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SHORT COMMUNICATION

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Oxidases production by *Trametes versicolor* grown on green waste and on polyurethane foam in solid-state fermentation: A comparative study

Producción de oxidasas por *Trametes versicolor* crecido sobre residuos verdes y sobre espuma de poliuretano en fermentación en estado sólido: Un estudio comparativo

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Abstract

Green waste (GW) is generated by the maintenance of public or private green spaces. It is necessary to find ecological alternatives for GW utilization, aiming to avoid accumulation of this material at the environment. In this research, the production of laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP) and unspecific peroxygenase (UnP) produced by *Trametes versicolor* grown on GW as a substrate and on polyurethane foam (PUF) as an inert support in solid state fermentation was evaluated. *T. versicolor* showed higher values of Lac, MnP, UnP and LiP activities (34, 943, 1023 and 766 U/gS, respectively) when grown on GW than when grown on PUF (10.9, 588, 559 and 229 U/gS, respectively). These results suggest that *T. versicolor* produced Lac inducible and constitutively, while LiP, MnP and UnP were induced by GW at the beginning of fungal growth, however, these enzymes were constitutive and inducible during the rest of the fermentation. The production of oxidases and peroxidases was induced and increased by

GW. It is suggested that LiP is involved (as a constitutive enzyme) at the beginning of the exponential phase, while MnP and UnP participate in fungal growth at the end of fermentation. To our knowledge, this is the first detailed study on the main lignincellulose-degrading fungal enzymes involved in GW degradation by fungi. In particular, the relevance of UnP was showed as peroxidase involved in lignocellulosic substrates biodegradation.

Keywords: Green waste, oxidases, peroxidases, solid-state fermentation, *Trametes versicolor*.

Resumen

Los residuos verdes (GW) se generan debido al mantenimiento de espacios verdes públicos o privados. Con el objeto de evitar la acumulación de este material en el medio ambiente, es necesario buscar alternativas ecológicas para su aprovechamiento. En esta investigación se estudió la producción de lacasa (Lac), lignin peroxidasa (LiP), manganeso peroxidasa (MnP) y peroxigenasa inespecífica (UnP) producidas por *Trametes versicolor* crecido sobre GW como sustrato y sobre espuma de poliuretano (PUF) como soporte inerte en fermentación en medio sólido. *T. versicolor* mostró valores más altos de actividades de Lac, MnP, UnP y LiP (34, 943, 1023 y 766 U/gS, respectivamente) en GW que en PUF (10,9, 588, 559 y 229 U/gS, respectivamente). Estos resultados sugieren que *T. versicolor* produjo Lac de manera inducible y constitutiva, mientras que LiP, MnP y UnP fueron inducidas por GW al inicio del crecimiento fúngico, sin embargo, estas enzimas fueron constitutivas e inducibles durante el resto de la fermentación. La producción de oxidasas y peroxidasas fue inducida e incrementada por GW. Estos estudios sugieren que LiP está involucrada (como enzima constitutiva) en el crecimiento fúngico desde el inicio de la fase exponencial, mientras que MnP y UnP participan en el crecimiento del hongo al final de la fermentación. Hasta donde sabemos este es el primer estudio detallado sobre la participación de las principales enzimas fúngicas lignocelulolíticas en la degradación de GW. En particular, se mostró la relevancia de UnP como peroxidasa involucrada en la biodegradación de sustratos ligninocelulósicos.

Palabras clave: Desechos verdes, fermentación en estado sólido, oxidasas, peroxidasas, *Trametes versicolor*.

