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Use of Nutshells Wastes in the Production of Lignocellulolytic Enzymes by White-Rot Fungi

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HIGHLIGHTS

- Nutshells and pine needles are potential substrates for solid-state fermentation.
- *G. lucidum* was able to use nutshells and pine need as substrates for growing.
- G. lucidum produced the highest yield of lignocellulolytic enzymes.
- *L. edodes* and *P. ostreatus* produce cellulases and xylanases less efficiently that *G. lucidum*.

Abstract: Agricultural wastes are an environmental and economic problem that countries must strive to solve as soon as possible. White-rot fungi can take advantage of these wastes through an efficient process involving the bioconversion of lignocellulosic materials. Therefore, this work has used pine needles (*Pinus pseudostrobus*) and nutshells (*Carya illinoinensis*), relevant wastes in Mexico, as substrates for growing three fungal strains (*Ganoderma lucidum*, *Lentinula edodes,* and *Pleurotus ostreatus*) on PDA plates supplemented with these materials. Besides, the capacity to produce lignocellulolytic enzymes by these three fungi through solid-state fermentation has been evaluated using pine needles and nutshells along with barley straw. The relevant results suggest that nutshells are a potential substrate for growing *G. lucidum* (mycelial growth rate of 0.758 cm d^{-1}). Albeit pine needles waste has allowed *G. lucidum* growth (0.402 cm d^{-1}), *L. edodes* and *P. ostreatus* growth on PDA supplemented plates were not observed. Further to this, nutshells or pine needles combined with barley straw were used as substrates through solid-state fermentation to produce lignocellulolytic enzymes (cellulase, xylanase, and laccase). *G. lucidum* presented the highest cellulase productivity (3.03 ± 0.34 IU/gdm·d) using nutshells and barley straw (BS_NS) as substrates, while

it was half using pine needles and barley straw (BS_PN) (1.35 ± 0.20 IU/gdm·d). The results were similar for xylanase productivity because *G. lucidum* produced 4.80 ± 0.61 IU/gdm·d using BS_PN, slightly higher than the BS_NS (3.79 ± 0.29 IU/gdm·). Finally, nutshells might be a suitable substrate for the growth of white-rot fungi, mainly the *G. lucidum* strain, making it an ecological alternative for future biotechnological applications.

Keywords: Lignocellulosic enzymes; *Ganoderma lucidum*; *Lentinula edodes*; *Pleurotus ostreatus*; nutshells waste; pine needles waste.

INTRODUCTION

Agricultural wastes are rich in lignocellulosic compounds beneficial for many biotechnological applications, but if these are not controlled cause environmental pollution. Several of the leading lignocellulosic wastes from wood, agriculture, and forestry, are particularly abundant and have a potential for bioconversion, which is one way to take advantage of these residues with significant environmental and economic benefits [1–3] Mexico possesses large areas with pine vegetation where the pine needles represent an essential organic residue but cannot serve as fodder. In contrast, these wastes could prevent the passage of light to the lower plants and increase the soil's acidity. It is also highly flammable, representing a high-risk factor for forest fires. Besides, it contains soluble phenolic compounds responsible for their slow decay and decreased soil fertility [4].

In the same way, nut production in Mexico is abundant, 171 372 tons in 2020 (SIAP; http://nube.siap.gob.mx), of which nutshells constitute 50% of the total weight sticking around and becoming a pollutant. Although shells have many bioactive compounds important for cosmetics, food, and pharmaceutical products [5], many organic wastes are not used, becoming a critical source of pollution and ecosystem risk. Therefore, this work is focused on utilizing agricultural wastes, such as pine needles and nutshells, as an alternative in search of substrates for lignocellulolytic enzyme production, which represents an advantage of these residues with significant biotechnological benefits [6,7].

The cell wall organic biomass composition differs between these agricultural wastes [8–10], being fungal strains beneficial because they can improve their bioavailability through solid-state fermentation (SSF). Fungi produce several lignocellulolytic enzymes with the ability to degrade lignin, which is known as white-rot fungi. The production of the enzymatic complexes depends on the type of substrate, strain, and cultivation conditions [11,12]. Despite this, enzymatic production has several advantages as requiring minimal cost, low water content, reduces bacterial contamination, and recycles several agricultural wastes, becoming an alternative to decrease the environmental impact [13]. Then, the biodegradation of lignocellulosic residues by white-rot fungi is a process that is gaining momentum in the biotechnology sector, reducing the environmental impact generated by the inappropriate disposal of such residues [14], not only but also are postulated as biological resources with high nutritional value because of having a high content in carbohydrates, phenolic compounds, vitamins, minerals, and protein, which could be an alternative to many rural communities' deficient intake [15].

