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FIBROLYTIC ACTIVITY OF *PODAXIS PISTILLARIS* FUNGUS IN SUBMERGED CULTURE

ACTIVIDAD FIBROLYTICA DEL HONGO *PODAXIS PISTILLARIS* EN CULTIVO SUMERGIDO

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ABSTRACT

Podaxis pistillaris is a fungus commonly found in most desert areas worldwide. The oval shaped peridium, the rigid woody stipe, plus a 10-15 µm spore size stand out among its morphological features. Even though this fungus is used for human consumption and for several traditional remedies, a lack of knowledge regarding its fibrolytic enzymatic system still prevails. This fungus was collected from the central region of the Sonoran desert (29° 07.23´ 97" LN and 110° 53.58´ 02" LW, 238 masl). In order to study its enzymatic system on common fibers, *P. pistillaris* was grown in a specific submerged culture in order to determine total cellulases, xylanases and laccase**s**. The maximum cellulolytic (501.7 U·mg^{-1}), as well as xylanolytic (157.8 U·mg^{-1}) activities, were detected after 18 cultivation days, whereas the highest laccase specific activity (179.6 U·mg^{-1}) was registered after 15 days at 40° C. The thermostability of total cellulases, xylanases and laccases was found within temperatures ranging from 40 to 60° C. The present study represents the first report of *P. pistillaris* fibrolytic activity in submerged culture.

Keywords: cellulases, xylanases, laccases, thermostable enzyme, enzymatic activity.

RESUMEN

Podaxis pistillaris es un hongo que se encuentra comúnmente en la mayoría de las áreas desérticas del mundo. Entre sus características morfológicas se destacan su peridio de forma ovalada, estípite leñoso y rígido, y esporas entre 10-15 µm. A pesar de que este hongo se usa para el consumo humano y varios remedios tradicionales, el conocimiento sobre su sistema enzimático fibrolítico es aún escaso en la literatura. Este hongo fue recolectado en la región central del Desierto de Sonora (29° 07.23'97" LN y 110° 53.58' 02" LO y 238 msnm) y, para estudiar el sistema enzimático, se cultivó en un medio sumergido específico para determinar la actividad de celulasas totales, xilanasas y lacasas. La máxima actividad celulolítica (501.7 U·mg^{-1}), así como xilanolítica (157.8 U·mg^{-1}), se detectó después de 18 días de cultivo, mientras que la mayor actividad específica para lacasa (179.6 U·mg^{-1}) se registró después de 15 días de incubación a 40° C. La termoestabilidad de celulasas, xilanasas y lacasas se encontró en el rango de temperaturas entre 40 y 60° C. Este estudio representa el primer reporte de actividad fibrolítica de *P. pistillaris* en cultivo sumergido.

Palabras clave: celulasas, xilanasas, lacasas, enzima termoestable, actividad enzimática.

INTRODUCTION

Podaxis pistillaris (L.) Fr. fungus (*Agaricaceae*, Basidiomycota) is a widely distributed and frequently cited species for both arid and semi-arid areas around the world, where temperatures can be as high as 60° C and soil nutrients rather limited (Herrera and Ulloa 1998; Esqueda *et al*., 2012). The uses for spores of this species include makeup for women and for skin health care by the Wayuu aboriginal peoples in the upper Colombian Guajira (Villalobos *et al*., 2017). In addition, bioactive compounds have been attributed to this species (Feleke and Doshi, 2017). Recently, Vásquez-Dávila (2017) reviewed nutraceutical (edible), medicinal and cosmoceutic traditional uses for *P. pistillaris*, and reported morphology in Durango and in Oaxaca, Mexico although only little is known about this species enzyme system. It is generally known that most wood degrading fungi transform the complex carbohydrates from plant tissue, such as the hemicellulose, cellulose and lignin, into soluble products through the action of fibrolytic enzymes (Dashtban *et al.*, 2010), but *P. pistillaris* is found in poor desert soils where these macromolecules are scarce. The oxidation of phenolic compounds from lignin is catalyzed by laccases (EC. 1.10.3.2); in filamentous fungi in general, approximately 30 types of these enzymes have been identified, which are mainly involved in conidia pigmentation, lignin degradation, pathogenicity and fruiting body formation. Furthermore, they are used in the industry as part of the delignification and decolorization within the pulp and paper industry and for textile care in food and beverage processing, cosmetic, etc. (Hollmann *et al*., 2008). On the other hand, the cellulases responsible for degrading cellulose by breaking hydrogen bonds and releasing glucose and cellobiose chains (Atreya *et al.*, 2016), are widely used in the paper industry, detergent manufacture, fruit and vegetable juices extraction and filtration, as well as in the edible oil processing (Chander *et al.,* 2011). In addition, cellulases have been used in animal feed supplementation, as they help

