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The utilization of lignocellulosic wastes for laccase production under semisolid-state and submerged fermentation conditions

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Abstract: The aim of this study was to produce laccase enzymes by using various lignocellulosic wastes (LCWs) under semisolid-state (SsF) and submerged fermentation (SF) conditions. White rot fungi are the best laccase-producing organisms and they can be easily grown on LCW. Thus, in this work, *Trametes trogii* (Berk.) ATCC 200800 and *Trametes versicolor* (L.) ATCC 200801, well-known laccase-producing white rot fungi, were used as laccase-producing organisms. According to the literature, some of the LCWs such as sunflower receptacle, apricot seed shell, and bulrush were tested first as substrates for laccase production by these fungi. In SsF, the maximum laccase activities were 2206 U/L for *T. trogii* incubated in a medium containing walnut shell and 387 U/L for *T. versicolor* incubated in a medium containing corncob. In SF, the highest laccase activities, 386 U/L and 1216 U/L, were obtained from *T. trogii* grown in a medium containing pulverized apricot seed shell and *T. versicolor* grown in a medium containing pulverized bulrush, respectively. Because laccase is an important biotechnological enzyme with widespread applications, it can be useful to utilize these natural substrates for laccase production by these fungi.

Key words: Lignocellulosic waste, white rot fungi, fermentation, laccase, enzyme activity

1. Introduction

The renewable material lignocellulose is the major component of biomass and consists of lignin, hemicellulose, and cellulose (1). It is the most abundant natural material in the world (2). Large quantities of lignocellulosic wastes (LCWs) are released from various industries such as food, agricultural, forestry, paper pulp, and timber. These wastes cause serious environmental pollution, but can be reused constructively rather than burned due to their rich sugar contents (3,4). The chemical properties of these lignocellulosic wastes make them a crucial and cost-effective fermentation medium for biotechnological applications (3). However, while the hemicellulose and cellulose components of lignocellulosic materials are used by numerous microorganisms, the lignin, which is the most resistant material to microbial degradation, is converted efficiently by only a limited number of organisms, such as white rot fungi (1). Lignin serves as a barrier that protects cellulose and hemicellulose from enzymatic attack; however, white rot fungi can attack this barrier in order to obtain energy from cellulose (5). These fungi produce different extracellular ligninolytic enzymes such as laccase, manganese peroxidase, and lignin peroxidase (6,7). Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) has received much attention because of its relatively low substrate specificity in comparison to most enzymes, and for its potential application in different biotechnological, industrial, and environmental fields. This enzyme can be used for biopulping, biobleaching, wastewater treatment, decolorization of various dyes, enzymatic removal of phenolic compounds in beverages, construction of biosensors, and bioremediation (8–14). Laccase can be produced at varying rates by using a wide range of organisms grown on different substrates and by using several methods of fermentation, such as solid state, semisolid state, and submerged (15–18). However, for effective laccase production, it is very important to use efficient laccase-producing organisms, suitable fermentation methods, and cheap and widespread sources. Accordingly, one of the most suitable approaches for the production of this enzyme is to use the most efficient agricultural wastes for increasing the production of the ligninolytic enzymes (19).

Agricultural wastes such as corncob and sunflower receptacle were tested as fungal fermentation media for laccase production in this study. Every year, approximately $2–3.10^6$ and 85.10^5 t of corn and sunflower are produced in Turkey, respectively (20). In addition, approximately 26.10^6

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t of wheat straw (21) and 15.10^4 and 35.10^4 t of walnut and hazelnut shells are produced annually in Turkey (22). Large amounts of apricot seed shells are also obtained from Malatya, which is the most important apricot production center not only for Turkey, but in the world. Annually, 35.10^3 t of apricot pits are obtained in Turkey. The pit consists of the kernel and its encasing shell; however, the kernel is the only edible part of pit. After washing, sorting, breaking, and separation processes, nearly all of the apricot kernels are exported (23). However, most apricot seed shells are usually burned or left in the field to rot.

Since LCWs are an abundantly available renewable carbon and energy source, many researchers have tested them for laccase production by using different fungi under various fermentation methods. Many different LCWs, such as orange bagasse (17), tree leaves (*Fagus sylvatica*) (19), mandarin orange peel (24), reed grass (25), wheat straw (26,27), corncob (28), and groundnut shells and seeds (29), have been used to produce laccase with the help of several fungi in solid-state fermentations.