Currently, we can find multiple reports mentioning several white-rot fungi that produce a high number and yield of lignocellulolytic enzymes, among which may be named *Pleurotus ostreatus*, *Ganoderma lucidum*, and *Lentinula edodes* [1,16–18]. However, some agricultural wastes have been explored poorly under specific conditions, such as pine needles (*Pinus pseudostrobus*) and nutshells (*Carya illinoinensis*). Therefore, this work evaluated these two relevant agricultural wastes for white-rot fungi cultivation and the production of lignocellulolytic enzymes such as cellulases, xylanases, and laccases.

MATERIAL AND METHODS

Fungal and agricultural wastes

The white-rot fungi *P. ostreatus* was donated by the Setas Monarca farm, while *G. lucidum* and *L. edodes* strains were used from the Molecular Biology Laboratory of the Instituto de Ciencias Agropecuarias, UAEH. The agricultural wastes were pine needles (*Pinus pseudostrobus*) and nutshells (*Carya illinoinensis*), these obtained from two regions in Tulancingo de Bravo and Barranca de Metztitlán, both in Hidalgo state, Mexico. The samples were collected, dried, and grounded in a blender. After that, the samples were sieved through 5 mesh/4000 microns and ten mesh/2000 microns and stored at room conditions until their use.

Radial mycelia growth rate

The radial growth of the mycelium was evaluated on Petri plates using agricultural wastes mixed with PDA. Three treatments were evaluated: pine needles and PDA (PDA_PN), nutshells and PDA (PDA_NS), and PDA as a control treatment. Firstly, pine needles and nutshells were ground and sifted to a particle size of approximately ten mesh/2000 microns. Based on Coello-Loor and coauthors (2017), 60 g of pine needles or 20 g of nutshells was boiled with 500 mL of distilled water for 15 min; after that, added 19.5 g of PDA and the suspension was homogenized and boiled for 5 min [19]. It is important to mention that the number of nutshells used was the maximum possible to allow solidification of the agar. The solutions were autoclaved at 121 °C for 15 min. Three replicate plates of each medium were inoculated centrally with a 5 mm mycelial disc cut from a 7-day old plate of *P. ostreatus*, *G. lucidum*, and *L. edodes*. Then, plates were incubated in a static incubator at 28 °C for up to 10 days. The radial mycelial growth was measured with a vernier caliper every 24 hours until the total growth of the mycelial [20].

Solid-state fermentation (SSF) and enzyme extraction

SSF experiments were made using barley straw mixed with agricultural wastes to evaluate the growth of the fungi. Each experimental unit was prepared in Erlenmeyer flasks, using 66% barley straw and 34% pine needles or nutshells. The substrates were hydrated, reaching 80% moisture. The flasks were autoclaved at 121 °C for 1 h. The experiments were started by adding five disks (5mm) with mycelium into each Erlenmeyer flask. Then, the experimental units were incubated in a static incubator at 24 °C and a constant humidity of 80% in dark conditions.

After five days of incubation, three flasks were randomly selected for each strain of the different substrates to obtain the crude enzyme extract; this procedure was repeated every three days until seventeen days. To obtain the crude enzyme extract, 100 mL of distilled water was added to Erlenmeyer flasks and kept agitation shaking for 30 minutes at 4 °C. After that, the medium was filtered through Whatman No. 40 and centrifuged at 4000 rpm at 4 °C for 30 min. After centrifugation as above, the clear supernatant was collected as the enzyme crude extract (ECE) and was used in the next step to determine the enzyme activity [21].

