El Colegio de la Frontera Sur

Biodegradación del bagazo de agave comiteco por *Pleurotus* spp.: fuente de celulasas para el tratamiento hidrolítico en la producción de bioetanol

TESIS
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Por

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hacemos constar que hemos revisado y aprobado la tesis titulada:

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para obtener el grado de **Maestro en Ciencias en Recursos Naturales y Desarrollo Rural.**

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Resumen

El objetivo de este estudio fue determinar la producción de celulasas de cinco cepas de *Pleurotus* spp., durante su cultivo sobre bagazo de agave comiteco y la viabilidad de utilizar los extractos celulíticos en la elaboración de caldos fermentables para la producción de bioetanol. Tras el cultivo, los parámetros de producción de basidiomas variaron entre 41.2-65,7% en eficiencia biológica, 0.41-0,65 en el rendimiento de producción de basidiomas, 0.60-0.90% Tasa de Producción, 16.4- 41.1% Bioconversión y 9.4- 21.3 g Peso Promedio de los Hongos.

De los extractos celulolíticos obtenidos, el correspondiente al día 15 de crecimiento de *Pleurotus djamor* mostró mayor actividad β-glucosidasa (21.97±2.27 UI/ml); y en el día 33 y por la misma cepa se detectó mayor actividad endoglucanasa (10.56±0.25 UI/ml). Ambos extractos se purificaron parcialmente y los parámetros cinéticos V_{max} y K_M fueron de 20.83 µmol/ml seg y 232.01 µmol/ml para la β-glucosidasa y de 685.01 µmol/ml seg y 1 240.34 µmol/ml para la endoglucanasa. En la hidrólisis enzimática, se obtuvo mayor concentración de azúcares reductores (43.134±1.09 g/L) (21.55 g/g) con la mezcla sinérgica de ambas enzimas, tras 48 h y un ajuste de pH. Los resultados permiten proponer el aprovechamiento del bagazo de agave comiteco para el cultivo de hongos comestibles, a la vez que para obtener extractos celulolíticos, como una alternativa de tratamiento en la producción de bioetanol y de reducción de residuos.

Palabras claves: setas, β-glucosidasa, endoglucanasa, purificación de proteínas, hidrólisis enzimática, azúcares reductores.

Capítulo 1. Introducción

En la actualidad un tema discutible que aqueja y compete a la humanidad es la crisis ambiental global. Hoy se sabe que los recursos son utilizados a un ritmo mayor a las capacidades de la naturaleza por producirlos; “esencialmente se trata de una contradicción entre el ritmo de los ciclos biogeoquímicos y el ritmo de los ciclos de producción humana, para un nivel determinado de desarrollo de las fuerzas productivas” (Tommasino et al. 2001). El uso de combustibles fósiles forma parte de esta problemática ambiental; dado que son energéticos no renovables, la producción es insuficiente para el crecimiento exponencial de la población, genera altas emisiones de CO_2, además de los altos precios de extracción, las tensiones geopolíticas, la competencia por acceso a nuevas regiones y la devastación ambiental por las técnicas de extracción usadas, como el fracking (facturación hidráulica) (André et al. 2012). Por todo lo anterior surge el interés de generar alternativas biotecnológicas para producir biocombustibles que sea acorde con la situación socio-ambiental que enfrenta la humanidad. Estos representan una alternativa de seguridad energética; debido a que pueden producirse a partir del polisacárido más abundante de la naturaleza, la celulosa (Chandrakant y Bisaria 1998; Sánchez 2010). Los biocombustibles han evolucionado integrando nuevas tecnologías así como varias técnicas y procedimientos, con el fin de obtener mejores rendimientos de etanol, métodos sostenibles, a la vez que más rápidos y sencillos (Cuervo et al. 2009); por lo que, actualmente la propuesta de los biocombustibles incluye hasta la cuarta generación (Guzmán-Moreno et al. 2016); no obstante, dadas las características socioambientales y agroproductivas de México, los de segunda generación se adaptan mejor; dado que utilizan como materia prima residuos lignocelulósicos (Cruz et al. 2011).

En el altiplano chiapaneco se produce una bebida espirituosa (alcohólica) a partir del agave cultivado localmente, denominada “comiteco”. Esta bebida resulta interesante dada la diversificación del uso del pulque establecido en la región. Para obtenerla se realiza una fermentación de la savia (pulque) y posteriormente una destilación (Lara-Hidalgo et al. 2017), y representa una industria artesanal como actividad económica a pequeña escala; con un cultivo disperso en alrededor de 27 850 km^2 (Reynoso-Santos et al. 2016). La identidad de la planta utilizada aún se discute, pues se han encontrado en

ciertas regiones organismos con características intermedias entre *Agave americana* var. *americana* y *A. salmiana* por lo que posiblemente se trate de un hibrido regional, conocido como “agave comiteco” (Reynoso-Santos et al. 2012). De esta agroindustria, surge como principal subproducto el bagazo de agave, de difícil manipulación, por sus características físicas y químicas, dentro de los cuales sobresale su contenido en lignina 10-20%, hemicelulosa 20-40% y celulosa 20-60% (Balam et al. 2006; Carmona et al. 2017; Naranjo et al. 2016; Ramírez-Cortina et al. 2012; Santiago et al. 2002) correspondiente con un sustrato apto para el crecimiento de hongos del género *Pleurotus* (Mata et al. 2017).

El cultivo de *Pleurotus* spp en residuos de *Agave* spp., en México ha sido reportado en otras investigaciones, como parte de los estudios de aprovechamiento de los subproductos de la industria del tequila y el mezcal (Baena-González 2005; Bernabé-González et al. 2004; Chairez-Aquino et al. 2015; Soto-Velazco et al. 1989; Heredia-Solís et al. 2014; Moreno 2020; Muthangya et al. 2014); y representan una alternativa de valorización de este subproducto. Para el caso del presente estudio era de particular interés, además de evaluar una alternativa de aprovechamiento del principal subproducto de la industria del comiteco, conocer el potencial de las enzimas celulolíticas del género *Pleurotus* excretadas durante el desarrollo del hongo en el bagazo del agave comiteco, y el uso para la producción de biocombustibles.

La producción y uso de celulasas como transformadoras de polisacáridos en azúcares fermentables se lleva a cabo a través de la ruptura de los enlaces glucosídicos β-1,4 de la celulosa y la hemicelulosa; por la acción de la endoglucanasa, la exoglucanasa y la β-glucosidasa (Goyal et al. 1991). El uso de celulasas como tratamiento de lignocelulosa ha sido reportado en varias investigaciones donde la obtención de enzimas se ha llevado a cabo principalmente en el grupo de los hongos microscópicos anamorfos de los géneros *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, entre otros (Gupta et al. 2016; Lynd et al. 2002; Olajuyigbe 2017). Sin embargo las especies comestibles del género *Pleurotus* son una alternativa de producción enzimática viable dado que son organismos inocuos a diferencia de los anamorfos, los cuales es bien conocida su relación antagonista con plantas y animales (Ocara et al. 2018); aunado a esto, se conoce muy bien el proceso de producción de las especies de *Pleurotus* e incluso dicho proceso ha sido estandarizado

y mejorado para obtener mayores rendimientos (Royse y Sánchez 2017); esto puede aprovecharse en otras tecnologías como la de los biocombustibles. A su vez la habilidad de este género para producir celulasas ha sido demostrada en diversas investigaciones (Álvarez et al. 2016; Khalil et al. 2011; Goyal y Soni 2011; Okereke et al. 2017; Reddy et al. 2003). “El éxito de estas enzimas extracelulares se debe a que encuentran ampliamente distribuidas en la naturaleza, son catalizadores altamente específicos y trabajan en condiciones de reacción moderada, lo que se traduce en tasas altas de conversión de sustrato a producto, así como un menor consumo energético” (Casas y Sandoval 2014), por lo que dicho proceso biotecnológico resulta ser aceptable en la producción de bioetanol.

Con este contexto se planteó el objetivo de determinar la producción de celulasas de cinco cepas de *Pleurotus* spp., durante su cultivo sobre bagazo de agave comiteco *Agave americana* L. y la viabilidad de utilizar los extractos celulíticos en la elaboración de caldos fermentables para la producción de bioetanol.

Después del Capítulo 1 (Introducción), este trabajo cuenta con dos capítulos adicionales. El capítulo 2 consiste en un artículo científico titulado Biodegradación del bagazo de agave comiteco por *Pleurotus* spp., fuente de celulasas como tratamiento hidrolítico en la producción de bioetanol; finalmente, el capítulo 3 integra las conclusiones de este estudio.