1. Introduction

Green waste refers to the biodegradable garden waste and public park waste (Eades *et al*., 2020). This waste is generated by the maintenance of public or private green spaces and can include organic materials like pruning, grass clippings and leaves. Therefore, this material is considered heterogeneous lignocellulosic biomass (Boldrin and Christensen, 2010; Yousuf *et al*., 2020; Langsdorf *et al*., 2021). Improper disposal of green waste in landfills or open dumps has been reported to cause the generation and emission of greenhouse gases, increasing the impact of climate change. In addition, inappropriate handling of these wastes, such as burning of this material and disposed into waterways

and oceans, among others, can generate foul odors, and proliferation of pest and insects (Ayilara *et al*., 2020). Therefore, it is necessary to find ecological alternatives for utilization of diverse green wastes generated each year worldwide. In this context, the use of microorganisms as an eco-friendly alternative has much to offer. Agricultural crop products not used in human dietary intake are valuable substrates for microbial growth, due to their composition (complex carbohydrates, crude proteins and minerals) (Grujić *et al*., 2015). In particular, fungi are able to produce unspecific enzymes that catalyze a diversity of reactions, which makes them highly effective for the degradation of complex substrates (Martínez-Berra *et al.*, 2018; Hernández-Sánchez *et al*., 2019; Sánchez, 2020; Khan *et al*., 2023). These microorganisms grow in a filamentous manner and some of them can produce fruit bodies. Mycelial growth is generally coupled with increased enzyme production and respiration, hyphae absorb digestive products from the substrate which allow it to growth and branch to form a network of hyphae to invade and penetrate the substrate (Sánchez *et al*., 2006; Sánchez *et al*., 2020; Loftus *et al*., 2020). In particular, *Trametes versicolor* is a basidiomycete fungus found throughout the world growing on tree trunks, which is a well-known traditional medicinal mushroom. This organism has been reported as a promising fungus for the utilization of lignocellulosic wastes in a wide range of applications such as; production of biofuels, feeds, biofertilizers, and for biotransformation processes and wastewater treatments (Tišma *et al*., 2021). *T. versicolor* can efficiently degrade the lignocellulosic material due to the production of extracellular enzymes such as laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) among others (Sánchez, 2009; Aydinoğlu *et al*., 2013; Amara *et al*., 2018; Bari *et al*., 2020; Dao *et al*., 2023). In particular, unspecific peroxygenase (UnP) is produced by the basidiomycete *Agrocybe aegerita* (Karich *et al*., 2017). UnP has been reported to be capable of degrading the majority of USEPA (US Environmental Protection Agency) priority environmental pollutants, which is due to its ability to catalyze different types of reactions (Karich *et al*., 2017; Shin *et al*., 2018; Civzele *et al*., 2023).

In this context, the selection of the appropriate fermentation system using lignocellulosic materials is crucial for optimal invasion of the substrate and for an efficient enzyme production. It has been reported that enzyme production by fungi is greater when they are grown in solid-state fermentation than in submerged fermentation (Viniegra-González *et al*., 2003; Sandhya *et al*., 2005; Ferrer-Parra *et al*., 2018; Liu *et al*., 2020; Premalatha *et al*., 2023).

In this research, the production of Lac, LiP, MnP and UnP produced by *T. versicolor* grown on green waste (GW) as substrate and on polyurethane foam (PUF) as inert support in solid state fermentation were evaluated.

2. Material and Methods

2.1. Organism

T. versicolor was purchased from the culture collection at Colegio de Postgraduados Campus Puebla (Puebla, Mexico). The strain was kept at 4 °C until used. Colonies of *T. versicolor* were grown on potato extract agar (Merck, Mexico) for 12 d, then five fragments

of mycelium (10 mm in diameter) were taken from the colony periphery and used as inoculum.

2.2. Substrate preparation

A heterogeneous fresh GW mixture was obtained from the compost plant at the Polytechnic National Institute (Unidad professional Adolfo López Mateos) and dried at room temperature. GW was subsequently crushed with a hand mill and sieved for sample size homogenization (1-5 mm of length).

