Phytotoxicity reduction of walnut green husk by white-rot fungi under solid-state fermentation

Yu Zhang

Kunming University of Science and Technology

Xuejie Li

Kunming University of Science and Technology

En Yang

Kunming University of Science and Technology

Huini Xu

Kunming University of Science and Technology

Jinping Yan (✉ jpyan2019@163.com)

Kunming University of Science and Technology <https://orcid.org/0000-0002-5583-0912>

Research Article

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Abstract

Phytotoxicity reduction is one of the main aims of reusing walnut green husk (WGH) for cultivation purposes. This study was conducted to evaluate the efficiency of bio-detoxification by selected *Trametes* and *Schizophyllum* strains under solid-state fermentation. Ninety-two isolates of filamentous fungi were obtained from forest litter, and nine strains belonging to *Trametes* and *Schizophyllum* genus were selected due to their efficient laccase production and rapid growth using WGH and corncob (w/w, 1:1) as the carbon source under solid-state fermentation. Further, enzyme activities and phytotoxicity assays showed that water extract of WGH treated by *Trametes* strains 120301, 10288, and 10306 showed a vast lignocellulose enzyme system, higher laccase activity (about 620 U/g), and maximal germination rate (100%), compared with 20% germination rate of the untreated extract of WGH. Our results indicated that *Trametes* strains are suitable for the phytotoxicity reduction of WGH and laccase production, and showed the possible role of laccase in the detoxification of agricultural wastes rich in phenols such as WGH.

Introduction

Walnut (*Juglans regia* L.) is known as one of the world's four major dried fruits, and the planting area is expanding on the world scale owing to their different uses in the food industry (Ali and Ryszard 2018; Zheng et al. 2019; Soto-Madrid et al. 2021). The global walnut production until 2017 exceeded 3.8 million tons (Zheng et al. 2019). In the walnut processing industry, upon separating the kernel from the outer parts of the fruit, large amounts of husk and shell more than 60% of the walnut fruit are produced. Among them, the amount of walnut green husk (WGH), also named exocarp or mesocarp, is equivalent to 20% of the total walnut production (Izadiyan et al. 2019; Soto-Madrid et al. 2021). However, WGH is discarded as agricultural wastes, burned or antioxidant extract (Caballero et al. 2019; Izadiyan et al. 2019; Latos-Brozio and Masek 2019; Soto-Madrid et al. 2021), and the utilization efficiency of WGH biomass is still limited partly because of their scattered distribution and high transportation cost.

In the case of agricultural wastes, straightforward and easy disposal is by their reuse for cultivation purposes (Ciniglia et al. 2012). For example, WGH biomass as a fertilizer is one of its applications as it is rich in organic matter (Soto-Madrid et al. 2021). However, it is well known that walnut is allelopathic, and the walnut litters, such as leaves and green husks, is also concentration-dependent phytotoxic (Ciniglia et al. 2012; Wang et al. 2014). Green husks may serve as a fertilizer at low concentrations but have toxic effects against several crops at high concentrations (Ciniglia et al. 2012). In addition, the accumulation of green husks biomass results in large amounts of wastewater containing water-extractable components, such as water-soluble polyphenols, together with hull residues, which is a potential risk and health threat in the walnut-producing regions (Ciniglia et al. 2012). Previously, many studies focused on toxic substances in walnut, and the results showed that juglone, phenolic acids, flavonoids, terpenes, and other polyphenols present in walnut might be responsible for its toxicity (Ciniglia et al. 2012; Saba et al. 2013; Wang et al. 2014). Thus, reducing the toxin factor in WGH and its effluent is essential for its application and environment-friendly treatment.