Capítulo 2. Artículo: Biodegradación del bagazo de agave comiteco por *Pleurotus* spp.: fuente de celulasas como tratamiento hidrolítico en la producción de bioetanol

Biodegradation of agave comiteco *Agave americana* L. bagasse by *Pleurotus* spp.: A source of cellulases in hydrolytic treatment for bioethanol production

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Biodegradation of agave comiteco *Agave americana* L. bagasse by *Pleurotus* spp.: A source of cellulases in hydrolytic treatment for bioethanol production

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Abstract

The objective of this study was to determine the cellulase production of five strains of *Pleurotus* spp. during their cultivation on agave comiteco bagasse and the feasibility of using cellulolytic extracts in the production of fermentable broths for bioethanol production. After cultivation, the basidiome production parameters varied between 41.2-65.7% (biological efficiency), 0.41-0.65 (yield), 0.60-0.90% (production rate), 16.4- 41.1% (Bioconversion) and 9.4- 21.3 g (mean mushroom size). At day 15 of growth, *Pleurotus djamor* showed the highest β-glucosidase activity (21.97±2.27 IU/ml); on day 33, the same strain had the highest endoglucanase activity (10.56±0.25 IU/ml). Both extracts were partially purified, and the kinetic parameters V_{max} and K_M were estimated (20.83 µmol/ml sec and 232.01 µmol/ml for β-glucosidase and 685.01 µmol/ml sec and 1 240.34 µmol/ml for endoglucanase). At the hydrolysis step, the highest concentration of reducing sugars (43.134±1.09 g/L; 21.55 g/g bagasse) was obtained by a mixture of the two enzymes acting synergistically after 48 h and with a pH adjustment. The results suggest that the use of agave comiteco bagasse for the cultivation of edible mushrooms while obtaining cellulolytic extracts is an alternative treatment in the production of bioethanol and waste reduction.

Keywords: mushrooms, β-glucosidase, endoglucanase, protein purification, enzymatic hydrolysis, reducing sugars.

Declarations

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Conflicts of interest/Competing interests: The authors declare that they have no conflict of interest.

Ethics approval: The ethics committee of El Colegio de la Frontera Sur has confirmed that no ethical approval is required because this paper does not contain any studies with experimental animals.

Consent to participate: Not applicable. This paper does not contain any studies with human

participants.

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Availability of data and material: Transparency and full availability of data.

Code availability: Total code availability.

Authors' contributions: All authors contributed to the study conception and design, material preparation, data collection and analysis were performed by Miriam Lagunes Reyes, José E Sánchez, René Humberto Andrade Gallegos and Rubén Fernando Gutiérrez Hernández.

Introduction

On the plateau of the state of Chiapas in Mexico, an alcoholic beverage known as "comiteco" is produced from locally cultivated agave. This beverage is interesting given the diversification of the use of pulque in the region. To obtain it, the sap is fermented and then distilled [1]. The production of comiteco represents a cottage industry as a small-scale economic activity, with dispersed cultivation over approximately 27 850 km^2 [2]. The identity of the plant used is still under discussion, as organisms with intermediate characteristics between *Agave americana* var. *americana* and *A. salmiana* have been found; thus, it is possibly a regional hybrid known as agave comiteco [3]. The main byproduct of this agroindustry is agave bagasse, which is difficult to handle due to its physical and chemical characteristics, including its 10-20% lignin, 20-40% hemicellulose and 20-60% cellulose contents [4-8], corresponding to a substrate suitable for the growth of mushrooms of the genus *Pleurotus* [9]. The cultivation of *Pleurotus* spp. on *Agave* spp. residues in Mexico has been reported in other research papers as part of several studies on the utilization of byproducts from the tequila and mezcal industries [10-16] and represent an alternative for the valorization of this byproduct. For the case of the present study, it was of particular interest, in addition to evaluating an alternative use of the main byproduct of the comiteco industry, to know the potential use of cellulolytic enzymes of the genus *Pleurotus* that are excreted during the development of mushrooms in the bagasse of agave comiteco and their use in the production of biofuels. Cellulases have been utilized mainly by anamorphic microscopic fungi of the genera *Aspergillus, Penicillium, Fusarium, and Trichoderma*, among others [17-19]. However, *Pleurotus* species are a viable enzyme production alternative since they are harmless organisms, unlike anamorphs, which are known to be antagonistic to plants and animals [20]; in addition, the *Pleurotus* production process has been standardized and improved to obtain higher yields [21]. In turn, the ability of this genus to produce cellulases has been demonstrated in several investigations [22-27]; as a result, this bioprocess turns out to be acceptable in the production of bioethanol from enzymatic hydrolysis. In this context, the objective of this study was to determine the cellulase production of five strains of *Pleurotus* spp. during their cultivation on agave comiteco bagasse and the feasibility of using cellulolytic extracts in the production of fermentable broths for bioethanol production.

Materials and methods

Mushroom strains

Strains of the edible mushrooms *Pleurotus djamor* ECS-123, *P. pulmonarius* ECS-190 and ECS-196, and *P. ostreatus* ECS-1123 and ECS-152 were used; they were obtained from the mycological collection of El Colegio de la Frontera Sur (ECOSUR). They were reactivated on potato dextrose agar (PDA) (pH 6.4) and were incubated at 24 °C for 15 days.

Preparation of inoculum

The inoculum was prepared by transferring colonized mycelium on sorghum grains sterilized at 1.05 kg/cm^2 (121 °C) for 30 min, according to the method of Quimio [28]. They were then incubated at 24 °C for 15 days and stored at 5 °C.

Agave comiteco bagasse preparation

Bagasse fibers from the agave comiteco *A. americana* L. were used as substrates for mushroom cultivation and for obtaining enzymatic extracts from *Pleurotus* spp. Bagasse was collected in San José de la Montaña and San Rafael Jocom, Comitán de Domínguez, Chis. The preparation consisted of direct sun-drying of the agave leaves until a humidity of less than 10% was achieved; they were then crushed to a fiber size of 3-5 cm in length and stored until use.

Cultivation of *Pleurotus* spp. in the bagasse of agave comiteco *A. americana* L.

The bagasse with 60% moisture and 4% hydrated lime was pasteurized by self-heating (60-65 °C) in a wooden box (1 m^3). The substrate was removed at 46 h, and the process was completed at 67 h [29-30]. The five strains were spawned (3%) in 3-kg substrate portions and placed in polypropylene bags [31]. Indications stated by Zadrazil and Kurtzman [32] for basidiome production were followed; this consisted of incubating the bags at 24 °C for 2-3 weeks and then inducing fruiting by controlling relative humidity (80-90%), temperature (22 °C) and continuous ventilation. Three harvests were obtained over a period of 72 ± 2 days.

Production variables

The evaluation of mushrooms was based on the biological efficiency (BE), which is the fresh weight of the carpophores divided by the dry weight of the substrate multiplied by 100; the production rate (PR), which is the quotient of the BE between the days from spawning to the last harvest; and the yield (Y), which is the quotient between the dry weight of the carpophores and the dry weight of the substrate [33]. The mean mushroom size (MMS) was also determined as follows: total weight of fresh mushrooms harvested/total number of mushrooms harvested and finally

bioconversion (B), which is shown as follows:

$$\%B = \frac{Iw - Fw}{Iw} \times 100$$

Iw: Initial dry weight of substrate

Fw: Final dry weight of substrate

Obtaining enzymatic extracts

The strains that showed the highest percentage of bioconversion in the previous experiment (ECS-123, ECS-152, ECS-196 and ECS-1123) were used for this stage. A colonized sorghum grain (0.5 cm in diameter) was inoculated into the center of a Petri dish containing 10 g of bagasse (70% moisture and 4% hydrated lime) sterilized at 1.05 kg/cm^2 (121 °C) for 30 min. Sterilized bagasse without inoculation was used as a negative control (C-). Petri dishes were incubated at 24 °C. Nine measurements were established over time (days 9, 12, 15, 18, 21, 24, 27, 30 and 33) for the detection of β-glucosidase and endoglucanase activity. Subsequently, 10 g of substrate colonized by each fungus was extracted, to which sodium citrate extraction buffer 0.05 M pH 4.8 was added for the detection of β-glucosidase; furthermore, for endoglucanase activity, sodium citrate extraction buffer 0.05 M pH 5.3 was used at a 1:2 ratio (substrate:buffer). Each mixture was homogenized and macerated for 20 min. The mixtures were then filtered with filter paper and centrifuged at 16 000 rpm for 20 min [34]. Cell-free extracts were kept refrigerated at 5 °C for no more than three days.

Determination of β-glucosidase and endoglucanase activities.

For the determination of β-glucosidase, the reaction consisted of 900 µl of 4-Nitrophenyl β-D-glucopyranoside 1.0 mM as substrate (with sodium citrate 0.05 M pH 4.8 as buffer) and 100 µl of enzyme extract. The reaction mixture was incubated for 10 min at 50 °C. It was then stopped with 500 µl of Na_2CO_3 1.0 M and measured spectrophotometrically (L6S UV-VS spectrophotometer) at 400 nm [34]. For endoglucanase, 250 µl of carboxymethyl cellulose substrate (2% CMC in sodium citrate buffer 0.05 M pH 5.3) and 250 µl of the enzyme extract were taken. The reaction mixture was incubated for 30 min at 50 °C [35-36]. Finally, the reaction was detected with 3,5-dinitrosalicylic acid (DNS), stopped with $KNaC_4H_4O_64H_2O$ 2.0 M and measured spectrophotometrically (L6S UV-VS spectrophotometer) at 540 nm [37]. One unit of enzyme activity (IU) was defined as the amount of enzyme required to release 1 µmol of glucose or 4-Nitrophenyl β-D-glucopyranoside per minute under the specified conditions.