2.3. Culture media and culture conditions

A mineral medium was prepared containing (in g/L) the following composition: $CuSO_4.5H_2O$, 0.127; $MgNO_3$, 0.5; and $CaCO_3$, 1.5, which was used to prepare culture media. 125-mL sterile Erlenmeyer flasks containing 27 mL of mineral medium were used. The GW-supplemented culture medium contained 7.5. g of dry GW, while the PUF-containing culture medium had 1 g of dry PUF and 10 g of glucose per litter. PUF was cut into 0.5-cm cubes and treated with 1 N HCl and 1 N NaOH solutions for 24 hours each. Then, it was dried in an oven (AR-290D) at 40 °C for 48 h and used.
Flasks were autoclaved at 120 °C for 15 min, cooled to room temperature and then inoculated with five mycelial fragments (of 10 mm diameter) taken from the periphery of 7-day-old *T. versicolor* colonies grown in potato extract agar (Merck, Mexico). Cultures were incubated for 14 d at 25 °C under static conditions. Analyses were carried out on samples taken at 48-h intervals and performed in triplicate.

2.4. Enzymatic assays

Extracellular enzymatic extract was obtained from cultures, after adding 25 mL of distilled water and shaken at 120 rpm for 1 h.
Lac activity was evaluated using 2,6-dimetoxyphenol (DMP) as a substrate. The reaction mix comprised 900 µL of DMP dissolved in 0.1 M acetate buffer at pH 4.5 and 100 µL of supernatant and incubated at 40 °C for 1 minute. Absorbance was measured at 468 nm (ε_{469} = 49600 M^{-1} cm^{-1}) using a Jenway 7305 UV-Vis spectrophotometer (Stone, Staffs, UK) (Camacho-Morales et al., 2017).
The MnP activity was measured using guaiacol as the substrate. The reaction mix comprised 75 µL of 1 mM MnSO4, 790 µL of 0.1 M tartrate buffer (pH 4.2), 50 µL of 40 mM H_2O_2, 75 µL 10 mM guaiacol, and 10 µL of supernatant. The mix was incubated at 25 °C for 5 minutes. Changes in absorbance were measured at 334 nm (ε_{334} = 18300 M^{-1} cm^{-1}) using a Jenway 7305 UV-Vis spectrophotometer (Camacho-Morales et al., 2017).
LiP activity was evaluated using veratryl alcohol as a substrate. The reaction mixture comprised 200 µL of 40 mM veratryl alcohol, 200 µL of 40 mM H_2O_2, 580 µL of 0.1 M tartrate buffer (pH 4.2), and 20 µL of supernatant. The mix was incubated at 25 °C for 5 minutes. Changes in absorbance were measured at 310 nm (ε_{310} = 9300 M^{-1} cm^{-1}) using a Jenway 7305 UV-Vis spectrophotometer (Arora and Gill, 2001).
The UnP activity was determined using veratryl alcohol as the substrate. The reaction mixture comprised 100 µL 40 mM veratryl alcohol, 50 µL of 40 mM H_2O_2, 840 µL of 0.1 M

citrate buffer (pH 4.5), and 10 µL of supernatant. The mix was incubated at 25 °C for 5 minutes. Changes in absorbance were measured at 310 nm (ε_{310} = 9300 M^{-1} cm^{-1}) using a Jenway 7305 UV-Vis spectrophotometer (González-Rodríguez et al., 2023).
In all cases, enzyme activity was reported as units of enzyme per g of dry substrate (U/gS). One unit (U) of enzyme activity was defined as the amount of enzyme required to obtain 1 µmol of product per minute.
Enzyme parameters are reported as maximum enzymatic activity (E_{max}), which corresponds to the maximal enzyme activity value observed during the fermentation (U/gS), and enzyme productivity (*P*), which is the maximum enzymatic activity value per hour.

2.5. Statistical analysis

Data for analysis were obtained from three independent samples. Data were evaluated by one-way ANOVA, and Tukey's test was used. SAS® OnDemand for Academics (SAS Institute, Inc., Cary, NC) was used.

3. Results

Lac production by *T. versicolor* grown on GW and on PUF is shown in Fig. 1. In GW-supplemented cultures, the greatest Lac activity (34 U/gS) was observed at 96 h of growth (Fig.1, Table 1). Lac activity decreased at both 144 and 240 h, showing a slight increase at 192 and at the end of the fermentation. Cultures of *T. versicolor* grown on PUF showed a low and relatively constant Lac production during the fermentation, showing the highest Lac activity at 144 h (10.9 U/gS).

