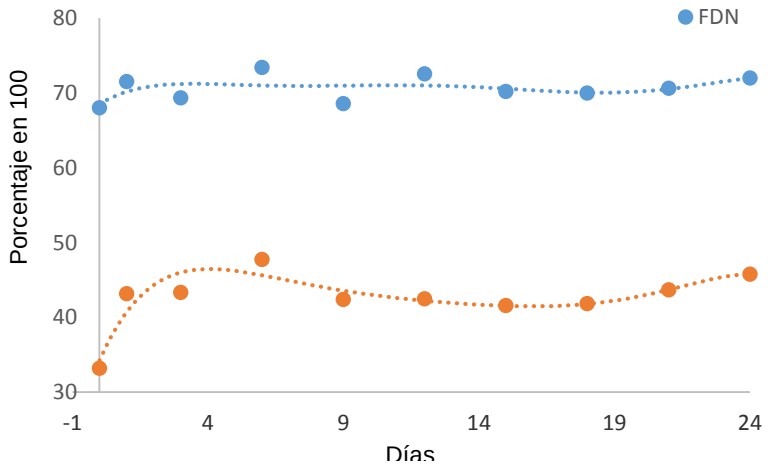

Figura 4. Cambio en el contenido de fibra detergente neutro (FDN) y fibra detergente ácido (FDA) en un sustrato con rastrojo de maíz a distintos días de tratamiento con *Fomes* sp EUM1.

El mayor contenido de HC y CEL también se presentó en este día. Para la variable CEL, se observó una interacción sustrato*día. El contenido de HC se vio afectado por *Fomes* (Figura 5), donde se observan valores de 34.91 para el control, hasta de 24.48 en el día 3 en el sustrato CM; y valores de 34.71 a 24.99 para el control y para el día 6 en el sustrato SM. Estos días coinciden con la mayor actividad enzimática. En investigaciones realizadas con sustratos con alto contenido de lignocelulosa, tratados con hongos de la pudrición blanca, la pérdida de HC también ha sido reportada (Tuyen *et al.*, 2012) en donde observaron una fuerte correlación (r=0.96) entre la pérdida de lignina y hemicelulosa por efecto de la incubación fúngica, aunque la correlación entre la pérdida de lignina y celulosa fue baja (r=0.41).

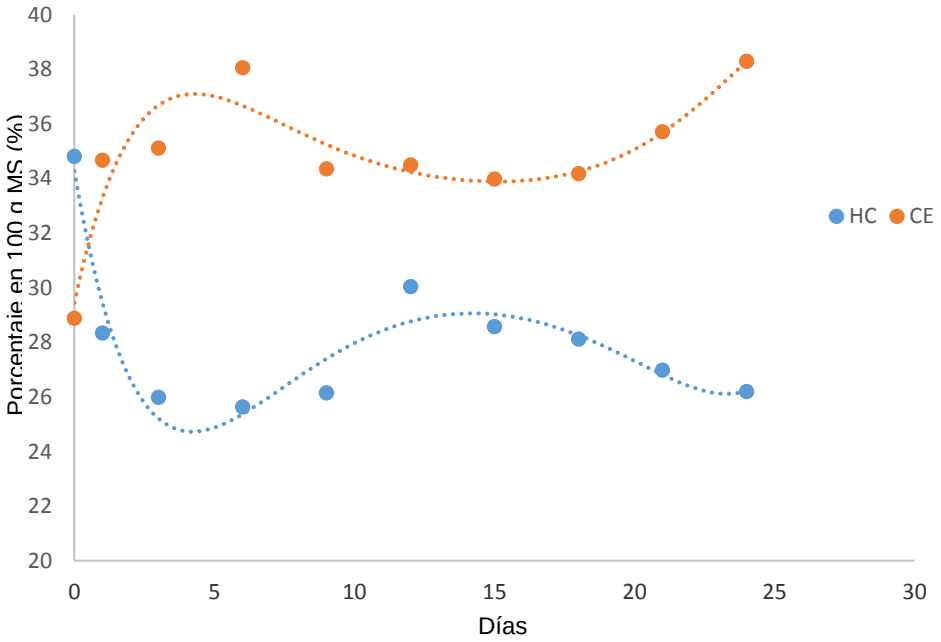

Figura 5. Cambio en el contenido de celulosa (CEL) y hemicelulosa (HC) en un sustrato con rastrojo de maíz a distintos días de tratamiento con *Fomes* sp EUM1.

Esto puede indicar que durante el crecimiento vegetativo del hongo, el proceso de deslignificación en paja de trigo está acompañado de una degradación de celulosa. Estos resultados concuerdan con la alta producción de xilanasas de *Fomes*, ya que altas

actividades de xilanasas se relaciona con la degradación de la hemicelulosa (Tuyen *et al.*, 2012). Esto podría explicar que al producir el hongo mayores cantidades de xilanasas, el contenido de HC se ve disminuido.

En conclusión, esta investigación muestra que no hay efecto de sustrato para la producción de enzimas, por lo que es mejor utilizar el rastrojo de maíz sin mazorca, pues es mucho más económico. El rango de días para obtener un extracto enzimático del hongo *Fomes* sp EUM1 bajo las condiciones señaladas anteriormente, se encuentra entre el día 6 y 9 de fermentación en estado sólido. El Vm se redujo linealmente en ambos sustratos, indicando que el tratamiento con *Fomes* sp EUM1 reduce la fermentación ruminal potencial del sustrato, debido a la asimilación de los carbohidratos por el hongo. Además, la principal reducción del gas de fermentación coincidió con el tiempo de mayor actividad celulolítica y xilanolítica. Por otro lado, los sustratos fueron modificados en su composición química a medida que el hongo fermentó dichos materiales a través del tiempo. El contenido de HC se vio afectado por *Fomes* donde se observaron reducciones de HC en los días 3 y 6 de fermentación, los cuales coinciden con la mayor actividad enzimática.

1.6. Agradecimientos

Este proyecto fue financiado por la Universidad Autónoma Chapingo (DGIP-135501012). Los autores también agradecen a CONACyT por el apoyo financiero para el primer autor durante sus estudios de doctorado.

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