Solid state fermentation (SSF) is an efficient strategy for biological detoxification and the bio-conversion of lignocellulosic wastes using fungal strains (Brand et al. 2000; Madeira et al. 2011; Zhang et al. 2021b). For example, *Paecilomyces variotii* can convert the toxic waste of castor beans into animal feed material (Madeira et al. 2011). *Aspergillus* sp. and *Mucor circinelloides* showed good prospects in the detoxification of coffee husk (Brand et al. 2000) and *Jatropha curcas* L. cake (Zhang et al. 2021b), respectively. In addition, many kinds of useful products can be obtained in the process, such as enzymes (including laccase, tannase, and phytase), polysaccharides, and active small molecules (Madeira et al. 2011; Wang et al. 2019). Among those fungal strains, wood-rotting fungi have potential due to their ability to degrade phenolic compounds (Wang et al. 2019), which is toxic and abundant in WGH (Ciniglia et al. 2012; Saba et al. 2013; Wang et al. 2014). Wood-rotting fungi along with their extracellular enzymes, such as laccase and MnP, showed great potential in phenolic compound degradation (Wang et al. 2019; Di Lella et al. 2021). For example, *T. versicolor* under SSF can efficiently degrade BPA along with high laccase activity (Wang et al. 2019; Wang et al. 2020), and *Hericiumerinaceus* showed 47% total phenol decay associated with laccase and manganese peroxidase activities when cultured in olive mill wastewater (Koutrotsios et al. 2016; Wang et al. 2019). Based on these results, we inferred that wood-rotting fungi might be a potential candidate for bio-detoxification and bio-conversion of WGH.

In recent years, China has become the world's largest walnut producer with 1.9 million tons in 2017, but there are still limited ways to effectively process or utilize large amounts of WGH. Moreover, walnut is usually planted in mountainous area in China, which possesses potential environmental risk to the rivers and lakes nearby. In the present study, we aimed to (i) screen wood-rotting fungi capable of detoxifying WGH; (ii) establish a phytotoxicity assessment model for the water extract of WGH; and (iii) evaluate the efficiency of WGH bio-detoxification by selected strains and explore the role of laccase in this process.

Materials And Methods

Chemicals and fungal strains

Enzyme substrates, 2,2'-azino bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and oats xylan and veratryl alcohol (VA), were purchased from Sigma (USA). The fungal strains used in this study were stored at the strain collection of Biotechnology Research Center of Life Science and Technology College, Kunming University of Science and Technology. Strains were routinely maintained on a GYP slant (2% glucose, 0.5% yeast extract, 0.5% tryptone, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.5% agar power) at 4°C. Dried WGH and corncob biomass were collected from walnut growing area in Baoshan of Yunnan Province, China.

Isolation and identification of fungal strains from forest litter

The decayed branches and leaves with fungal fruit body or hyphae was collected from the forest in Baoshan, China. After the elimination of surface contamination, a small piece of wood or fruit body (3-5

mm²) were placed on GYP plate for incubation at 28°C until the visible colony formed. The pure cultures were obtained from hyphal tip and transferred to another GYP plates for further isolation and purification. Morphological and molecular identification were performed according to our previous study (Yan et al. 2015). The ITS region was amplified using the primer pairs: 5'-GGAAGTAAAAGTCGTAACAAGG-3' (forward) and 5'-TCCTCCGCTTATTGATATGC-3' (reverse).

Solid-state fermentation (SSF) and linear grown test

After dried at 60°C for 4 d, WGH and corncob biomass was milled using a micro-grinder and sieved a screen with 18-40 mesh. Then, the blended powder of WGHs and corncobs (2:1, w/w) biomass was used as solid support for SSF. Each 100 g mixture was humidified with 200 mL of a liquid medium containing CaCO₃ (1.5%, w/v) and CaSO₄ (0.8%, w/v). The initial pH and relative humidity of the medium was adjusted to 7.0 and 60%, respectively. 40 g of the above mixed biomass was transferred to a 28 × 150 mm test tube and sterilized at 121°C for 90 min. This medium was used for SSF and linear grown test. After 8 d incubation at 30°C, the mycelia of GYP plates were used as inoculation. Each test tube contained 4 mycelial plugs (1 cm in diameter) and continued to grow at 28°C in darkness. The length of the colonized substrate was recorded every three days. Solid fermented substrates were harvested in three equal layers (the upper, middle and the lower layer) when the mycelium of all strains spread the whole tubes (about 15 days). For linear grown test, substrate colonization rate per day was calculated according to the method described by Postemsky et al. (2015). Instead, wet weight of the sterilized substrate in each tube was employed in our study.