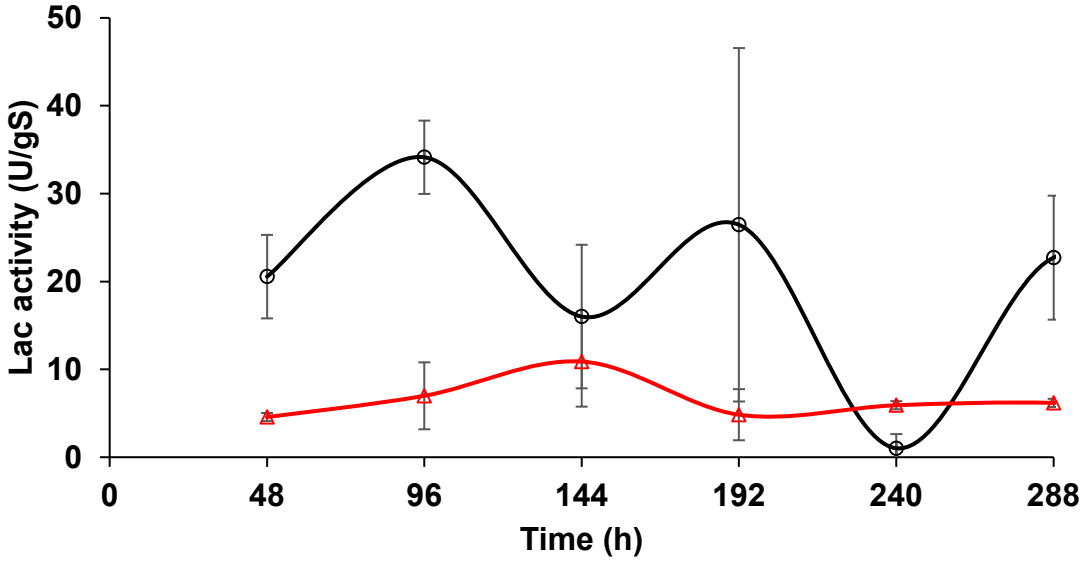

Fig. 1. Lac activity of *T. versicolor* grown on GW (circle) as a substrate and on PUF (triangle) as an inert support in solid-state fermentation.

Figure 2 shows MnP production by *T. versicolor* grown on GW and on PUF. GW-supplemented cultures had the highest MnP activity at 48 h (943 U/gS), which decreased at both 144 and 240 h, and then increased at the end of the fermentation (419 U/gS) (Fig. 2, Table 1). In PUF-added cultures, no MnP activity was detected during the first 96 h of growth, however, this enzyme activity was observed from 144 h, reaching the highest production level at 192 h (588 U/gS), which decreased at the end of the fermentation.