Enzyme assay

Cellulase and xylanase activity was determined by measuring the amount of glucose liberated [22]. For cellulase activity, 0.1 mL of the ECE solution was mixed with 0.9 mL of 1% carboxymethyl cellulose (Sigma-Aldrich) solution in 50 mM sodium citrate buffer (pH 5.0); meanwhile, 0.1 mL of the ECE solution was mixed with 0.9 mL of 0.5% birchwood xylan (Sigma-Aldrich) solution in 50 mM sodium citrate buffer (pH 5.3) for xylanase activity. The assays were carried out at 50 °C for 20 min for cellulase activity and 5 min for xylanase activity [23]. The reaction was stopped by adding 1.5 mL of DNS solution and heating the tube in a boiling water bath for 5 min [24]. The amount of sugar released was measured by absorbance at 540 nm using a spectrophotometer (UNICAM HEλIOS). In both cases, one international unit (IU) of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose or xylose per minute under the assay conditions. A calibration curve of reducing sugars constructed from different glucose or xylose concentration was used to quantify the enzyme activity.

Laccase activity was determined by measuring the oxidation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Ɛ420= 36000 $M^{-1}cm^{-1}$) as substrate [25]. First, 0.5 mL of ECE was incubated in a water bath at 40 °C for 5 min in a tube. At the moment of the measurement, 0.5 mL of ABTS solution in 50 mM citrate buffer pH 5.0 was added and mixed with a vortex. The amount of substrate oxidated was measured each 10 s for to up 90 s by absorbance at 420 nm in a spectrophotometer (UNICAM HEλIOS). Laccase activity was reported as international units (IU), where 1 IU was defined as the amount of enzyme that produces 1 µm of oxidized ABTS per min under the assay conditions [26]. All enzymatic activities are reported as international units per initial gram of dry matter (gdm).

Statistics analysis

The experiments were done in triplicate and with a random design. Linear regression was made for the three fungi to compare the relationship between the radial growth rate base on the substrate using the SSPS software and R package. The figures show the mean ± standard deviation. Statistical Analysis of Variance (ANOVA) was used to evaluate the relative influence of substrates. The images were made using the RStudio software.

RESULTS

Effect of agricultural wastes in the white-rot fungi growth.

The mycelial growth of the three fungal strains (*G. lucidum*, *P. ostreatus,* and *L. edodes*) was evaluated using Petri plates prepared with nutshells and pine needles as substrates added to the PDA media. Interestingly, the mycelial growth was affected by adding pine needles waste because we did not observe mycelium on the plates. Otherwise, the three fungal strains were able to grow by adding nutshells. However, the growth velocity was better on the plates supplemented with nutshells than those supplemented with pine needles (Figure 1). Nutshells substrate showed potential effects, especially for the *G. lucidum* strain (Figure 1B), obtaining a velocity rate of 0.758 cm d^{-1} in the PDA_NS medium, which means two times more than the growth obtained on PDA alone. It is important to mention that *G. lucidum* started its growth on the second day after the initial incubation, and the total mycelium invasion happened up to 6 days after, which was the fastest growth observed for the three cases. However, this strain was slower than other strains grown without agricultural wastes, strengthening the benefits of adding these substrates.

Likewise, *P. ostreatus* grew better in PDA_NS than PDA without agricultural wastes; however, the *P. ostreatus* growth was late, compared to *G. lucidum*, with a corresponding velocity rate of 0.684 cm d^{-1} (Figure 1A). Similarly, *L. edodes* grew better on PDA_NS (0.684 cm d^{-1}) than on PDA (0.578 cm d^{-1}) (Figure 1C), which confirms the positive effect of supplementation with this substrate.