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fibers degradation, increasing the forage nutritional value (Murad and Azzaz, 2010).

Finally, xylanases are responsible for hydrolyzing the xylans glycosidic linkages, which is the most abundant polysaccharide in nature after cellulose (Ho and Iylia, 2015). Xylanases are used in the filtering process by the brewing industry, paper bleaching and by the bread bakery as a texturizer (Kanwar and Devi, 2012).

Extensive studies are available regarding the use of thermophilic fungi, as an important source of thermostable and thermotolerant enzymes with applications in biotechnology and industry. Nevertheless, new enzyme sources are needed for catalytic features and thermal stability. In this regard, and due to the extreme conditions in which *P. pistillaris* thrives, the present study explores its capability of fibrolytic enzymes production and their thermostablility, in submerged culture.

MATERIALS AND METHODS

Isolation

The *P. pistillaris* fruiting bodies were collected in Sonora, Mexico (29° 07.23´ 97"LN and 110° 53.58´ 02"LW, 238 masl); three fruiting bodies were sampled, and recorded both the macroscopic and microscopic characteristics, as proposed by Villalobos *et al.* (2017) for species identification. Spores were prepared for examination by light and scanning electron microscopy using a JEOL JSM-5410LV (Peabody, MA, USA). Isolation was performed in two commercial media, potato dextrose agar (PDA) and malt extract agar (MEA), from Difco, incubated at 27° C for 15 days. Working cultures were made on PDA.

Molecular identification

Podaxis pistillaris identification was done through molecular techniques using Internal Transcribed Spacer (ITS) sequences analysis. The genomic DNA was isolated from this species fructification, as described by Plaza *et al*. (2004). The ITS sequences PCR amplification was carried out using ITS1 (5´ TCC GTA GGT GAA CCT GCG G 3'), ITS4 (5´ TCC TCC GCT TAT TGA TAT GC 3´) and ITS5 (5´ GGA AGT AAA AGT CGT AAC AAG G 3´) primers. The combinations ITS1/ITS4 and ITS5/ITS4 target both the ITS1 and ITS2 regions, including the 5.8S rRNA gene. A total of 50 ng of DNA, PCR GoTaq Green Master Mix (Promega) and 0.4µM of each primer, in a final volume of 25 µL, were used for amplification reactions. The PCR amplification was carried out in a DNA Engine BioRad thermocycler (BIORAD) using an initial denaturation step at 94° C for 3 min, followed by 30 cycles at 94° C for 1 min, 55° C for 1 min and 72° C for 2 min, with a final extension of 72° C for 10 min. PCR products were observed by means of a 1% agarose gel electrophoresis, cloned into PCR 2.1 vector (Invitrogen) and plasmid DNA was obtained by alkaline lysis method (Green y Sambrook, 2012). The nucleotidic sequences determination was performed at the Genomic Analysis and Technology Core (GATC) at the University of Arizona (Tucson, AZ. USA). Sequences were analyzed by comparison with reference to *P. pistillaris* sequences contained in the GenBank nucleotide sequence database using the BLAST algorithm (Altschul *et al*., 1990).