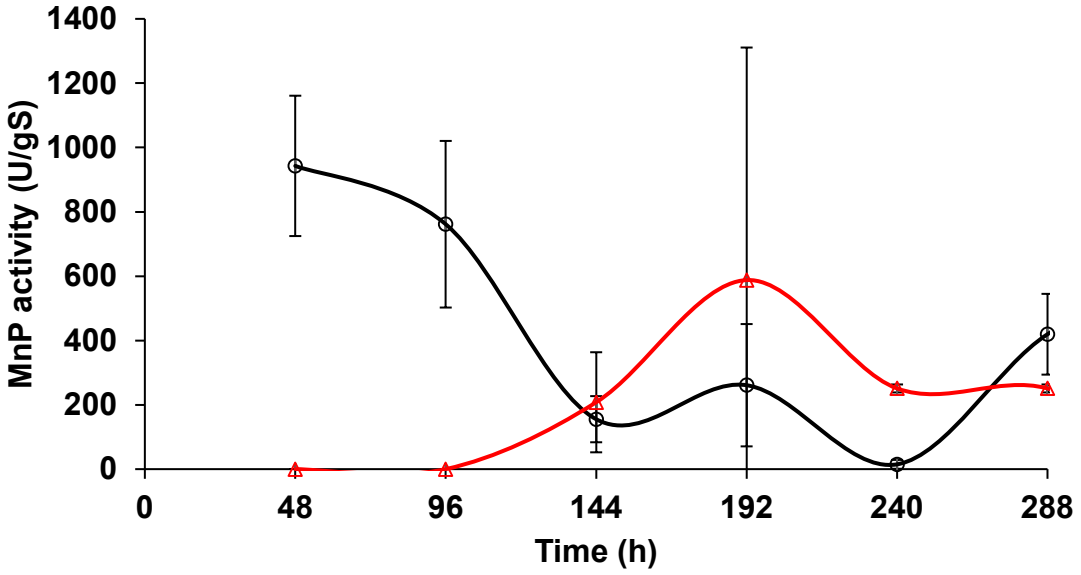

Fig. 2. MnP activity of *T. versicolor* grown on GW (circle) as a substrate and on PUF (triangle) as an inert support in solid-state fermentation.

UnP production by *T. versicolor* grown on GW and on PUF is observed in Fig. 3. In GW-supplemented cultures, UnP showed the highest activity value at 48 h (1023 U/gS) and the lowest at 240 h (26 U/gS), showing a slight increase in production at both 192 h and 288 h (228 and 326 U/g/S, respectively). In PUF-added cultures, UnP activity was observed at 144 h (229 U/gS), reaching the highest activity value at the end of fermentation (559 U/gS).

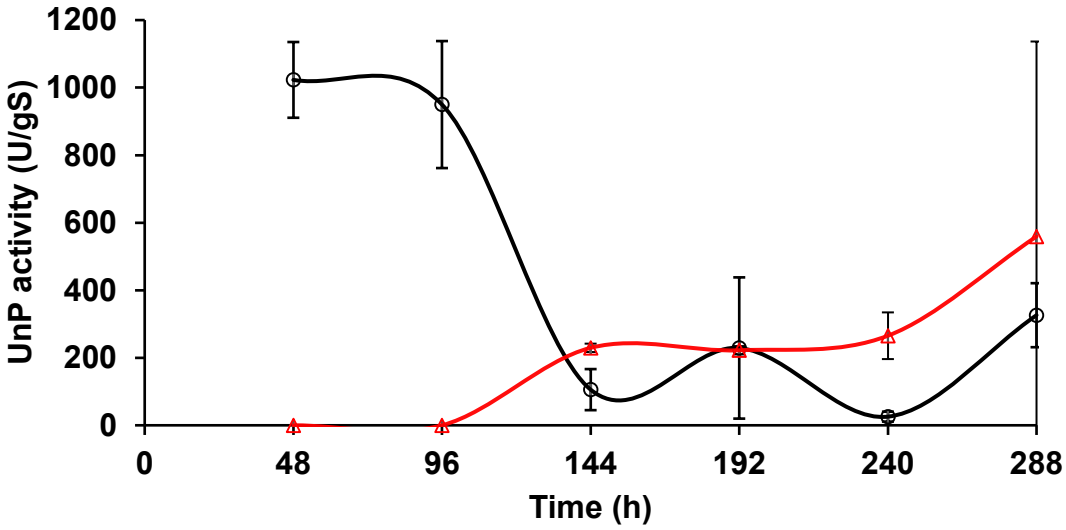

Fig. 3. UnP activity of *T. versicolor* grown on GW (circle) as a substrate and on PUF (triangle) as an inert support in solid-state fermentation.

Figure 4 shows LiP production by *T. versicolor* grown on GW and on PUF. GW-supplemented cultures had the highest activity at 48 h (766 U/gS), which decreased until at minimum of 30 U/gS at 240 h, however, LiP activity had a slight increase at 192 (198 U/gS) and 288 h (152 U/gS). PUF-added cultures showed a low LiP production during the fermentation. LiP production was detected at 96 h and increased at 144 h (75 U/gS), reaching the highest activity value at 288 h (229 U/gS).
In general, it was observed that all the enzymes had the highest activity during the first 96 h in cultures grown on GW.