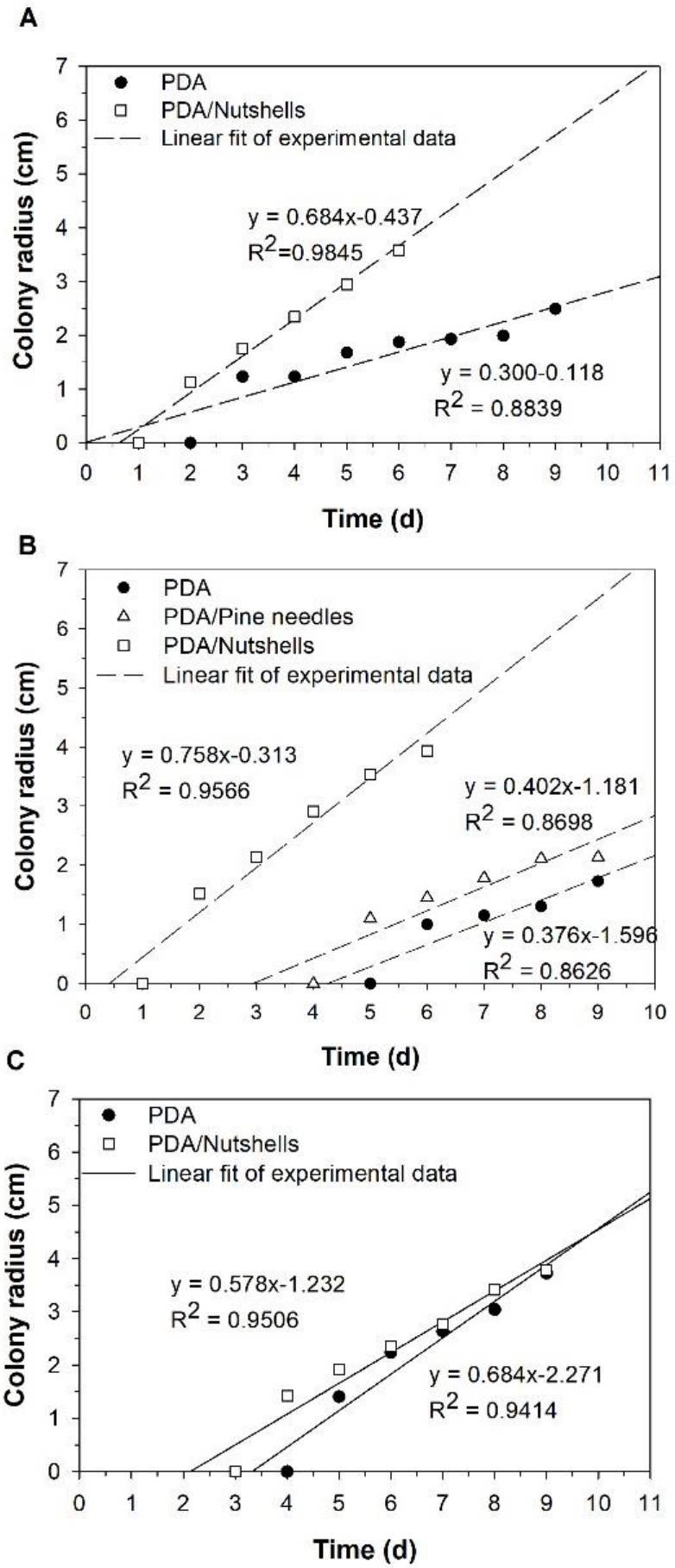

Figure 1. The mycelium growth rate of white-rot fungi on agricultural wastes combined with PDA media. A) *Pleurotus ostreatus*, B) *Ganoderma lucidum*, C) *Lentinula edodes*. Each graphic shows the mean ± sd. The results were adjusted to a linear regression model. (PDA: Potato Dextrose Agar, NS: nutshells, and PN: pine needles).

Effect of agricultural wastes on enzyme activities obtained by solid-state fermentation of white-rot fungi

Solid-state fermentation (SSF) represents an attractive process that allows obtaining crude fermented products through bioconversion using fungal, which could be used directly as enzyme sources. Fungal lignocellulolytic enzyme production was evaluated using the nutshells or pine needles combined with barley straw as agricultural wastes under SSF. Two treatments were established per fungal strain (*G. lucidum*, *P. ostreatus,* and *L. edodes*) to know if they could carry out fungal growth and enzyme production.

The results showed that the three fungal strains could grow on these agricultural wastes combined with barley straw producing cellulase, xylanases, and laccase enzymes (Figure 2 and Table 1). The cellulase activity was continued for up to 17 days of sampling in the flask BS_NS (51.47 UI/gdm, Figure 2A). However, *P. ostreatus* and *L. edodes* have shown similar enzyme activity on different sampling days (Figure 2A).