Partial purification of the enzymatic extracts

The extracts obtained were subjected to precipitation with ammonium sulfate ($(NH_4)_2SO_4$) at 40% and 90% saturation at 5 °C for 1 h. The mixtures were centrifuged at 16 000 rpm for 30 min. They were then dialyzed on a high-retention cellulose membrane (32 mm, MWCO 12 400, Sigma-Aldrich) and suspended in a larger volume of sodium citrate 0.05 M pH 4.8 for β-glucosidase and pH 5.3 for endoglucanase for 24 h at 5 °C. The dialyzed sample was fractionated on a Macro-prep weak ion exchange column (Bio-Rad) previously equilibrated with citrate buffer 0.05 M (pH 5.3 and 4.8, respectively); a polyester fabric filter was used. Proteins were eluted by a NaCl gradient (0.5 M, 1.0 M, 1.5 M, 2.0 M and 3.0 M) at a drop rate of 1 drop/4.7 min (endoglucanase), 1 drop/3.3 min (β-glucosidase) and 1 drop/3.3 min (β-glucosidase). Subsequently, 3-ml fractions were collected, and those showing β-glucosidase and endoglucanase activity were collected and concentrated again on a cellulose membrane (32 mm, MWCO 12 400, Sigma-Aldrich) (the purification procedure was performed at 5 °C). Protein concentration was determined by the Bradford method [38].

Determination of kinetic parameters

The maximum velocity (V_{max}) and affinity constant (K_M) for CMC and 4-Nitrophenyl β-D-glucopyranoside were determined based on substrate hydrolysis at different concentrations at 50 °C and pH 4.8 for β-glucosidase and pH 5.3 for endoglucanase. This determination was based on the Lineweayer-Burk model [39].

Substrate preparation for enzymatic hydrolysis

For the hydrolysis experiment, two types of substrates were established; one consisted of dried and crushed agave comiteco leaves with an alkaline chemical pretreatment, and the other consisted of spent agave comiteco substrate (after 90 days of growth of *P. ostreatus*). Both substrates were reduced to a fine powder (less than 25-µm particle size).

Chemical pretreatment

To hydrolyze the lignin of *A. americana* bagasse, hydrogen peroxide (33%) with 2% hydrated lime was added to the two types of substrates at a ratio of 1 g of substrate in 20 ml of alkaline solution (1:20 w/v). The pH of the mixtures was adjusted to 11.5 (NaOH 5.0 M) and incubated (Lab-Line 3525 Orbit Incubator Shaker) at 50 °C for 3 h and 120 rpm. Finally, the mixtures were successively washed with distilled water until clear water and a neutral pH (7.0) were obtained [40-41].

Detoxification of compounds generated in the chemical pretreatment of the substrate

Both types of substrates were subjected to an extraction of lignin hydrolysis products [42]. For this purpose, they were immersed in hot water (80 °C) for 15 min. Subsequently, they were filtered and immersed in an alcohol-acetone solution (1:2) for 15 min more; finally, they were filtered and washed with distilled water until clear water was obtained. They were then dried at 60 °C for 12 h.

Enzymatic hydrolysis of cellulose with partially purified extracts

To determine the degradation efficiency of the cellulases on the agave comiteco substrate, a reaction was carried out with the partially purified extract of each enzyme and with the mix of both cellulases. A commercial cellulase (from *Trichoderma viride* -Sigma-Aldrich) was taken as a positive control (C+), and a negative control (C-) without enzyme was also used. The reaction consisted of 5 IU/ml, 200 mg of pulverized substrate (spent by a *Pleurotus* species and/or alkaline pretreated substrate) and 1 ml of sodium citrate buffer 0.05 M. The reaction was incubated (Lab-Line 3525 Orbit Incubator Shaker) at 50 °C for 2 h and 24 h at pH 5.0 and 120 rpm. One more assay was performed at 48 h with the same reaction mix; in this case, a pH of 5.0 was adjusted for controls and enzyme mix, with a pH of 4.8 for β-glucosidase and a pH of 5.3 for endoglucanase. Hydrolysis was detected by reducing sugar analysis by the DNS method [37].

Experimental design

The experimental design for the analysis of the parameters BE, Y, PR, MMS and B consisted of a single-factorial randomized design with five levels (strains), and 10 replicates were established so that 50 experimental units were obtained. For the determination of the enzymatic activities of β-glucosidase and endoglucanase, a unifactorial randomized experimental design with five levels (4 strains and a negative control) was established. Three replicates and nine measurements were established over time (days 9, 12, 15, 18, 21, 24, 27, 30 and 33). A total of 270 experimental units were obtained. In the hydrolysis experiment, a randomized bifactorial experimental design was established as follows: factor one was composed of five levels (C+, endoglucanase, β-glucosidase, mix and C-), while factor two was composed of two levels (spent substrate and alkaline pretreated substrate). In addition, three replicates and three measurements were made over time (2 h, 24 h and 48 h); a total of 90 experimental units were obtained.

Statistical analysis

For the analysis of BE, Y, PR, MMS and B, one-way analysis of variance (ANOVA) was performed, and significant differences were analyzed by Tukey's HSD multiple comparisons test (α=0.05). For β-glucosidase and endoglucanase activities, repeated measures analysis of variance

(ANOVA) was performed, and a Tukey HSD (α=0.05) multiple comparisons test was applied. In the enzymatic hydrolysis experiment, a multivariate analysis of variance (MANOVA) was performed, accompanied by a Tukey HSD test (α=0.05). Statistical analyses were performed with the help of R software version 4.0.3, and graphs were generated with STATISTICA 8 software.

Results

Production parameters of *Pleurotus* spp. cultivation on agave comiteco *A. americana* L. bagasse

The highest value of BE% (65.78 ± 16.47) was obtained with strain *P. ostreatus* ECS-1123. This strain was similar (statistical group "a") to strains *P. ostreatus* ECS-152 (57.22 ± 7.05), *P. pulmonarius* ECS-190 (53.80 ± 13.15) and *P. pulmonarius* ECS-196 (52.41 ± 7.37). All of them were significantly different from *P. djamor* ECS-123 (41.24 ± 10.07), which showed the lowest BE% ($p<0.05$) (Table 1). Concerning Y, the highest value was obtained with strain ECS-1123 (0.658 ± 0.064), while *P. djamor* ECS-123 obtained the lowest value (statistical group "c", 0.412 ± 0.039) ($p<0.05$). In the case of PR%, no significant differences were found in almost all the strains evaluated (PR% range 0.901 ± 0.22 to 0.708 ± 0.09), except for strain ECS-123 (0.606 ± 0.14), which was significantly lower ($p<0.05$). The MMS obtained was the highest for strains ECS-1123 (21.37 ± 5.74 g) and ECS-190 (16.17 ± 3.41 g), which were placed in statistical group "a". Strains ECS-196 (13.86 ± 4.99 g) and ECS-152 (13.46 ± 2.44) in group "b" were significantly higher than strain ECS-123 (9.42 ± 3.97), which was placed in group "c" ($p<0.05$). Finally, the data directly related to the biodegradation (B) of agave comiteco indicated that the strain that most converted lignocellulose was strain ECS-123 (41.09 ± 0.10), followed by strains ECS-152 (19.06 ± 0.06), ECS-196 (18.05 ± 0.08), ECS-1123 (16.44 ± 0.08) and ECS-190 (14.30 ± 0.06) ($p<0.05$).

β-glucosidase activity

Analysis of variance showed that the highest β-glucosidase activity was present for the four strains during the first 18 days after spawning (8.70 ±0.54 IU/ml to 21.97 ±2.27 IU/ml) and then showed a reduction in activity from day 21 until day 33 of growth (6.34 ±0.13 IU/ml to 8.90 ±0.45 IU/ml) (Fig. 1). The negative control showed a significantly lower β-glucosidase activity of 4.49 ±0.03 IU/ml ($p<0.05$). No significant differences were found between the strains evaluated from day 21 and up to 33 days of growth ($p>0.05$). The highest β-glucosidase activity was detected on day 15 in the *P. djamor* ECS-123 strain (21.97 ±2.27 IU/ml) ($p<0.05$); therefore, this extract was chosen for partial purification (Fig. 1). In turn, the analysis of variance showed significant differences in

β-glucosidase activity between the strains evaluated and the negative control on different days of growth (F=37.405, p=0.0000).