Submerged culture medium

For total cellulolytic activity, the composition per liter was as follows: 20 g Carboxymethyl cellulose (CMC), 2 g of KH_2PO_4, 0.5 g of $MgSO_4$-$7H_2O$, 0.1 g of $CaCl_2$, 2 g of NH_4NO_3, adjusted to pH 8. For xylanolytic activity, the composition per liter was as follows: 20 g Xylan (Birch xylan), 2 g of KH_2PO_4, 0.5 g of $MgSO_4$- $7H_2O$, 0.1 g of $CaCl_2$, 2 g of NH_4NO_3, adjusted to pH 8. Thirdly, for laccases, the composition per liter was as follows: 20 g CMC, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$- $7H_2O$, 0.1 g of $CaCl_2$, 2 g of NH_4NO_3, adjusted to pH 8. Cu_2SO_4 up to a final 0.001 mM was added as inductor (Mazumder *et al.*, 2008). All cultures were incubated under constant stirring at 120 rpm in an Orbital Shaker (Lab-Line). The submerged culture was inoculated with three disks of PDA working cultures and incubated for a 24 days period at 120 rpm at 40° C, being sampled every 3 days. The biomass production was reported as dry weight.

Fibrolytic enzyme activity

The total cellulolytic activity was determined by monitoring the release of the reducing sugars as a function of time in the reaction mix containing: 5 mg of Whatman paper filter No. 1 as substrate and 250 µL crude extract (culture medium supernatant). Reducing sugars were determined with 1 mL of phenol at 5%, and 5 mL of concentrated sulfuric acid (Dubois *et al.*, 1956). Computed from the glucose concentration expressed as ($mg \cdot mL^{-1} \cdot min^{-1}$), the enzyme activity unit (U) was defined as 1 µmol of glucose released per minute.

On the other hand, total xylanolytic activity was determined by monitoring the release of reducing sugars to the medium from xylan and quantified by the phenol-sulfuric acid method (Dubois *et al.*, 1956), using a xylose standard curve ($mg \cdot ml^{-1}$). The reaction mix contained 250 µL crude extract and 15 mg xylan as substrate. For each sample, 1 mL of phenol (5%) and 500 µL concentrated sulfuric acid was used for sugar release (Dubois *et al*., 1956). The enzymatic activity unit (U) was defined as the amount of enzyme that catalyzes the release of 1µmol xylose$\cdot min^{-1}$.

Concerning laccase activity for this study, it was assayed spectrophotometrically by measuring the syringaldazine oxidation at 530 nm for 3 min (Manole *et al*., 2008). The reaction mixture contained 167 µL crude extract and 64 µL syringaldazine 0.216 mM in a 730 µL 0.1 M citrate-phosphate buffer, pH of 5.5. The crude extract soluble protein was quantified by Bradford's method (Bradford, 1976).

Thermostability

The extracts were incubated at 40, 50, 60 and 70° C, for 4 hours each, in order to measure the enzyme thermostability. Extract samples were obtained every 60 min, to determine the corresponding enzymatic activity, and reported as percentage of remaining activity from the control test.

RESULTS

Identification and isolation of *P. pistillaris*

The *P. pistillaris* fruiting bodies collected in the Sonoran desert had a height ranging between 12 and 15 cm with a white oval peridium and flaky texture on mature sampled specimens (Fig. 1a), as well as a spore diameter between 13 and 15 µm, and characteristically distal ridge (Fig. 1b).

Figure 1. Morphology of *P. pistillaris*. a) Oval white and scaly peridium, the stipe is woody and rigid. b) Spores observed by scanning electron microscopy with 13 and 15 µm diameter, and characteristic distal ridge.
Figura 1. Morfología de *P. pistillaris*. a) Peridium escamoso blanco y ovalado, estípite leñoso y rígido. b) Se observaron esporas mediante microscopía electrónica de barrido con un diámetro de 13 y 15 µm.

The morphological characteristics featured by the collected specimens in the study hereby, such as height, peridium shape, texture and color, are consistent with those reported by Vásquez-Dávila (2017), who reported the same morphological characteristics in *P. pistillaris* specimens collected in North America. This species has been found as part of the Sonoran mycobiota, thriving in the desert in poor saline soils and limited humidity of this Mexican region.

The species morphological identification was complemented with a molecular identification. The latter due to the influence on the aspect of fruiting bodies exerted by climatic conditions, under which such organism grows and presents different morph types that may occasionally hinder its identification.

When comparing the PCR products sequences obtained using ITS primers, to those reported in the GenBank data base; the collected specimens molecular identification in this study, was attained with a 99% identity value compared to other *P. pistillaris* ITS sequences reported (Johnson and Vilgalys, 1999).