Enzyme extraction

Crude enzyme was obtained by mixing a 5 g of each solid fermented substrate by different strains with 20 mL sodium acetate buffer (pH 4.8, 50 mM). Subsequently, the mixture was stirred continuously at 200 rpm for 30 min at 25°C, and centrifuged at 10000 rpm for 10 min at 4°C. The resulting supernatant were designated as the crude enzyme for further enzyme assays.

Enzyme assays

The activities of the lignocellulolytic enzymes including filter paper activity (FPA), laccase, xylanase, LiP and MnP was determined under standard conditions as described previously (Liu et al. 2019, Zhang et al. 2021a). LiP activity was determined with veratryl alcohol (VA) as substrate in sodium tartrate buffer (pH 2.5, 50 mM) containing 2 mM VA and 0.1 mM H₂O₂. One unit of LiP activity was defined as the amount of enzyme that oxidized 1 µmol of VA per min. The activity of MnP was measured with MnSO₄ as substrate in 100 mM sodium tartrate buffer (pH 5.0) containing 0.1 mM MnSO₄ and 0.1 mM H₂O₂ at 238 nm for 1 min at room temperature. One unit of MnP was defined as the amount of enzyme required to oxidize 1 µmol of substrate in 1 min. Meanwhile, laccase activity was determined in a reaction mixture

containing 0.5 mL of appropriately diluted crude enzyme and 1.1 mL of 2 mM ABTS in phosphate citrate buffer (100 mM pH 4.0) at 420 nm for 3 min. One unit of the enzyme activity was defined as the amount of enzyme that oxidized 1 mmol of the ABTS per min. The activities of FPA and xylanase were determined by 3, 5-dinitrosalicylic acid (DNS) method using filter paper and oats xylan as the substrates. The amount of reducing sugars released was determined by DNS method against boiled enzyme as a control and D-glucose as a standard. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μ mol of reducing sugars per min.

Phytotoxicity assay

An equal amount of solid fermented substrate from three layers of each treatment was harvested and mixed well. Subsequently, each 30 g mixture was humidified with 396 mL sterile water and stirred continuously at 200 rpm overnight at 4°C, making a water extract with 5% WGH (w/v). The filtrates obtained by filtration using four layers of cheesecloth were used for phytotoxicity assay. The water extracts were obtained according to the methods above with the final WGH concentration of 0.5%, 2.5%, 5%, 10%, and 20% (w/v) to prepare the water extract from untreated WGH and corncob biomass.

To assay phytotoxicity, a seed germination experiment was carried out by filter paper plating method. In brief, 10 mL of the water extract was added to a Petri dish with one layer of filter paper, and then Chinese cabbage sterilized seeds were sowed evenly on the surface of the wet filter paper. The Petri dishes were maintained in a greenhouse for four weeks, and seed germination, seedling growth, and root length were recorded every 24 h. Each treatment contained three plates with 20 seeds each.

Statistical analyses

Statistical analyses were performed using GraphPad Prism v8.3. The results of all experiments were based on the average of three independent experiments $\pm$ standard deviation (SD), and the statistical significance was determined by unpaired t-test and one-way Anova.

Results

Isolation and identification of fungal strains from forest litter

Ninety-two isolates of filamentous fungi were obtained from forest litter, which grew normally at 28°C. Their ability to produce laccase and the growth rate was evaluated on GYP solid medium with 2 mM ABTS, and the results showed that most strains could form green or purple staining zones. Nine strains belonging to *Trametes* and *Schizophyllum* genus were chosen based on higher growth rate and enzyme production on solid GYP medium towards ABTS (Table 1). Among them, four strains (120301, 10306, 10288, and 8607) belonged to *Trametes* genus, and others were *Schizophyllum S. commune* (Table 1).