Endoglucanase activity

The analysis of variance showed that strains *P. djamor* ECS-123 and *P. ostreatus* ECS-152 in the first 21 days of colonization had significantly higher endoglucanase activity (1.73 ±0.30 IU/ml to 3.75 ±0.88 IU/ml) than strains *P. pulmonarius* ECS-196 and *P. ostreatus* ECS-1123 (0.28 ±0.04 IU/ml to 1.73 ±0.25 IU/ml) ($p<0.05$) and the negative control, which showed lower activity on all days of measurement (lower than 0.75 ±0.51 IU/ml) ($p<0.05$) (Fig. 2). From day 21 onwards, there were gradual increases in enzyme activity in all strains, and up to day 27 of growth, all four strains showed no significant differences in endoglucanase activity (1.68 ±0.87 IU/ml to 7.74 ±0.95 IU/ml) ($p>0.05$). On day 33, the *P. djamor* strain ECS-123 showed the highest significant endoglucanase activity (10.56 ±0.25 IU/ml) ($p<0.05$) among all days and all strains evaluated; therefore, the extract obtained with this strain was chosen for partial purification (Fig. 2). Finally, the analysis showed significant differences in endoglucanase enzymatic activity between the four strains evaluated and the negative control on different days of growth (F=12.841, p=0.0000).

Partial purification of enzyme extracts

The purification procedure showed that β-glucosidase recorded an initial 1.11-fold increase in specific activity, and 75.6% was recovered after precipitation with ($(NH_4)_2SO_4$) at 40%. Subsequently, after concentration with 90% ($(NH_4)_2SO_4$), a 1.70-fold increase and 50.0% recovery were recorded (Table 2). Finally, in the last purification step, the β-glucosidase profile recorded an increase in β-glucosidase activity (4,608 IU/ml, 5,250 IU/ml, 5,050 IU/ml and 3. 965 IU/ml) corresponding to fractions 4, 5, 6, and 7, respectively, which coincide with increases in total proteins (0.524 mg/ml, 0.642 mg/ml, 0.959 mg/ml and 0.570 mg/ml, respectively), whereby β-glucosidase was eluted at NaCl concentrations of 0.5 M, 1.0 M and 1.5 M (Fig. 3). Such fractions were collected to be used in the hydrolysis experiment. From this step, a specific activity of 11,838 IU/mg was recorded, with a 2.16-fold increase in activity, and 27.3% of the activity was recovered (Table 2). In the case of endoglucanase, a 1.15-fold increase in specific activity was observed, and 84.4% was recovered after precipitation with ($(NH_4)_2SO_4$) at 40% saturation; it subsequently recorded a 1.32-fold increase, and 59.2% was recovered after precipitation at 90% ($(NH_4)_2SO_4$) (Table 3). In the last purification step (ion exchange column), the elution profile (Fig. 4) showed peaks of increased endoglucanase activity (3,487 IU/ml, 3,406 IU/ml, 2,217 IU/ml and 2. 522

IU/ml) in fractions 8, 9, 10 and 11, which in turn corresponded with peaks of increased total proteins (1.00 mg/ml, 0.910 mg/ml, 0.620 mg/ml and 0.610 mg/ml, respectively); thus, endoglucanase was eluted at NaCl concentrations of 1.5 M and 2.0 M (Fig. 4). From this procedure, a specific activity of 9,388 IU/mg, a 2.42-fold increase in enzyme activity and a recovery of the specific activity of 44.9% were recorded (Table 3).

Kinetic parameters

The kinetic parameters obtained indicated a higher affinity to the substrate of β-glucosidase, with a K_M value of 232.018 µmol/ml, lower than that of endoglucanase, with a K_M value of 1 240.34 µmol/ml. In contrast, endoglucanase showed a higher reaction rate V_{max} of 685.016 µmol/ml sec, which was higher than that of β-glucosidase of 20.833 µmol/ml sec (Table 4).

Enzymatic hydrolysis

Multiple analysis of variance did not register significant differences in either substrate or among the different enzymatic treatments ($p>0.05$) at 2 h of reaction and showed yields lower than 25.93±0.03 g/L (12.96 g/g of dry substrate used) (Fig. 5). In turn, at 24 h of reaction, significant increases in reducing sugars were recorded for almost all enzymatic treatments, which were higher in the alkaline treatment than in the spent substrate (26.23±0.05 to 33.17±0.03 g/L) (13.37-16.58 g/g) ($p<0.05$). The positive control showed significantly higher concentrations of reducing sugars at 2, 24 and 48 h of reaction (with pH adjustment) ($p<0.05$), which were related to the purification quality of the commercial product. Multiple analysis of variance also revealed that the pH adjustment for each enzyme at 48 h promoted significant increases in reducing sugars ($p<0.05$) at all the established reaction times; in turn, the mix of the two enzymes was one of the best treatments for obtaining reducing sugars. Although the highest concentration of sugars was obtained with the commercial enzyme (C+) (52. 61±0.90 g/L) (26.30 g/g), the mix of both enzymes showed units of 39.84±0.59 g/L (19.92 g/g) for the spent substrate and with the alkaline treatment of 43.13±1.09 g/L (21.55 g/g) ($p>0.05$) (Fig. 5). Multiple analysis of variance indicated a significant response of reducing sugars produced by the partially purified extracts, the mix and the controls at different reaction times and different pH values for the two types of substrates used (spent agave comiteco *A. americana* L. substrate and substrate with alkaline pretreatment; $F=2.911$, $p=0.0117$) (Fig. 5).

Discussion

This study confirms that the bagasse of agave comiteco *A. americana* L. is suitable for the growth and fructification of the edible species *P. djamor*, *P. ostreatus* and *P. pulmonarius*, without the

need for supplementation. Additionally, during the cultivation of these species, lignocellulose biodegradation takes place; therefore, it is possible to obtain enzymes that can hydrolyze the substrate into fermentable sugars, which can be used to produce bioethanol. The production parameters indicate that the strains presenting the highest BE, Y, PR and MMS presented the lowest B. The bioconversion percentage indicates the amount of substrate consumed by the fungus during its cultivation and is mostly related to the production of degradation enzymes [17]. The strain *P. djamor* ECS-123 presents the highest significant percentage of bioconversion and the lowest BE, Y, PR and MMS. In this sense, this strain represents a good alternative for cellulolytic enzyme production, although it presented low basidiome production parameters (with respect to the other strains tested). The results obtained are comparable to those reported by Cayetano et al. [43] in rice straw and coconut fruit residues, by Chaubey et al. [44] in wheat straw, by Hasan et al. [45] in sugarcane bagasse, by Motato et al. [46] in banana stems and pseudostems and by Salmones et al. [47] in coffee pulp, which present similar BE values of 24.1-61.8%. This in turn indicates that the strain *P. djamor* ECS-123 has a main enzymatic potential but can also be used as food. The strains *P. ostreatus* ECS-152 and ECS-1123 and *P. pulmonarius* ECS-190 and ECS-196 had higher BE values (52.4-65.78), Y (0.577-0.658), PR% (0.70-0.90) and MMS (13.46-21.37) and represent a viable alternative crop and food where agave comiteco bagasse is abundant. These results are comparable to those obtained by other authors when cultivating *Pleurotus* spp. [46, 48-55], among others.

In turn, it was also confirmed that the *Pleurotus* spp. strains studied (ECS-123, ECS-152, ECS-196 and ECS-1123) use the hydrolytic enzymes endoglucanase and β-glucosidase to biodegrade the cellulose of *A. americana* bagasse to obtain energy. Strain *P. pulmonarius* ECS-190 was discarded for obtaining cellulases because it presented the lowest bioconversion percentage. This strain has been reported to possess an interesting ligninolytic capacity that is useful for the degradation of chlorothalonil and endosulfan [56-57]. The expression of both enzymes is related to the cellulosome, multienzyme complexes whose components act synergistically and in a coordinated manner to hydrolyze cellulose from a substrate [58]. The cellulosome is composed of three types of enzymes, namely, endoglucanase, β-glucosidase and exoglucanase, and together, they degrade cellulose; therefore, although only two types of enzymes were studied in this experiment, the presence of the three types of cellulases should not be overlooked [59-60]. The *P. djamor* ECS-123 strain showed the highest activity for both enzymes on almost all days of measurement

compared to the other strains studied. This response may be related to the induction and activation of cellulases by the presence of the bagasse cellulose structure after efficient lignin hydrolysis. Since a low conversion rate of cellulose to simple sugars by the presence of lignin has been demonstrated, this is due to the nonspecific binding of cellulases to lignin by hydrophobic interactions [61]. Indeed, other studies have shown that *P. djamor* ECS-123 presents significant ligninolytic activity [62-63], which could indicate why its cellulases present higher activity, as they are less protected by the remaining lignin. However, the enzymatic analysis showed an initial trend of β-glucosidase production in the first 18 days of colonization and a subsequent decrease until day 33 of growth. At the same time, endoglucanase showed an opposite trend, i.e., a low production in the first 18 days and then a constant increase until day 33, when it showed the highest production. This trend could be related to the products of the coordinated activity of the cellulosome after recognition of the main cellulose structures (amorphous cellulose, crystalline cellulose to cellobiose and finally glucose) [60, 64]. The ranges of β-glucosidase and endoglucanase activity obtained do not differ considerably from those reported in other investigations [65-69] in which agro-industrial wastes and *Pleurotus* species were used; such differences are related to the different substrates used, the different species and strains, and the conditions in which the cultures of the organisms were developed. Studies agree that cellulase activity is related to the first stages of colonization; however, this enzyme expression has been mainly related to the recognition of cellulose as well as sophorose, xylobiose, lactose, D-xylosa and L-sorbose [70] and not necessarily to the cycles or life stages of the fungi, indicating the low substrate specificity of cellulases, which confers the potential to be used in other processes of plant matter degradation [71].