Once identified, a submerged culture in a liquid medium at 40° C and pH 8 was established for this species in order to determine the enzymatic activity in the supernatant.

Submerged culture enzyme production

Concerning the kinetics of the three aerobic fermentations, the fibrolytic activity for each of the *P. pistillaris* cultures, and their yield as related to carbon source, is shown in Table 1. The biomass yield (Yx/s) obtained for all media showed values close to 0.2 gX/gS. The culture age for the highest cellulases and xylanases enzymatic activity are 18 days from media inoculation, with values of 501.7 and 157.8 $U{\cdot}mg^{-1}$ of protein, respectively. The highest laccase activity reached, was 179.6 $U{\cdot}mg^{-1}$ of protein, which is a quite remarkable performance. These results are not optimized and only reflect *P. pistillaris* production of fibrolytic enzymes in submerged culture conditions specific to this study. Regarding growth in the different media assayed, the biomass yield as the relation between the product obtained and the substrate consumed (usually the carbon source), was similar. No differences were found within values of different carbon sources submerged cultures. The latter reflects in the specific growth rate (µ) as the cellulose carbon source was changed to hemicellulose, the values are fairly similar; both substrates utilization and conversion ability was comparable, with $Y_{x/s}$ values close to 0.2 gX/gS. Nevertheless, it is necessary to mention that in the wild, the conditions reported here are not the same, as *P. pistillaris* is not found in ponds, but dry poor saline soils in the desert. The basis for the study of the enzymatic system in submerged culture is set in the study to ease excreted enzymes recuperation. Still far from becoming an optimized process however, it shows the feasibility to grow this fungus in submerged culture conditions in a basal medium, suitable for enzyme purification. The above show us that this species is capable of secreting enzymes, but they have not yet been characterized.

Total cellulases, xylanases, and laccases activity

Figure 2 shows total cellulase, xylanase and laccase enzymes specific activities in the *P. pistillaris* crude extract and the biomass profile. The profile for laccase activity (Fig. 2A) evidently presents increasing values during the first 15 inoculation days, reaching maximum peaks by the 15th day (179.6 $U{\cdot}mg^{-1}$). Interestingly, this activity decreases when biomass profile reaches a plateau; which strongly suggests an association between the enzyme activity and growth. The

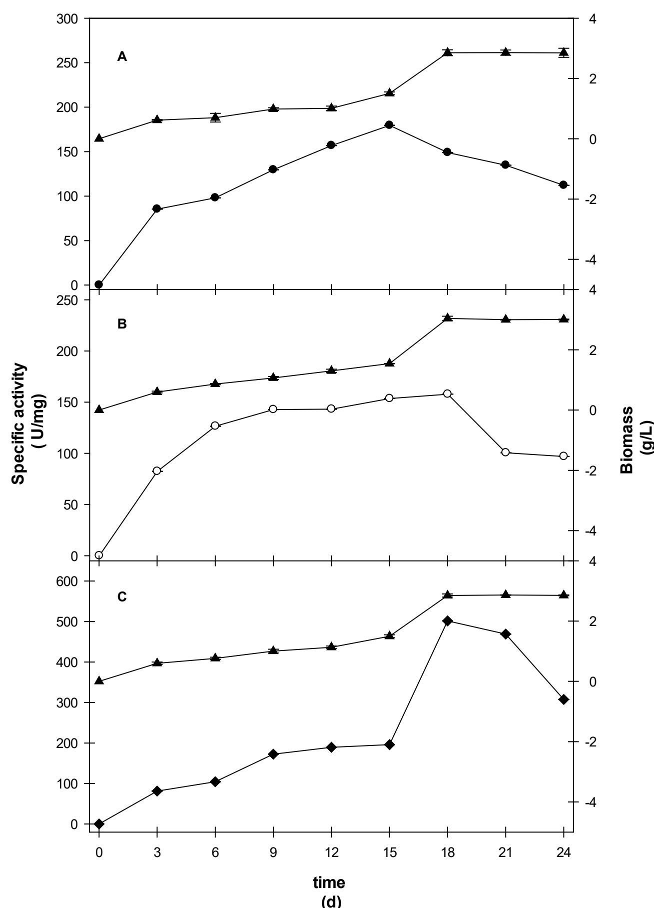

Figure 2. Enzymatic activity in a submerged culture batch supernatant of *P. pistillaris*: (A) laccase (●); (B) xylanase (○), and (C) cellulase (◆). The biomass was determined as g/L and shown for each culture (▲). The enzymatic activity units are defined as μmol/min of glucose for cellulases, and xylose for xylanases; for laccases, the oxidation of μmol/ mL of syringaldazine is used to define units. The experiments were performed in triplicate.