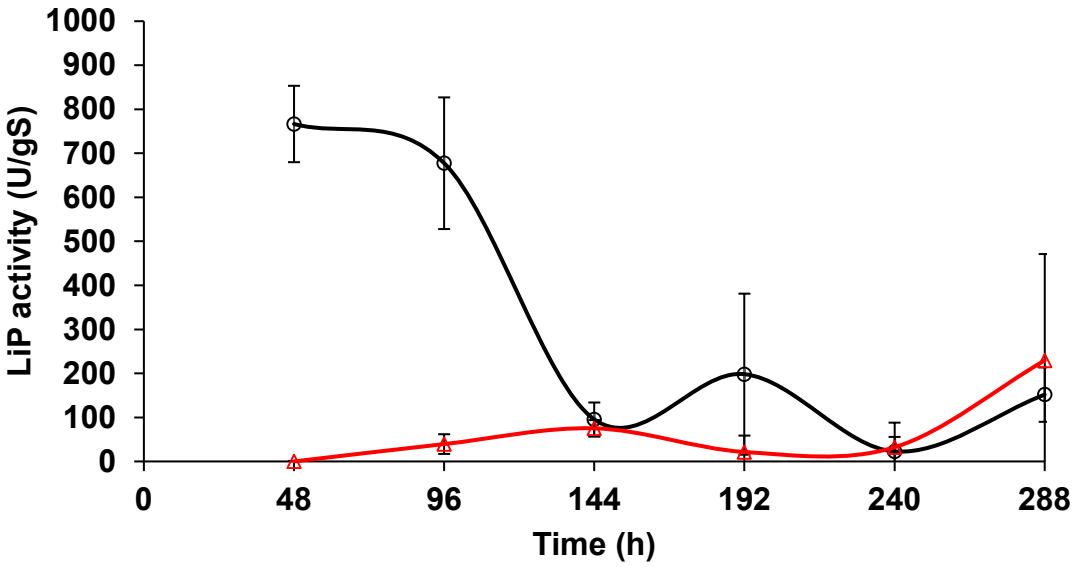

Fig. 4. LiP activity of *T. versicolor* grown on GW (circle) as substrate and on PUF (triangle) as inert support in solid-state fermentation.

Table 1. Enzymatic yield parameters of *T. versicolor* grown on GW as a substrate and PUF as an inert support in solid-state fermentation.

Parameters	Enzymatic activity							
	Lac		MnP		UnP		LiP	
	GW	PUF	GW	PUF	GW	PUF	GW	PUF
E_{max} (U/gS)	34.137^{ab}	10.9^{b}	942.881^{ab}	587.81^{ab}	1022.92^{a}	559.14^{ab}	766.52^{ab}	229.39^{ab}
P (U/gS/h)	0.355^{b}	0.075^{b}	19.643^{a}	3.06^{b}	21.31^{a}	1.94^{b}	15.97^{a}	0.79^{b}

E_{max}, maximum enzymatic activity; P_{ro}, enzyme productivity (E_{max}/h). Values are expressed as means (n=3). Means within the same row that do not share superscripts letters differ significantly at 5% level.

T. versicolor showed higher E_{max} and *P* values for all the enzymes tested in cultures grown on GW in comparison to that E_{max} values observed in cultures grown on PUF (Table 1). Lac and UnP activities were significantly higher in GW-supplemented cultures than in PUF-added cultures. In general, UnP showed the highest activity, followed by MnP, LiP and Lac in all *T. versicolor* cultures (Table 1).

4. Discussion

These results showed that *T. versicolor* produced Lac, MnP. LiP and UnP during all the fermentation when GW was used as a substrate. MnP and UnP activities were not detected during the first 96 h of growth, whereas LiP activity was observed after 48 h of fermentation in media containing PUF as an inert support. In addition, the higher enzymatic activities were observed in GW-supplemented cultures than in PUF-added cultures. These results showed that *T. versicolor* produced constitutive and inducible enzyme activities, which production was enhanced by GW. It is suggested that Lac is a constitutive enzyme and was produced during the fermentation in both PUF-added and GW-supplemented cultures. It has been reported that Lac production is required during the fungal growth for providing hydrogen peroxide to the peroxidases, which act synergistically during the catalytic degradation of the substrate (Santacruz-Juárez *et al*., 2021). In PUF-added cultures, LiP was detected after 48 h of growth, which might be associated (as constitutive enzymes) to fungal growth at the beginning of the exponential phase, whereas MnP and UnP were produced after 96 h, suggesting that these peroxidases are produced as constitutive enzymes at the end of fermentation.