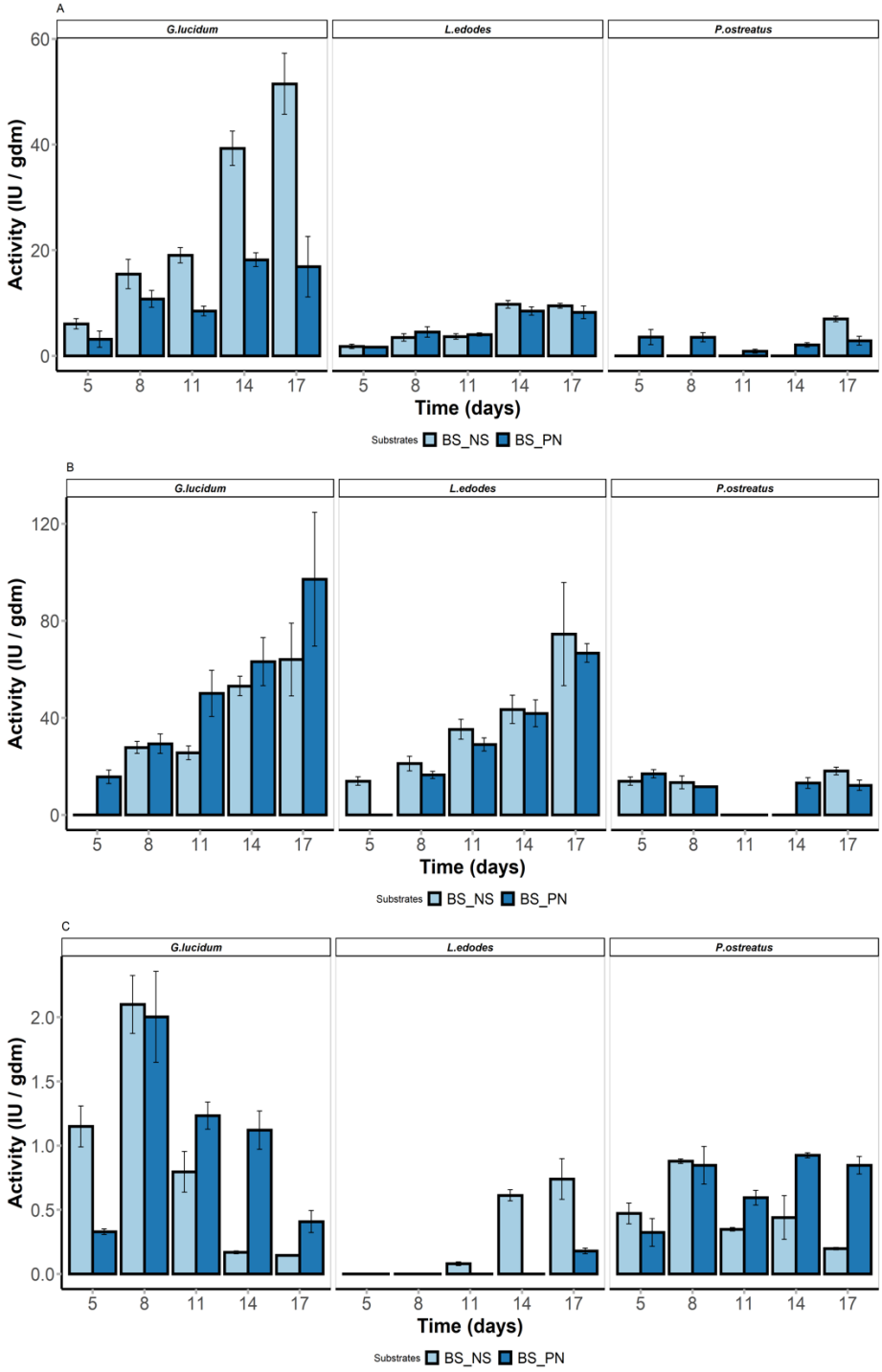

Figure 2. Lignocellulolytic enzyme activity was determined by SSF using three fungal strains. A) Cellulase activity, B) Xylanase activity, and C) Lacasse activity. The data show the mean ± sd of three determinations. (BS: Barley Straw, NS: nutshells, and PN: pine needles).