Figura 2. Actividad enzimática en el sobrenadante del cultivo discontinuo sumergido de *P. pistillaris:* (A) lacasa (●); (B) xilanasa (○) y (C) celulasa (◆). La biomasa determinada como g /L también se muestra para cada cultivo (▲). Las unidades de actividad enzimática se definen como μmol / min de glucosa para celulasas y xilosa para xilanasas; para las lacasas, la oxidación de μmol / ml de siringaldazina se usa para definir unidades. Los experimentos se realizaron por triplicado.

P. pistillaris specific activity values are significantly lower for those reported for diverse white rot fungi, where the laccase enzyme plays an important role in wood pulp delignification (Inalbon *et al*., 2015). On the other hand, the soil where this species usually grow shows much lower amounts of lignin, than that present in the substrate for any white rot fungi. Consequently, it is hypothesized by the authors that this enzyme is utilized by *P. pistillaris* for the building of the fruiting body to protect it from the sun, since it thrives in sandy soils and clearly with no mandatory association to plants that may provide constant shading. As reported by García-Oduardo *et al*. (2017), laccase participates in the fungus *Pleurotus spp*. mineralization, as well as in some degree of its lignification. Therefore, a comparable function is proposed by the authors for *P. pistillaris*. Further research is needed to fully explain the relatively high values found for laccases in this species.

Accordingly, in Fig. 2B, total xylanases activity values of 82.3 U·mg^{-1} of proteins can be appreciated by the 3rd incubation day, reaching maximum values by the 18th day with 157.8 U·mg^{-1} of proteins (almost twice higher than initial), with a comparable pattern, dropping in magnitude when biomass values reach a plateau. Concerning the case of the xylanase activity in *P. pistillaris* culture, higher values to those reported for *Pleorotus ostreatus* were observed by Elisashvili *et al*. (2008). Xylanase activity was also reported by these same authors with 56 U·mg^{-1} of proteins for *P. ostreatus*. It is significant that these are white rot fungi, which grow on wood. *P. pistillaris* xylanolytic activity is consistent; being a fungus found in soil, showing a lower ratio in terms of xylanolytic activity compared to wood decay related fungi was expected. Nevertheless, xylanase activity of this species was greater than that reported by Zambare *et al*. (2011) with 485 U·L^{-1} for a soil consortium, while in the present work, *P. pistillaris* showed 1001 U·L^{-1}, after a two weeks incubation period (Table 1).

Table 1. Fibrolytic activity of *P. pistillaris* in minimal broth media.
Tabla 1. Actividad fibrolitica de *P. pistillaris* en medio de cultivo mínimo.

Culture medium	Time (d)	X (g·l^{-1})	μ (d^{-1})	$Y_{x/s}$	Enzymatic activity (U·l^{-1})	Specific activity (U·mg^{-1})
Cellulase	18	2.849	0.12	0.189	4134.01	501.7
Xylanase	18	3.04	0.13	0.202	1001.05	157.8
Laccase	15	2.855	0.12	0.190	1459.84	179.6

X= mass of micelia; μ = specific growth rate; Y x/s= biomass yield.