Solid state fermentation of WGH and corn cob lignocelluloses by the selected strains

The selected strains were further screened for the mycelial growth and efficiency of enzyme production (LiP, MnP, Lac, filter paper enzyme activity, xylanase) under solid state fermentation using WGH and corncob (w/w, 1:1) as the base carbon source. All strains successfully colonized on the mixed substrates of WGH and corncob, and linear growth occurred along the tubes (Fig. 1A). Among all the selected strains, strains 10306, 10288 and 100301 were slower in linear growth and more dense mycelia, while other strains were favorable to cover quickly to the whole tubes and exhibited a substrate colonization rate of about 2500 mg d^{-1} , compared to about 1800 mg d^{-1} of strains 10306 and 10288 (Fig. 1B).

The activities of the lignocellulolytic enzymes per dry weight of substrates in these samples are shown in Fig 2. Strains 10288 and 10288 were found to be the most promising strains with higher FPA production compared to other isolates (Fig. 2A). The FPA activity of strains 10288 and 10288 were 130.9 and 137.5 U g^{-1} after 15 days of incubation, respectively. Whereas the FPA activity was found to be less for other strains, like strain 11193 which produced 22.3 U g^{-1} . All strains showed higher xylanase activity except strain 10306, which produced 17.7 U g^{-1} , while the xylanase activity of strains 10288 and 12031 reached above 120 U g^{-1} (Fig. 2B). The higher laccase activity was observed in strains 10306, 10288, and 120301 with the activities of 353.7 , 648.3 , and 392.7 U g^{-1} , respectively, and followed by strain 12028 (93.7 U g^{-1}), whereas the laccase activity of other strains was less than 2 U g^{-1} (Fig. 2E). During the incubation period, LiP and MnP activities could be observed in all the tested strains (Fig. 2C and D), strains 120301 and 10288 were the most promising strains for LiP (3.8 U g^{-1}) and MnP (4.5 U g^{-1}), respectively.

Toxicity analysis of the water extract of WGH on plant growth

The water extract of WGH at different concentrations (0.5%, 2.5%, 5%, and 10%) showed different effects on plant growth, including seed germination, the elongation of hypocotyls and roots when the seeds of Chinese cabbage were used as the tested plant (Fig. 3). The WGH water extract with a concentration lower than 2.5% showed no effect on seed germination, while seed germination was completely inhibited when the concentration of water extract increased to 5%. On the other hand, the WGH water extract had double effects on the elongation of hypocotyls by the concentration-dependent manner (Figs. 3 and 5). The WGH water extract with 0.5% concentration could obviously promote the elongation of hypocotyls. In addition, the water extract concentration higher than 2.5% restrained root growth.

Disinhibition effect of solid-state fermented WGH

To explore whether solid state fermentation by these white-rot stains can affect the inhibition of the WGH water extract on plant growth, seed germination experiment in plates containing different WGH water extract was employed. The results showed that the water extract of WGH treated with the selected white-rot strains showed different effects on seed germination in a strain-dependent way (Fig. 4). On plates with the treated water extract of WGH by strains 120301, 10288 and 10306, the seed germination was 45%, 60%, and 60% within 2 d, respectively, compared with a 10% germination rate in the water extract at the same concentration (5%) (Fig. 4). On day 5, the more obvious disinhibition effects by strains 120301, 10288 and 10306 were observed with the maximal germination rate of 100%, 100%, and 85%, respectively, while the untreated extract showed only 20% germination rate. However, other strains did not show obvious effects on seed germination.

Further, we also observed that the treated water extract of WGH by strains 120301, 10288 and 10306 could remarkably promote the growth of Chinese cabbage seedlings (Fig. 5). When the water extract of WGH treated by strains 120301, 10288 and 10306 was added, we can observe that the hypocotyls of all seedlings were 1.5 times as long as the control plate, along with larger leaves. Moreover, the root length of seedling treated by strain 120301, 10288 and 10306, was slightly longer than that control and almost four times longer than the untreated water extract of WGH (Fig. 5). These results indicated that solid state fermentation of WGH by some white-rot fungi, such as strain 10288, can reduce the toxicity of WGH and promote plant growth.