Table 1 summarizes the productivity data obtained per strain for both treatments, but only the time of the sample that produces the highest enzyme activity in each case. Overall, *G. lucidum* showed the highest cellulase productivity (3.03 ± 0.34 IU/gdm·d) using the BS_NS treatment. Conversely, the cellulase production from *P. ostreatus* and *L. edodes* showed a productivity of 0.41 IU/ gdm·d and 0.70 IU/ gdm·d for the BS_NS treatment. According to the treatment, BS_PN promoted the cellulase activity mainly by *G. lucidum*, producing 1.35 IU/ gdm·d.

Along with xylanase activity, these were determined for both treatment and three fungi (Figure 2B). Similarly to cellulase activity, the higher xylanase productivity was obtained by *G. lucidum* (Table 1), being better on BS_PN (4.80 IU/ gdm·d) than BS_NS (3.79 IU/ gdm·d). *L. edodes* produced a similar xylanase activity in both treatments, being better on BS_PN (3.92 IU/ gdm·d) than BS_NS (3.20 IU/ gdm·d), whereas *P. ostreatus* produced 3.39 IU/ gdm·d on BS_PN and 2.78 IU/ gdm·d on BS_NS.

In all cases, the behavior was very different for the laccase enzymes (Figure 2C). Firstly, maximum activity was obtained by *G. lucidum* on the first eight days for both treatments, being 1.61 UI/gdm (BS_PN) and 1.44 UI/gdm (BS_NS), and productivity of 0.25-0.26 IU/ gdm·d (Table 1) for this strain. After that, the laccase activity decreased down to 0.4 UI/gdm. *P. ostreatus* also produced the higher activity on the eight days (0.11 IU/ gdm·d), although the values were half of those obtained by *G. lucidum* (0.26 UI/gdm·d (BS_PN) and 0.25 UI/gdm·d (BS_NS). On the other hand, *L. edodes* expresses laccase enzymes late since the higher laccase activity was determined up to the 17 days of sampling (Figure 2C), being the values obtained on BS_NS (0.73 UI/gdm) four times higher than those obtained on BS_PN (0.17 UI/gdm) per time of sampling, which means the productivity of 0.04 IU/ gdm·d and 0.010.04 IU/ gdm·d (Table 1).

Table 1. Lignocellulolytic enzyme productivity of three fungal strains obtained by SSF using nutshells and pine needles wastes.

Cellulase activity (IU/gdm·d)			
	P. ostreatus	*G. lucidum*	*L. edodes*
Pine needles	0.65 ± 0.11 [a]	1.35 ± 0.20 [b]	0.61 ± 0.10 [a]
Nutshells	0.41 ± 0.03 [a]	3.03 ± 0.34 [c]	0.70 ± 0.05 [b]
Xylanase activity (IU/gdm·d)			
Pine needles	3.39 ± 0.34 [a]	4.80 ± 0.61 [b]	3.92 ± 0.23 [a]
Nutshells	2.78 ± 0.34 [a]	3.79 ± 0.29 [b]	3.20 ± 0.37 [a]
Laccase activity (IU/gdm·d)			
Pine needles	0.11 ± 0.02 [b]	0.25 ± 0.04 [c]	0.01 ± 0.001 [a]
Nutshells	0.11 ± 0.002 [b]	0.26 ± 0.03 [c]	0.04 ± 0.003 [a]

Means with different lowercase letters within the same row are significantly different ($p < 0.05$).

DISCUSSION

Agricultural wastes are commonly used as substrates in SSF because they have low costs, are easily obtainable, and represent a concentrated source of nutrients for fungal growth. Several reports have shown the importance of fungal adaptation using different agricultural wastes with or without pretreatments [27]. The above has been evaluated by measuring the radial growth rate, indicating strains' adaptability to the growth conditions [28].

Due to Mexico produces a considerable amount of nutshells and pine needles, Ozcariz-Fermoselle and coauthors (2018)[29] assessed the mycelial growth rate of different *Pleurotus* strains utilizing PDA broth combined with these wastes, concluding that the presence of agricultural wastes stimulates the growth rate. Then, this work evaluated the adaptability of *P. ostreatus*, *G. lucidum,* and *L. edodes* using these wastes. The results obtained (Figure 1) have shown that the nutshells waste improves the growth of the fungal strains. However, the highest growth was observed with *G. lucidum* (0.758 cm d^{-1}) using nutshells (PDA_NS) as lignocellulosic waste-based media, while *P. ostreatus* and *L. edodes* grew lately. These results suggested that nutshells can be considered suitable options. Interesting, the results may be explained due to the chemical composition of substrates used since nutshells waste contains 55.7% of holocellulose (which is the mixture of cellulose and hemicellulose in the cell walls of plants) and 29.4% of lignin [10], while pine needles

wastes have been reported that contains 27.8% of crude fiber [30] and a higher content of polyphenols that could affect the fungal growth. The less fiber content of pine needles was justified by increasing the amount in the medium, but we did not discard the possibility of testing a lower amount of pine needles or exploring different strains for future experiments.