Finally, Fig. 2C shows total cellulases activity, increasing 6 times its initial value by the 18th incubation day (501.7 U·mg^{-1}); then, the activity decreased to a 307.5 U·mg^{-1} total cellulase activity on the 24th day, again when biomass reached and steadily balanced a plateau. Although this were crude extracts and not purified enzymes, these results clearly evidence the capacity of *P. pistillaris* to produce fibrolytic enzymes associated to growth. Further research on purification and characterization is on its way to generate more conclusive results, since activities are for total activity and the endo, exo, enzymes involved for each enzymatic system are yet to be determined for this fungus. The combined action of cellobiohydrolase, endo and exoglucanases present within the medium are reflected by the total cellulase activity assay. Additionally, 69.17 U·mg^{-1} of protein (Márquez *et al*., 2007) specific activity was shown for the fungus *Trametes* spp. It is noteworthy that these fungi does not belong to the same family, so these comparisons with *P. pistillaris* are meant to be taken with due caution. However, it was shown that this species has higher values than those previously reported for other comparable soil fungi. A cellulase activity ranging between 201 and 344 U·L^{-1}, at the moment of inoculating a consortium of soil microorganisms in different substrates, was reported by Zambare *et al*. (2011) while in our study, a much higher total cellulases activity was revealed by *P. pistillaris* (4134 U·L^{-1}, Table 1). However, the activity assessment conditions differ slightly from the authors.

Thermostability

The criteria used to classify microorganisms as thermophilic has led to also consider the optimum temperature under which certain metabolites are produced, including enzymes (Blumer-Schuette *et al.*, 2008). *P. pistillaris* is not an extremophile fungus; however, its hydrolytic enzymes thermostability becomes interesting for its ability to grow under high desert temperatures (40-50° C). Results obtained from the thermostability study of the enzymes activity present in the extract, mainly cellulases, xylanases and laccases, are shown in Fig. 3. The *P. pistillaris* total cellulolytic activity values, determined for the first hour of incubation, were close to an 80% residual activity at 40 and 50° C incubation temperature (Fig. 3A). After incubation for 2 hours, the remaining activities were below 70%; and at 70° C, the remaining activity decreased lower than 40%.

Thermostability was assayed for *P. pistillaris* xylanolytic activity (Fig. 3B), and showed similar values close to 80% of residual activity at 40 and 50° C temperatures; such activity decreased further, when the extract was incubated at 60 and 70° C. During the following incubation hours, this activity kept on decreasing. These results are limitedly comparable to those reported by Li et al. (2010), who studied the effect of temperature on the xylanase activity of *Streptomyces rameus*, in purified native and recombinant enzymes, finding 60% of the initial activity at 70° C after 30 min. Finally, the laccase enzymes activities were thermostable at 40, 50 and 60° C for an incubation period of 4 hours as shown in Fig. 3C. The supernatants measured activities showed residual enzymatic activity well above 50% at 60° C for one hour. Special behavior in laccase activity was found up to two hours at 60° C. Although these were crude extracts and not purified enzymes, these results clearly put in evidence mild thermal stability for the system of enzymes produced by *P. pistillaris*. While the values for specific activity reported by Zhu et al. (2011) were lower for the laccase produced by *Trametes versicolor* than the present work on *P. pistillaris, T. versicolor* laccase is much more stable at 50, 60 and 70º C for 120 minutes, showing relative activity by 95, 90 and 55% respectively. Nevertheless, our results are similar to those determined for *Chaetomium* sp laccase, at 50° C and 60º C for 90 min, the enzyme retained is around 85% and 50% respectively (Mtibaà et al., 2017), and with *Myceliophtora thermophila* laccase which is stable between 50 and 60º C for 1 hour, especially at pH 6 (Mate and Alcalde, 2017).