Discussion

Recently, along with the spread and increasing of walnut growing area, functional applications of WGH, the main by-product of walnut industry, become the concerns of scientists all around the world (Kachlishvili et al. 2014; Ali and Ryszard 2018; Zheng et al. 2019; Soto-Madrid et al. 2021). Bio-transformation of waste biomass using fungal strains can produce a variety of high-value products along with the reduction of phytotoxicity or anti-microbial components (Madeira et al. 2011; Sadh et al. 2018; El-Sayed et al. 2020). For example, the detoxification and the higher production activity along with higher activity of the production of tannase and phytase were observed simultaneously when castor bean residues were treated under solid-state fermentation by *Paecilomyces variotii* (Madeira et al. 2011).

The WGH biomass is usually composed of crude fiber (44.7%), protein (4.7%), lipids (1.4%) (Soto-Madrid et al. 2021), which is regarded as a low-cost nutrient substitute for the bio-transformation by fungal strains (Elisashvili and Kachlishvili 2009; Kachlishvili et al. 2014). On the other hand, WGH is rich in juglone, tannins, phenolic acids, and other polyphenols, and the presence of phytotoxic or anti-microbial components may show an inhibitory effect in the bio-transformation process (Soto-Madrid et al. 2021). White-rotting fungi growth in olive mill wastewater with high content in polyphenolics resulted in a significant reduction of phenolics (by 64–67% and 74–81% for *Ganoderma* and *Pleurotus* spp., respectively) along with the increase of laccase activity (Ntougias et al. 2012), which revealed a significant correlation between laccase activity and the reduction of phenolics. In addition, phenolics are effective inducers of fungal laccase (Wang et al. 2019).

In this study, ninety-two isolates of filamentous fungi were obtained from forest litter, and nine strains were selected due to their efficient laccase production and rapid growth (Table 1). Those strains all can colonize and grow when WGH and corncobs were used as the carbon source under solid state fermentation (Fig. 1), and during the solid state fermentation process, the activities of lignocellulose degrading enzymes can be detected (Fig. 2), which suggests that WGH is a potential substrate for the solid state fermentation by *Schizophyllum* and *Trametes* spp. However, strains *Schizophyllum commune* showed a rapid substrate colonization rate but sparse mycelia, which was contrary to those of strains *Trametes* spp. (Fig. 2). One possible reason is that *Schizophyllum commune* strains tend to utilize corncob preferentially, which can be confirmed by their higher xylanase activity (Fig. 2). Strains showed dense mycelia and considerable levels of the lignocellulolytic system, especially higher activity of laccase and MnP. This result is in good agreement with observations in *Cerrena unicolor* (Kachlishvili et al. 2014) and other *Trametes* strains (Elisashvili and Kachlishvili 2009). In this respect, the ligninolytic system of *Trametes* strains differed from that of *Schizophyllum commune*, and the former is a potential source of dominant strains for WGH fermentation.

Except for LiP, the MnP, laccase, FPA, and xylanase activities of *T. hirsuta* strain 10288 during cultivation on WGH were higher than other selected strains, with the activities of about 4.5 U g^{-1} , 620 U g^{-1} , 125 U g^{-1} , and 110 U g^{-1} , respectively (Fig. 2). These results demonstrated that *T. hirsuta* 10288 possesses a wide lignocellulose enzymes system. Compared with laccase production by different white-rotting fungi grown on natural supports under SSF (Madeira et al. 2011; Phengnuam and Suntornsuk 2013; Wang et al. 2019), *T. hirsuta* 10288 has high ability to produce laccase in the tested conditions. Whether the high yields of laccase in *T. hirsuta* 10288 due to the certain special components of WGH is still unknown. Moreover, *T. hirsuta* strain F13 exhibited much higher laccase activity under submerged cultivation conditions than solid-state cultivation conditions (Jović et al. 2018). Further studies focused on the conditions of laccase production on *T. hirsute* 10288 will be employed.