Regarding the partial purification of both enzymes by column chromatography, the enzymes were obtained through a gradient method at different molar concentrations due to their physicochemical characteristics. In the case of endoglucanase, the isoelectric point ranges from 3.3 to 6.7, and the molecular weight ranges from 30-55 kDa [72], while for beta-glucosidase, the isoelectric point ranges from 3.4 to 4.4, and the molecular weight ranges from 59 to 62 kDa [73]. Purification is possible through a gradient of higher ionic strength as the salt concentration increases; thus, proteins are unadsorbed from the matrix or column in order of binding strength. This binding strength is lower for high-molecular-weight molecules [74]. Thus, proteins weakly bound to the matrix elute faster and are the ones collected in the first fractions [75]. Since higher β-glucosidase activity was obtained at NaCl concentrations of 0.5, 1.0 and 1.5 M, it is inferred that the binding

strength is lower, which is corroborated by the high molecular weight with respect to endoglucanases, as it was unadsorbed at 1.5 and 2.0 M concentrations; according to Macarrón [73], it has a lower molecular weight. In the elution profile of endoglucanases, several peaks of enzyme activity are observed, which could represent the isoenzymes of endoglucanases. Ülker and Sprey [76] describe that these isoenzymes complement the reactions to degrade the different cellulose structures and are identified by their different molecular weights, isoelectric points, amino acid sequences, and posttranslational modifications, among other differences.

The V_{max} values of both enzymes are high. It has been documented that when this parameter is high, it means that the active centers of the enzyme are saturated with substrate at high substrate concentrations [77]. Regarding the K_M value, for endoglucanase, this parameter was high compared to β-glucosidase. This parameter determines the affinity of the enzyme with the substrate, so when low values are obtained, it indicates that the enzyme binds strongly to the substrate, quickly becoming saturating with it [78]. In this sense, there is higher affinity to the substrate for β-glucosidase compared to endoglucanase. The values reported for endoglucanase are similar to those reported by Sibanda et al. [79] and higher than those reported by Chinedu et al. [80] and Pham et al. [81]; in the case of beta-glucosidase, lower kinetic values are reported than those reported by Wei et al. [82] and Mallek and Belghith [83]. Such differences are related to the producing organism and the extraction and purification procedures performed. In the purification process, gradual increases in the purification factors of both enzymes were obtained, although the increases were not considerably large if a steady increase was recorded; after three different steps, it was possible to purify both enzymes almost twice, and a maximum recovery of 75-80% of the total enzymatic activity of both was obtained. The results suggest optimizing the established method to selectively eliminate elements that inhibit or repress the activity of these enzymes, as is the case for cellular parts of the hyphae, pigments or elements themselves from the lignin hydrolysis that arises at the same time [84-85]. Similar research proposes the use of stepwise chromatographic methods to optimize the purification of the cellulolytic enzyme complex, as well as selective cell fractionation and the use of protease inhibitors [73, 84, 86-90]. However, the ability to produce multienzyme complexes from *Pleurotus* spp. that degrade lignocellulose makes these species candidates for establishing simultaneous bioconversion protocols; that is, generating extracts rich in lignin-degrading enzymes (ligninases, peroxidases, and laccases), hemicellulose (xylanases), and cellulose (cellulases) [65]; thus, ligninolysis helps to increase the effect of cellulases.

The hydrolysis confirmed that the efficiency of cellulose degradation depends mainly on the coordinated actions of the different types of enzymes since in all times in which the enzymatic hydrolysis was carried out, the simultaneous use of both enzymes showed a higher content of reducing sugars compared to the treatments in which the enzymes acted alone. In turn, the analysis showed that the β-glucosidase activity for all the established times obtained lower reducing sugar yields than the endoglucanase treatment and the positive control. This behavior could be related to the fact that β-glucosidases are in charge of completing cellulose degradation after the actions of endoglucanases and exoglucanases, which cut the different regions of cellulose into cellobiose and cellooligosaccharides or cellodextrins. These products, in turn, recognize β-glucosidases as substrates and complete the degradation process [71]. Hydrolysis analysis also showed the susceptibility of enzyme activity to pH since enzyme reactions were enhanced when adjusted to the optimum pH of each enzyme according to Mandels and Reese [91], with a considerable increase at 48 h. The higher amount of reducing sugars obtained (43.13 g/L) by cellulases compared to other residues in a time of approximately 48 h was higher than those reported by Bonilla et al. [92] for banana peels and by Rodriguez and Piñeros [93] for palm residues and was equal to that reported by Zhu et al. [94] for rice residues; however, such amounts of reducing sugars are low compared to those reported by Cruz-Cardona et al. [95] for barley hulls, by Albernas-Carbajal et al. [96] for sugarcane bagasse, by Chen et al. [97] for corn straw, by Tejeda et al. [98] for pruning residues, by Xu et al.[99] for soybean straw and by Han et al. [100] for wheat straw. Comparing the hydrolysis performed with other byproducts of *Agave* species, lower yields were obtained with respect to time [101-104]. The differences presented are related to the chemical compositions of the lignocellulosic residues, and the methodological strategies to access cellulose, which promote a fast degradation rate and obtain reducing sugars. Some of the aforementioned research suggests the use of chemical pretreatments (acids and bases); however, they may present a problem, as they are a major source of environmental pollution. Finally, the analysis also showed higher concentrations of reducing sugars in the substrate with the alkaline chemical pretreatment than with the spent substrate; it is inferred that the substrate with the alkaline pretreatment had a higher hydrolysis of lignin, therefore promoting the expression of cellulases [9]. Moreover, it is inferred that in the substrate spent by the fungus, the sugars released from the enzymatic activity are absorbed by the fungus itself, so the concentration of sugars will be lower; however, if there is no organism consuming the reducing sugars, they will be released to the medium, and therefore, higher yields will be recorded [105].

The results suggest redirecting the hydrolysis method proposed to improve the yields of reducing sugars, which would be useful for fermentation to bioethanol.

Conclusions

The bagasse of agave comiteco *A. americana* allows the cultivation of the edible mushrooms *P. pulmonarius, P. djamor* and *P. ostreatus* and obtains BE values between 41.24-65.78%. Additionally, after its biodegradation, the residue acquires a potential interest in producing the enzymes β-glucosidase and endoglucanase in the ranges of 5.48-11.83 IU/mg and 3.86-5.13 IU/mg, respectively. Likewise, the carbohydrate composition of bagasse allows hydrolysates to be obtained with a considerable content of fermentable sugars (43.13 g/L) (21.56 g/g of dry substrate used) that can be used in bioethanol production. A finer study of the ligninolytic/cellulolytic potential of the edible fungal strains used can optimize the acquisition of fermentable sugars and, therefore, bioethanol. Finally, it is concluded that cultivation, enzyme production and enzymatic hydrolysis are bioprocesses that can be used for the revaluation of *Agave* spp. residues, with important beneficial effects on the conservation of a healthy environment, as well as alternatives for the use of native resources.

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Tables

Table 1. Production parameters BE, Y, PR, MMS and B of five *Pleurotus* spp. strains after 72 ± 2 days of cultivation on agave comiteco *A. americana* bagasse.

Strain	BE (%)	Y	PR (%)	MMS (g)	B (%)
***P. djamor* ECS-123**	41.24 ± 10.07^{b}	0.412 ± 0.039^{c}	0.606 ± 0.14^{b}	9.42 ± 3.97^{c}	41.09 ± 0.10^{a}
***P. ostreatus* ECS-152**	57.22 ± 7.05^{a}	0.577 ± 0.027^{b}	0.806 ± 0.09^{ab}	13.46 ± 2.44^{bc}	19.06 ± 0.08^{b}
***P. pulmonarius* ECS-190**	53.80 ± 13.15^{ab}	0.538 ± 0.051^{b}	0.769 ± 0.18^{ab}	16.17 ± 3.41^{ab}	14.30 ± 0.06^{e}
***P. pulmonarius* ECS-196**	52.41 ± 7.37^{ab}	0.524 ± 0.029^{b}	0.708 ± 0.09^{ab}	13.86 ± 4.99^{bc}	18.05 ± 0.08^{c}
***P. ostreatus* ECS-1123**	65.78 ± 16.47^{a}	0.658± 0.064^{a}	0.901 ± 0.22^{a}	21.37 ± 5.74^{a}	16.44 ± 0.08^{d}

Equal letters in the same column indicate no significant difference between strains $p<0.05$ (Tukey HSD multiple comparisons test). BE means biological efficiency, Y the yield, PR the production rate, MMS is the mean mushroom size and B the percentage of bioconversion.

Table 2. Activity and yields of the different purification steps of β-glucosidase from *P. djamor* ECS-123.