Moreover, the mycelium growth of these three strains has been evaluated with other wastes [31]. For instance, Saavedra-Molina and coauthors (2020)[32] used three strains of *Pleurotus* on barley straw extract agar. They determined their radial growth rate for each one, and *P. ostreatus* formed the highest radial growth rate (0.696 cm d^{-1} to 0.72 cm d^{-1}). A different work reported by Coello-Loor and coauthors (2017)[19], they have used rice husk residues to obtain a radial growth rate for *P. sapidus* and *P. ostreatus* of 1.05 cm d^{-1} and 1.13 cm d^{-1}, respectively; in which showed better adaptability than to use of passion fruit peel (0.9-0.98 cm d^{-1} by *P. sapidus* and *P. ostreatus*). In the same way, Martinez and coauthors (2015)[33] showed the importance of evaluating strains' ability to colonize agricultural waste as malt extract agar combined with pear marc, which showed a maximum growth rate of 1 cm d^{-1} for three different strains evaluated for *Pleurotus* sp. Then, Figure 1 showed that *P. ostreatus* could grow in the presence of added nutshells with a radial growth rate of 0.684 cm d^{-1}, which was similar to the results obtained with barley straw extract agar. Albeit it is not easy to compare the structural and morphological conformation between nutshells and barley straw, they have similar chemical compositions, containing 55.7% [10] and 56.3% [34] of holocellulose, respectively, and referring in lignin content is 29.4% [10] and 19.2% [34], respectively.

Furthermore, Rashad and coauthors (2019)[35] reported the growth of *G. lucidum* mycelia on six agro-wastes with the ability to grow in all of them with varying mycelial extension rates (0.242–0.519 cm d^{-1}). Cotton stalk appeared best among the substrates used, followed by sugarcane bagasse and rice straw for mycelial growth. However, the *G. lucidum* mycelial growth rate was better using nutshells waste-based media (Figure 1), which shows the importance of testing various wastes for growing white-rot fungi strains. On the other hand, *L. edodes* has been evaluated growing in barley straw, wheat straw, and vineyard pruning [8]. The authors mentioned that *L. edodes* has a high capacity to grow on non-conventional substrates. However, there is no information using nutshells or pine needles as substrates. Then, this work showed that the radial growth rate using nutshells was 0.578 cm d^{-1}(Figure 1), suggesting that nutshells could be accessible for growing but also affect a little bit. In line with this, Valenzuela-Cobos et. (2021) mentioned that *L. edodes* needs a long incubation time and specific substrates such as sawdust, rice straw, barley straw, and hazelnut husk, which suggests that following these experiments for a long time may allow obtaining better fungal growth [36].

In addition, the white-rot fungi have a high bioconversion potential for agricultural waste because they can efficiently produce complex lignocellulolytic enzyme systems [37]. According to Bharathiraja and coauthors (2017)[38] the enzyme-producing cost could be significantly reduced using low-priced biological substrates such as agricultural waste. Then this work evaluated the behavior of white-rot fungi on non-conventional endemic substrates in the Hidalgo State, Mexico, because not all white-rot fungi produce all lignocellulolytic enzymes simultaneously [39]. Lignocellulose degradation requires the synergistic activity of multiple carbohydrate-active enzymes through cooperative hydrolytic (lignocellulolytic enzyme) and oxidative enzymes (lignin degradation). The most relevant enzymes (cellulase, xylanase, and laccases) were produced through SSF using nutshells and pine needles wastes combined with barley straw (Figure 2), showing that they are good alternatives to produce these enzymes. We used barley straw because this substrate has been used to express lignocellulolytic enzymes in several fungi strains. Firstly, *Lentinula edodes* grow on barley straw by solid-state fermentation, decreasing from 25.5% to 15.6% the concentration of hemicellulose and 31.8% of the lignin, which is the result of lignocellulolytic enzymes produced [8]. Secondly, *Pleurotus* species are among the most efficient types of lignocellulolytic white-rot fungi which grow on barley straw, having a xylanase activity more than 10 times from the beginning of fermentation up to 16 d (200.54 UI/gdm), cellulose activity around of 2.59 UI/gdm and 4.57 UI/gdm of Lacasse activity at the same fermentation time [40], demonstrating their potential to prepare mixed substrates, especially in the case of poorly used and non-conventional substrates like nutshells and pine needles.