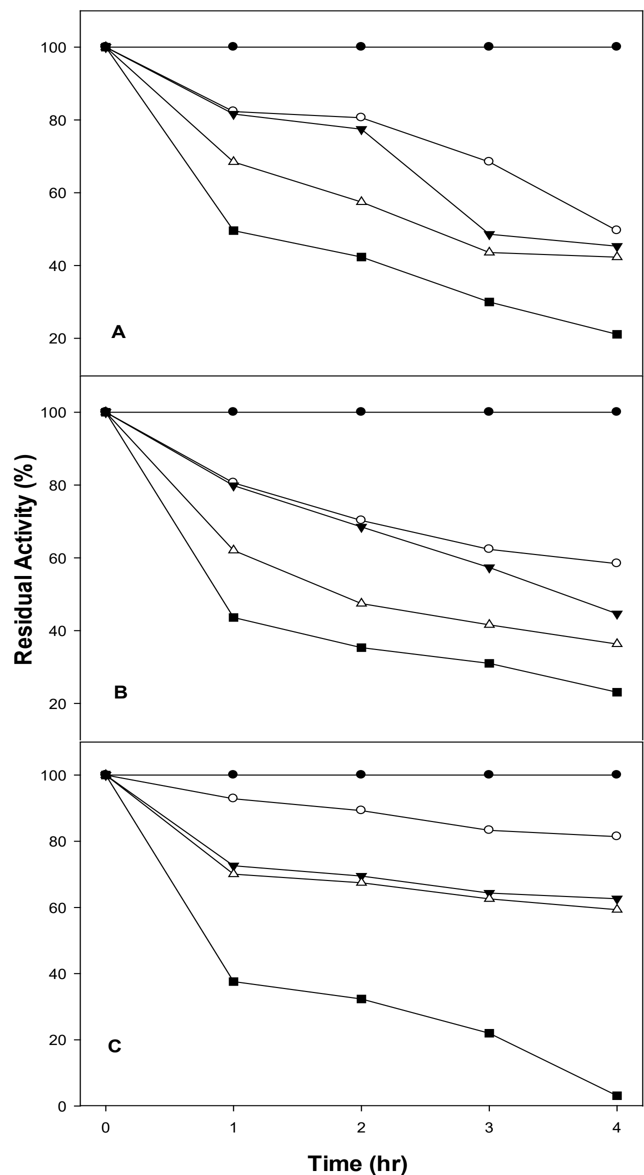

Figure 3. Residual enzymatic activity measured as a percentage on supernatant of *P. pistillaris* submerged in culture. (A) Total cellulases, (B) total xylanases, and (C) laccases. The temperatures assayed are represented as follows: (●) 25° C, (○) 40° C, (▼) 50° C, (Δ) 60° C, and (▪) 70° C. The experiments were performed in triplicate.

Figura 3. Porcentaje de actividad enzimática residual medida en el sobrenadante del cultivo sumergido de *P. pistillaris*. (A) celulasas totales, (B) xilanasas totales, y (C) lacasas. Las temperaturas ensayadas se representan de la siguiente manera: (●) 25 ° C, (○) 40 ° C, (▼) 50 ° C, (Δ) 60 ° C y (▪) 70 ° C. Los experimentos se realizaron por triplicado.

DISCUSSION

The cellulolytic activity reported for other fungi involve typically various enzymes known as cellulases, which include exo and endo glucanases, as well as cellobiohydrolases. The cellulolytic activity reported in this study relates to total cellulases activity o*f P. pistillaris* and was found to be lower to that reported for other organisms, such as the fungus *Trametes* spp. Interestingly the latter species grows on wood requiring to unfold complex carbohydrates into simple sugars for its feeding, while the former grows in soils with organic matter. This fact raises more questions than answers to whether the role of these enzymes have in this macrofungus life cycle. Furthermore, even though *P. pistillaris* xylanolytic activity is lower to that reported in extremophiles fungi, its potential in lignocellulosic materials degradation in arid ecosystems is quite remarkable. Finally, the laccase activity is not only comparable with white rot fungi, but they have a greater stability. The latter feature enhances *P. pistillaris* importance as a degrader in high salinity deserts with an adapted morphology and fibrolytic system worth studying.

CONCLUSIONS

After having taxonomically identified *P. pistillaris* in Northwest Mexico, it has been confirmed that it is the same organism globally distributed. The morphological differences in peridium aspect and height of the sampled specimens show that these are biotypes of a single species. Conclusively, *P. pistillaris* has a mild thermostable extra-cellular fibrolytic enzymatic system capable of degrading lignocellulose compounds. Such system includes cellulases, xylanases and laccases enzymes, which allow adaptation to high temperature conditions in their natural habitat. Regarding the presence of thermostable laccase activity in a desert macromycete, not associated to wood but with saline soils; it has been hypothesized that the role of this enzyme in *P. pistillaris* is involved in peridium building of the fruiting body, as well as in its melanization/lignification, aiming to protect it from solar radiation to which it is naturally exposed in its habitat. Further research is undergoing to support the latter.

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