Purification steps	β-glucosidase				
	Enzymatic activity UI/ml	Total protein mg/ml	Specific activity UI/mg	Yield %	Purification factor
Extraction	21.977	4.010	5.480	100	1
Ammonium Sulfate 40%.	16.631	2.728	6.096	75.67	1.11
Ammonium sulfate 90%.	11.003	1.179	9.332	50.06	1.70
Ion exchange column / Dialysis.	6.014	0.508	11.838	27.36	2.16

Table 3. Activity and yields of the different purification steps of endoglucanase from *P. djamor* ECS-123.

Purification steps	Endoglucanase				
	Enzymatic activity UI/ml	Total protein mg/ml	Specific activity UI/mg	Yield %	Purification factor
Extraction	10.563	2.732	3.866	100	1
Ammonium Sulfate 40%.	8.921	2.002	4.455	84.45	1.15
Ammonium sulfate 90%.	6.259	1.219	5.133	59.25	1.32
Ion exchange column / Dialysis.	4.746	0.505	9.388	44.93	2.42

Table 4. K_M and V_{max} kinetic parameters of endoglucanase from partially purified *P. djamor* ECS-123 with different concentrations of CMC at 50 °C, pH 5.3, and β-glucosidase using 4-Nitrophenyl β-D-glucopyranoside at 50 °C, pH 4.8.

Enzyme	K_M	V_{max}
β-glucosidase (pH 4.8, 50°C)	232.018 µmol/ml	20.833 µmol/ml sec
endoglucanase (pH 5.3, 50°C)	1 240.34 µmol/ml	685.016 µmol/ml sec

Captions to figures

Figure 1 β-glucosidase activity of four *Pleurotus* spp. strains and a negative control on different days of growth in agave comiteco *A. americana* L. bagasse at 50 °C, pH 4.8

Figure 2 Endoglucanase activity of four *Pleurotus* spp. strains and a negative control on different days of growth in agave comiteco *A. americana* L. bagasse at 50 °C, pH 5.3

Figure 3 Elution profile of β-glucosidase and total proteins of fractions obtained from *P. djamor* ECS-123

Figure 4 Elution profile of endoglucanase and total proteins obtained from fractions from *P. djamor* ECS-123

Figure 5 Reducing sugars from hydrolysis performed with partially purified extracts of *P. djamor* ECS-123 alone, mix and controls, under different reaction times (2 h, 24 h and 48 h) and at different pH values on agave comiteco bagasse as spent *Pleurotus* substrate and agave comiteco bagasse as substrate with alkaline pretreatment

Figures

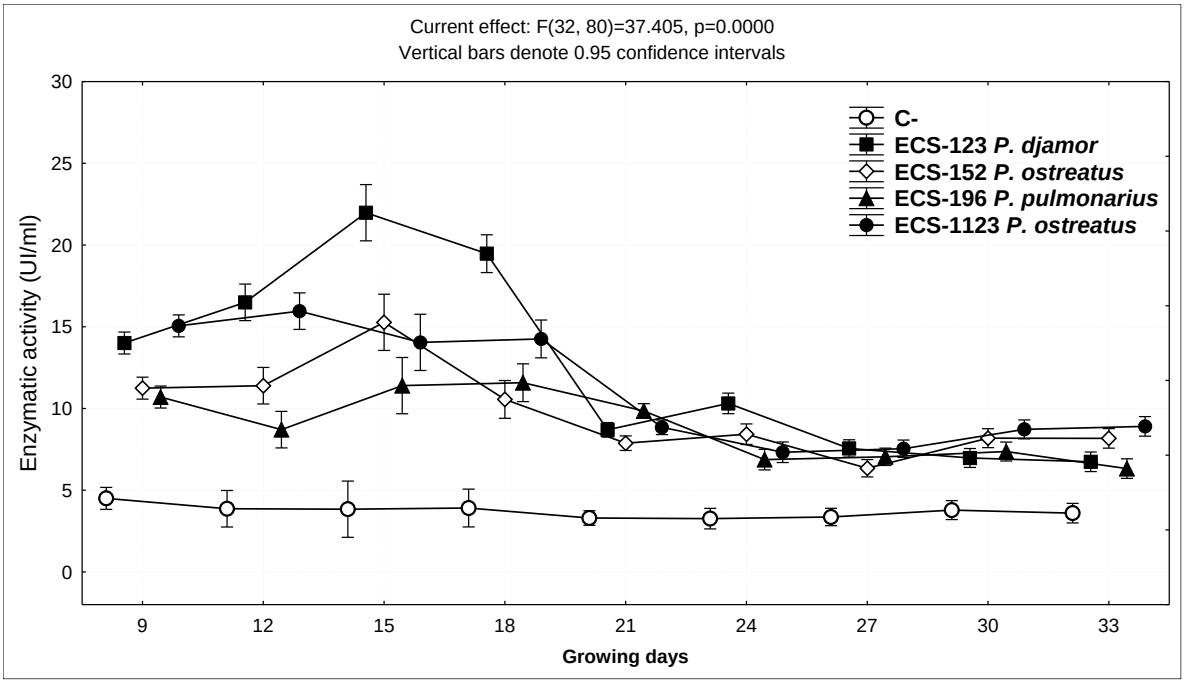

Fig. 1

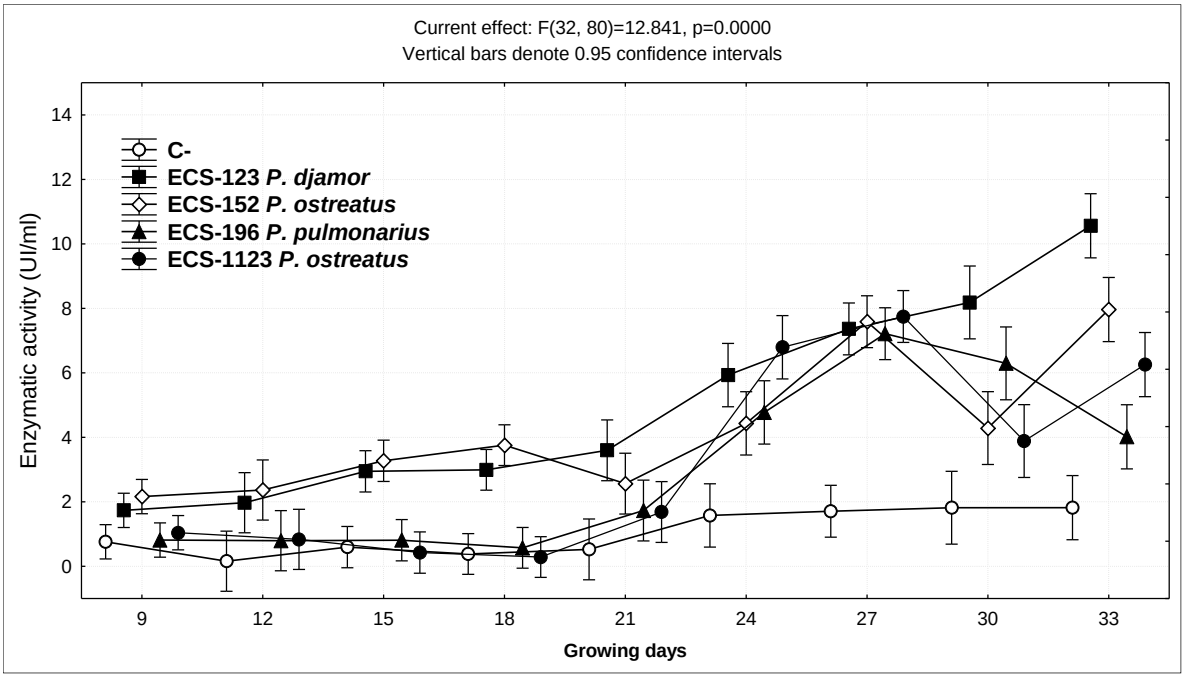

Fig. 2

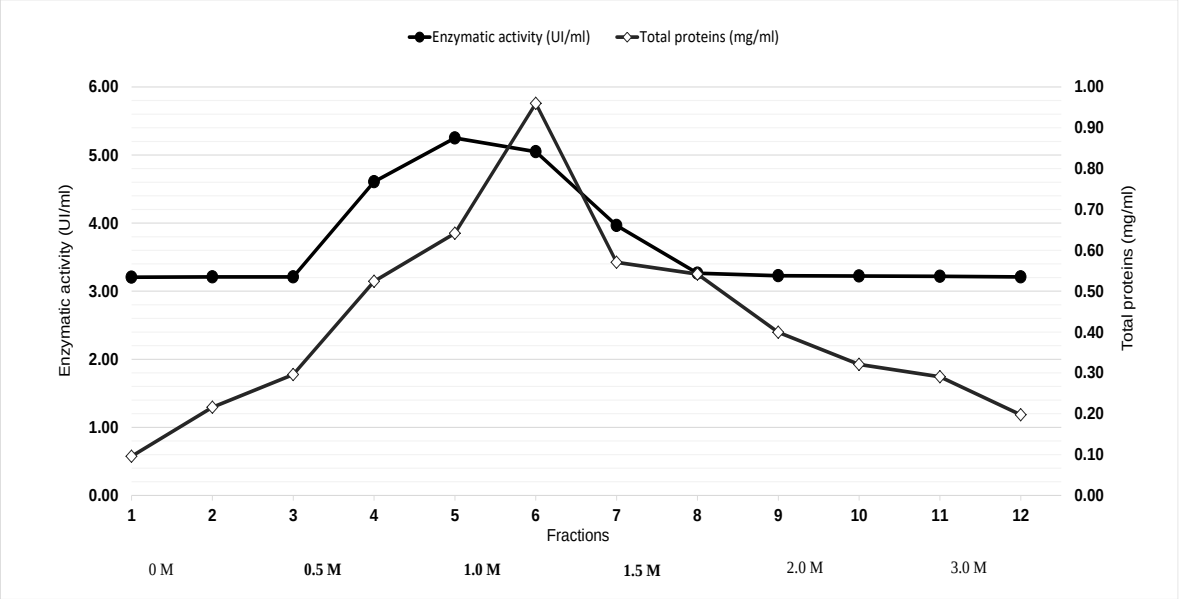
Enzymatic activity (UI/ml)
Total proteins (mg/ml)
Enzymatic activity (UI/ml)
Total proteins (mg/ml)
Fractions
0 M
0.5 M
1.0 M
1.5 M
2.0 M
3.0 M