Due to the presence of toxic substances in walnut, such as high concentration of polyphenols, reduction of phytotoxicity in WGH and its leachate is one of the main aims in their reuse for cultivation purposes (Ciniglia et al. 2012; Wang et al. 2014). To assess reduction of phytotoxicity by the selected strains under SSF, phytotoxicity assay was established using seeds of Chinese cabbage. Compared with the untreated water extract at the same concentration (5%), the obvious disinhibition effects on seed germination and seedling growth by *Trametes* strains 120301, 10288, and 10306 were observed (Figs. 4 and 5), which may suggest the detoxification effect by *Trametes* strains during SSF. The detoxification effects were also detected in the bio-treatment process of castor bean residues by *Paecilomyces variotii* (Madeira et al. 2011), effluents of the green olive by white-rotting fungi (Aggelis et al. 2002), *Jatropha curcas* L. cake by a soil-borne *Mucor circinelloides* (Zhang et al. 2021b). Thus, the tested *Trametes* strains 120301, 10288, and 10306 in this study are candidates for the phytotoxicity reduction in WGH.

In this study, we also found that the detoxification efficiency of WGH revealed a consistent pattern with the yields of laccase in those strains (Fig. 2 and 4), which was also detected in olive mill wastewater biodegradation by *Ganoderma* and *Pleurotus* spp. (Ntougias et al. 2012). The mechanism of

detoxification of WGH has not been fully deduced, though it is well known that laccase has ability to oxidize a broad range of structurally different substrates (e.g., monophenols, polyphenols, aminophenols,, aromatic amines), remove xenobiotic substances and generate polymeric products (Wang et al. 2019; Zhang et al. 2021a). Of course, it is very likely that other factors, such as some biological compounds or enzymes secreted by microbes, contribute to the detoxification effect. In our laboratory, the experiments on the laccase of *T. hirsuta* 10288 and its transformation towards polyphenols of WGH are underway.

Conclusion

Agricultural wastes are high-quality fertilizer resources, and the reuse of WGH as fertilizer is mainly limited by the presence of phytotoxic substances. We showed strains of *Trametes* genus, represented by *T. hirsute* 10288, were suitable candidates for the phytotoxicity reduction of WGH and also for laccase production using WGH as a low-cost nutrient substitute under solid state fermentation. Our study also suggested the possible role of laccase in the detoxification of agricultural wastes rich in phenols, such as WGH.

Declarations

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Authors' contributions

Yu Zhang and Xuejie Li: methodology, data curation and writing-original draft, En Yang: visualization and investigation, Huini Xu: methodology, software and conceptualization, Jinping Yan: methodology, supervision, writing—review and editing.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors mutually agreed to publish the work in this journal.

Conflict of Interest

The authors declare no conflict of interest.

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Tables

Tables are available in the Supplemental Files section.

Figures

Figure 1

Solid-state fermentation using WGH and corncob as the base carbon source by *Trametes* and *Schizophyllum* strains. A: Mycelium growth after 12 days of cultivation. B: The comparison of the linear growth rate among the selected strains.

Figure 2

Lignocellulolytic enzyme activities of nine *Trametes* and *Schizophyllum* strains under solid-state fermentation using WGH and corncob as the carbon sources. A: LiP activity. B: MnP activity. C: Filter paper activity (FPA). D: Xylanase activity. E: Laccase activity.

Figure 3

The effects of WGH water extract on seed germination using the seeds of Chinese cabbage as the tested plant.

Figure 4

Seed germination experiment using the solid fermented WGH by the selected white rot fungi after 15 days of cultivation at 28°C. As control, A and L were added with equal amounts of distilled water and water extract from untreated WGH, respectively.

Figure 5

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Seedling growth experiment using the solid fermented WGH by the selected white rot fungi. Control 1 and 2 are added with equal amounts of water extract from untreated WGH and distilled water, respectively.

Supplementary Files

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