In GW-supplemented cultures, *T. versicolor* produced the highest MnP, LiP and UnP activities during the first 96 h of growth, with UnP showing the highest activity of all. It has been reported that UnP is a relevant biodegradation and biocatalytic enzyme capable of transforming most organic US EPA priority pollutants (Karich *et al*., 2017). In the present research, UnP also showed to be an important enzyme during degradation of GW. Pinheiro *et al*. (2020) found that *T. versicolor* showed a Lac activity of 23 U/L and 80 U/L approximately, in sugarcane bagasse and barley bagasse, respectively, at 14 d of growth in liquid fermentation. In addition, Xu *et al*. (2020) studied Lac production by *T. versicolor* in solid-state fermentation using tea residues as substrate and found that this fungus has a Lac activity of 25.7 U/gS after 7 d of growth under optimum condition. In this research, *T. versicolor* produced 34 U/gS of Lac at 96 h in GW-supplemented cultures grown under solid-state conditions. In addition, Aydinoğlu and Sargin (2013) studied Lac production by *T. versicolor* using olive leaves as substrate under solid-state fermentation conditions. It was found that highest Lac activity (276.62 U/gS) was achieved optimizing moisture and particle size of the substrate supplemented with yeast extract as nitrogen source. Paice *et al*. (1993) reported that *T. versicolor* produce both Lac and MnP during bleaching of kraft pulp, while lignin peroxidase was usually not detectable. *T. versicolor* grown on wheat bran in optimizing and upscaling solid-state fermentation process had the highest Lac and MnP activities (820 and 23 U/gS, respectively) at day 21 of fermentation (Baker and Charlton, 2023). It has been reported that enzyme production by fungi is greater when they are grown in solid-state fermentation in comparation to submerged fermentation (Viniegra-González *et al*., 2003).

It is shown that production of lignincellulose-degrading enzymes by *T. versicolor* depends on the strain, nature of the substrate, fermentation system, and growth conditions. These enzymes are very important as concerns of biotechnology field, which can be used in areas such as paper and pulp, industry, food processing technology, and in the development of techniques for degradation of environmental pollutants among others (Singh and Gupta, 2020).

5. Conclusion

These results suggest that *T. versicolor* produced Lac inducible and constitutively, whereas, LiP, MnP and UnP were induced by the substrate at the earliest stage of fungal growth, however, these enzymes were constitutive and inducible during the rest of the fermentation. It is shown that oxidases and peroxidases production was induced and enhanced by GW. The present research demonstrates that the use of GW for fungal growth in solid-state fermentation is a promising method for the production or oxidases and peroxidases, which represent a low-cost and efficient technique for the production of enzyme, as well as an alternative for GW reduction from the environment. *T. versicolor* was capable to degrade GW due to its production of lignincellulose-degrading enzymes, which was induced by the components present in this lignocellulosic waste. To the best of our knowledge, this is the first detailed study on the involvement of the main lignincellulose-degrading enzymes in GW degradation by fungi. In particular, the relevance of UnP as a peroxidase involved in lignocellulose biodegradation was demonstrated. Further studies are required to define the optimal fungal growth conditions, nutritional supplementation of the substrate (nitrogen, calcium, magnesium sources, etc.), the size of the substrate particles and the type of inoculum to increase the yield of enzyme production in GW.

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Author contribution

Edgardo Ocaña-Romo performed the experimental work and analyzed data. Celestino Odín Rodríguez-Nava planned the experiments, conceived the initial idea, supervised the research and analyzed data. Carmen Sánchez planned the experiments, conceived the initial idea, supervised the research, analyzed data, and wrote the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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