Fig. 3

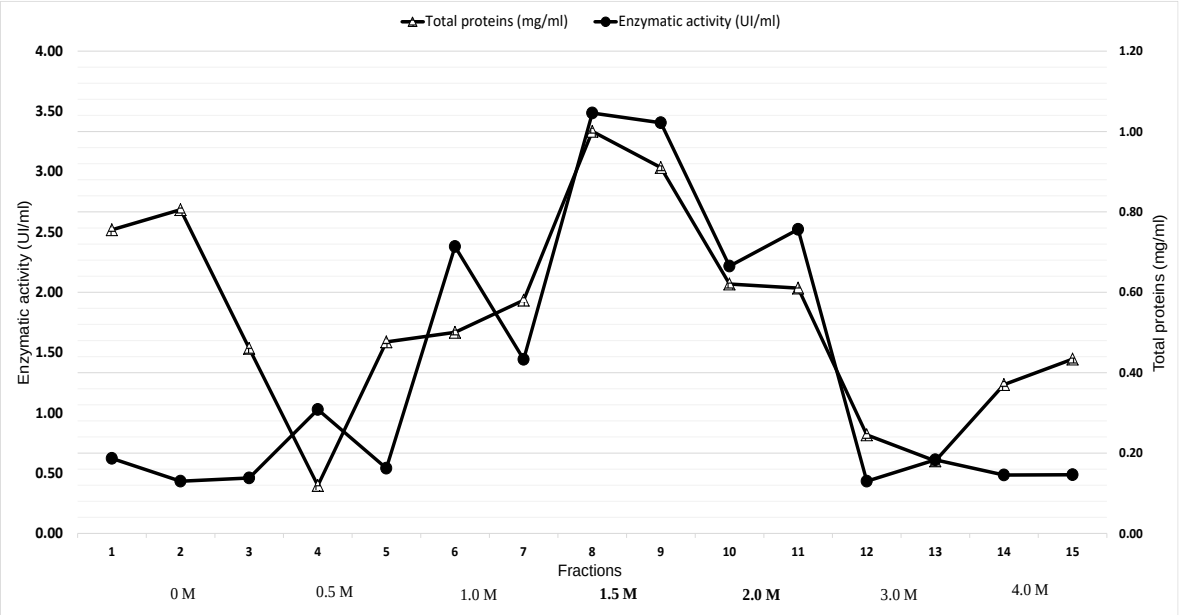
Total proteins (mg/ml)
Enzymatic activity (UI/ml)
Enzymatic activity (UI/ml)
Total proteins (mg/ml)
Fractions
0 M
0.5 M
1.0 M
1.5 M
2.0 M
3.0 M
4.0 M

Fig. 4

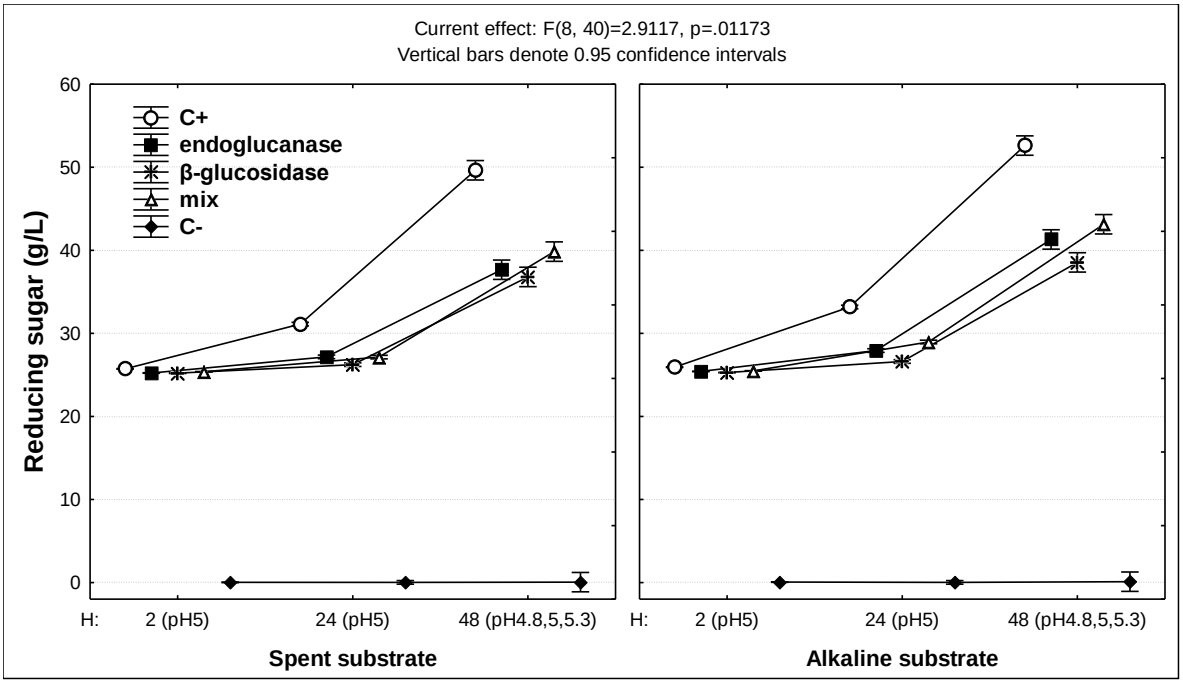
Current effect: F(8, 40)=2.9117, p=.01173
Vertical bars denote 0.95 confidence intervals
Reducing sugar (g/L)
60
50
40
30
20
10
0
C+
endoglucanase
β-glucosidase
mix
C-
H:
2 (pH5)
24 (pH5)
48 (pH4.8,5,5.3)
Spent substrate
H:
2 (pH5)
24 (pH5)
48 (pH4.8,5,5.3)
Alkaline substrate

Fig. 5

Capítulo 3. Conclusiones

El bagazo de agave comiteco *A. americana* permite el cultivo de los hongos comestibles *P. pulmonarius*, *P. djamor* y *P. ostreatus*, obteniendo valores de EB% entre 41.24-65.78. Adicionalmente, tras su biodegradación el residuo adquiere un interés potencial por la producción de las enzimas β-glucosidasa y endoglucanasa en un rango de 5.48-11.83 UI/mg y 3.86-5.13 UI/mg respectivamente. Así mismo, la composición de carbohidratos del bagazo permite obtener hidrolizados con un considerable contenido de azúcares fermentables (43.13 g/L) (21.56 g/g de sustrato seco utilizado) que puede ser utilizados en la producción de bioetanol. Los resultados sugieren optimizar el método establecido de purificación con el fin de eliminar selectivamente elementos que inhiben ó reprimen la actividad de estas enzimas como es el caso de partes celulares de las hifas, pigmentos o elementos mismos de la hidrólisis de lignina que surge a la par (Megha et al. 2015; Sánchez 2010). Investigaciones similares proponen el uso de métodos cromatográficos escalonados con el fin de optimizar la purificación del complejo enzimático celulolítico, así como un fraccionamiento celular selectivo y el uso de inhibidores de proteasas (Ahmed et al. 2009; Islam y Roy 2018; Macarrón 1992; Megha et al. 2015; Potprommanee et al. 2017; Rubio et al. 2005; Yassien et al. 2014). Sin embargo, la capacidad de producir complejos multienzimático de *Pleurotu*s spp. que degrade la lignocelulosa hace a estas especies candidatas para establecer protocolos de bioconversión simultanea; es decir, generar extractos ricos en enzimas que degradan la lignina (ligninasas, peroxidasas y lacasas), la hemicelulosa (xilanasas) y celulosa (celulasas) (Elisashvili et al. 2006); de esta manera, la ligninólisis coadyuva a incrementar el efecto de las celulasas. Por otra parte, un estudio más fino del potencial ligninolítico/celulolítico de las cepas de hongos comestibles utilizados puede optimizar la obtención de azúcares fermentable y por lo tanto de bioetanol.