Also, several reports have shown differential lignocellulosic enzyme activity from lignocellulosic raw materials by SSF through different fermentation conditions [31]. Still, it is difficult to predict the effect of waste utilization in enzyme production; then, it is necessary to explore what kind of condition could be more efficient.

In this work, *G. lucidum* produced the highest cellulase activity using nutshells plus barley straw as substrates from 14 d up to 17 d of sampling. Similarly, *G. lucidum* produced higher xylanase activity in both substrates (nutshells and pine needles) to 17 f of fermentation. The above is important because lignocellulolytic enzymes during *G. lucidum* growth have not been well studied [41]. Although it is evident that *G. lucidum* growth is determined by the degradation of the substrate through enzyme production, it is difficult

to know or find the best combination that can optimize their development and the factors that contribute to the global regulatory system.

On the other hand, the enzyme complexity and radical components involved in lignin degradation could only be done optimally in a fungal system [42]. However, the fungus-substrate combination shows a significant variation in the degree of delignification [11]. Ligninolytic enzyme activities are not always correlated with lignin degradation, leading to the ligninolytic enzyme complex's function not being fully understood [42]. In this work, *G. lucidum* was the strain that produced the higher laccase activity for eight days of sampling; after that, the activity decreased in both treatments. Otherwise, *L. edodes* and *P. ostreatus* produce laccase enzymes after 14 days and 17 days of sampling, suggesting a delay in expressing these enzymes by these strains under these fermentation conditions. Some reports indicate that *L. edodes* and *P. ostreatus* are lignin-degrading mushrooms that use laccases and at least one of the peroxidases. However, more studies are necessary to determine an efficient screening of fungal strains for laccase production from lignocellulosic wastes [42]. For instance, Elisashvili and coauthors (2008b) [43] used several strains of *L. edodes* and *P. ostreatus*, comparing their ability to produce the lignocellulolytic enzymes in SSF. The above indicated that fungi cultivation in identical conditions revealed wide differences among both species and strains of the same species, which suggests that the selection of residues for fungal growth and the synthesis of enzymes plays an essential role and requires more studies to improve our understanding of how the lignocellulosic enzyme production is regulated.

Finally, our results show an ecological proposal and the possibility of using the pine needles and nutshells to propagate and grow *G. lucidum*, *P. ostreatus*, and *L. edodes* without adding additives. Future work could evaluate culture parameters and include scaling and biochemical analysis of the enzymes obtained; it is also necessary to evaluate these wastes' selective degradation to incorporate them into another production chain (e.g., animal feed).

CONCLUSION

The use of agricultural wastes for bioconversion through the white-rot fungi is a relevant field of study that can decrease environmental and economic problems. The results obtained in this work suggested that pine needles (*Pinus pseudostrobus*) and nutshells (*Carya illinoinensis*) are helpful substrates for *G. lucidum*, *L. edodes,* and *P. ostreatus* mycelial growth, as well as lignocellulolytic enzyme production. Interestingly, *G. lucidum* generated the highest productivity of cellulases and xylanases using nutshells combined with barley straw. Besides, the laccases were expressed earlier using nutshells combined with barley straw than the enzyme obtained from *L. edodes,* and *P. ostreatus*. Those mentioned above suggested that nutshells waste may be an excellent component for solid-state fermentation, but it is necessary to design more studies to understand the enzyme production dynamics by white-rot fungal using these wastes.

Therefore, these results showed that it is essential to evaluate the use of lignocellulosic resources as a first step in proposing substrates and their combinations for fungal biomass and extracellular enzyme extracts with future applications.

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