Finalmente, se concluye que el cultivo, la producción de enzimas y la hidrólisis enzimática, son bioprocesos que pueden ser utilizados para la revalorización de residuos de *Agave* spp, con importantes efectos benéficos en la conservación de un ambiente sano; así como alternativas de aprovechamiento de recursos nativos.

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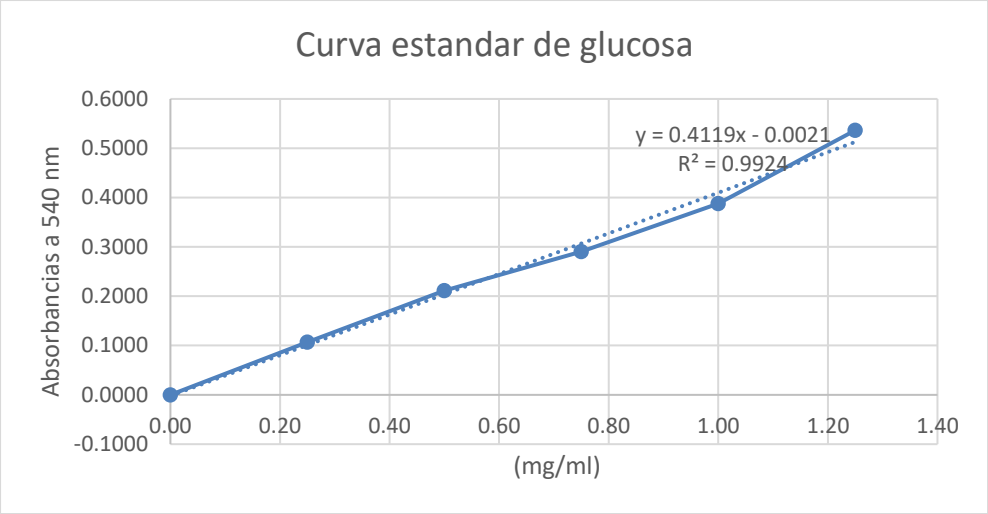
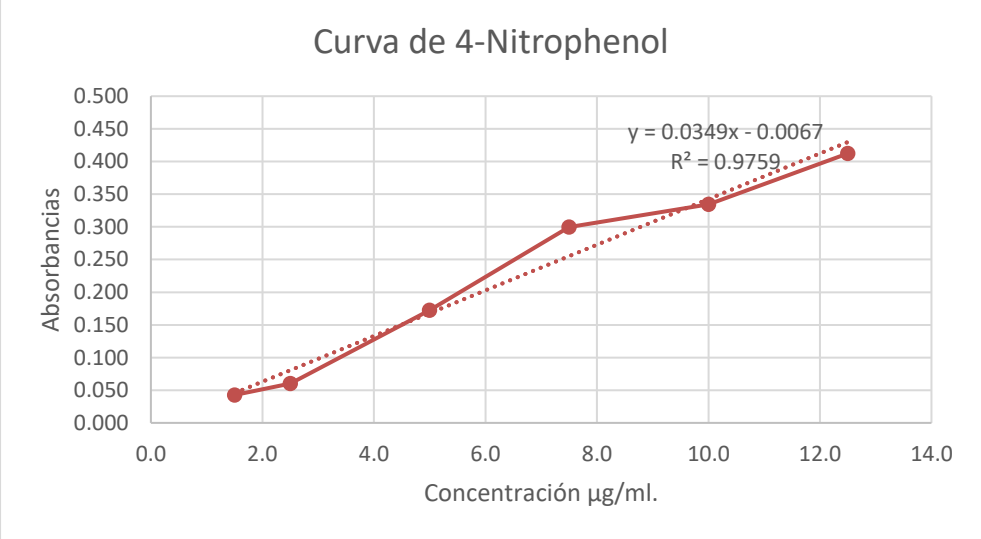
Anexos

Curva de calibración de glucosa para la determinación de la endoglucanasa por azúcares reductores.



Curva de calibración de 4-Nitrophenol para la determinación de la β-glucosidasa



Curva de calibración de Bradford para la determinación de las proteínas totales

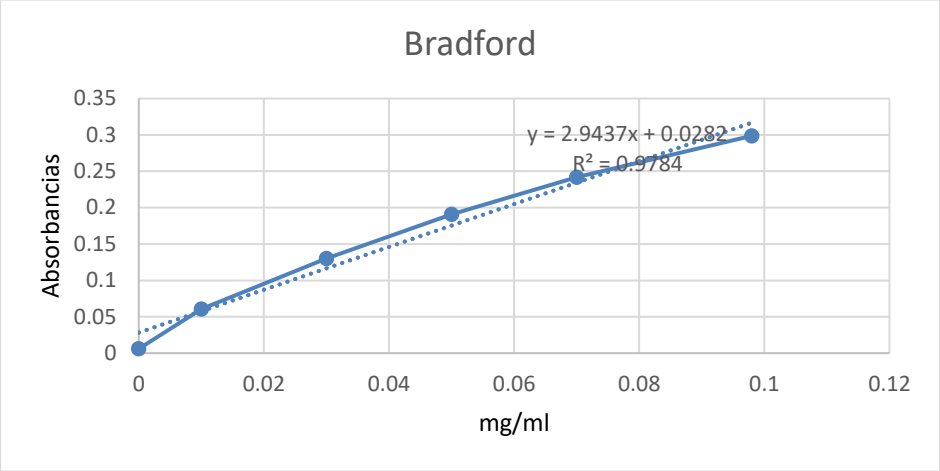


Diagrama de Lineweaver-Burk para la determinación de la K_M y la V_{max} de la endoglucanasa

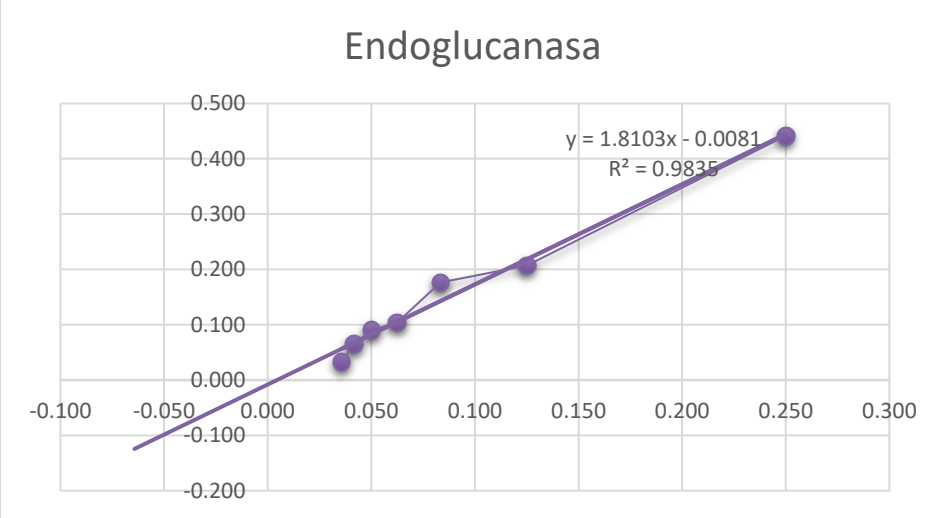
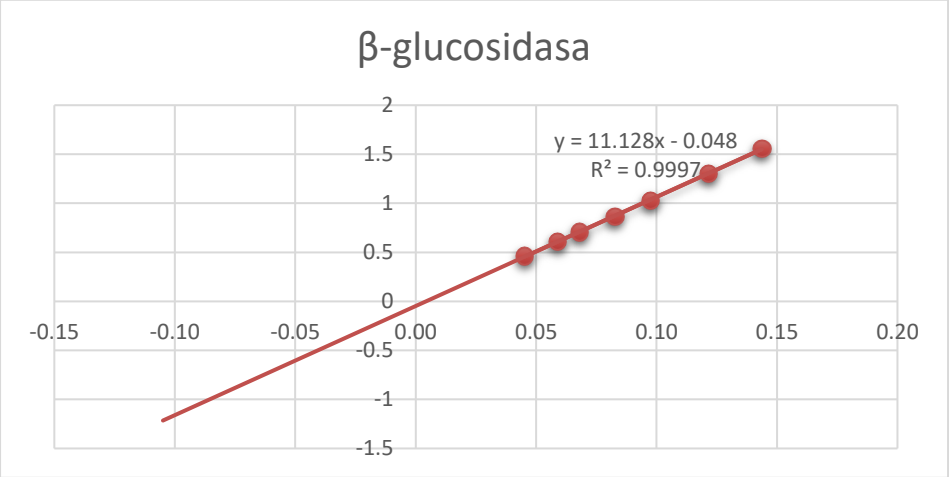


Diagrama de Lineweaver-Burk para la determinación de la K_M y la V_{max} de la β-glucosidasa



Curva de calibración de glucosa para la determinación de la hidrólisis enzimática