The main aim of this study was to investigate the feasibility of using LCWs, sunflower receptacle (SR), walnut shell (WS), hazelnut shell (HS), apricot seed shell (ASS), corncob (CC), wheat straw (WHS), pulverized walnut shell (PWS), pulverized bulrush (PB), pulverized apricot seed shell (PASS), and pulverized wheat straw (PWHS), as natural, low-cost substrates for laccase production by *Trametes trogii* ATCC 200800 and *Trametes versicolor* ATCC 200801 in 2 different fermentations in short time periods. Some of these LCWs (SR, ASS, and PB) were first used for laccase production by these fungi. A limited number of studies on laccase production using lignocellulosic materials and/or wastes have been performed in semisolid-state (SsF) (30) and submerged fermentation (SF) conditions (31–33) in comparison with solid-state fermentation (6,18,24,28). Therefore, *T. trogii* and *T. versicolor*, well-known laccase producers, were selected and the cultures were incubated separately in SsF and SF conditions for laccase production.

2. Materials and methods

2.1. Organisms

Two different species of the genus *Trametes*, known to be one of the most efficient lignin-degrading genera, were selected as laccase-producing organisms. *Trametes trogii* (Berk.) ATCC 200800 growing on *Populus* sp. and *Trametes versicolor* (L.) ATCC 200801 growing on *Cupressus* sp. were used in this study. *T. trogii* and *T. versicolor* were originally collected from Malatya and Adana, respectively, in Turkey. The white rot fungi are stock cultures at İnönü University, Faculty of Arts and Science, Department of Biology, Malatya, Turkey. The stock cultures were maintained on Sabouraud dextrose agar (SDA) plates at 4 °C. After that, a portion of each fungal stock culture was transferred to fresh sterile SDA plates, and they were periodically subcultured every 2–3 weeks in a static incubator at 30 °C for 5 days.

2.2. LCWs used in the study

Various LCW were used for laccase production in SsF and SF. Suitable amounts of SR (*Helianthus annuus*), WS (*Juglans regia*), HS (*Corylus maxima*), ASS (*Armeniaca vulgaris*), CC (*Zea mays*), and WHS (*Triticum sativum*) were used in SsF. In SF, different quantities of PWS, PB (*Typha domingensis*), PASS, and PWHS were used.

2.3. Preparation of inoculum

The mycelia of fungi were incubated at 30 °C on slant SDA. After 1 week, mycelial suspensions of *T. trogii* and *T. versicolor* were prepared by adding 10 mL of sterilized distilled water to the SDA slant culture and rubbing the mycelia with a sterilized loop. After that, 5 mL of suspensions were pipetted into 250-mL flasks containing 100 mL of Sabouraud dextrose broth. Both of the fungi were cultured at 30 °C and 150 rpm for 5 days in a shaker incubator. After that, the cultures were gently homogenized (Polytron PT 10–35) under aseptic conditions, and these homogenized mycelia were used as inocula (18).

2.4. SsF, SF, and sampling methods

The LCWs used in both SsF and SF were dried in air at room temperature for 5 days and then the air-dried wastes were mechanically pretreated by chopping or pulverization to reduce the particle size of the LCW and thus facilitate laccase production. Before starting the experiments, the most convenient dry weights of LCW were chosen according to our preliminary test. Because they had different structural characteristics, the most suitable amounts of LCW in the flasks were determined for both fermentation methods. For the SsF studies, dried LCW were first chopped into pieces of about 1 cm, and 2 g of SR, 3 g of WS, 2 g of HS, 3 g of ASS, 4 g of CC, and 0.25 g of WHS were added separately to 250-mL flasks containing 30 mL of stock basal medium (SBM) consisting of (g/L): KH_2PO_4 0.2, $CaCl_2.2H_2O$ 0.1, $MgSO_4.7H_2O$ 0.05, $NH_4H_2PO_4$ 0.5, $FeSO_4.7H_2O$ 0.035, glucose 2, and yeast extract 1. These growth media were then autoclaved at 121 °C for 20 min. After autoclaving, 2 mL of inocula were transferred into the flasks under sterile conditions, and the fungal cultures were incubated at 30 °C in a static incubator for 10 days. For the SF studies, first the dried LCWs were pulverized in a blender in order to increase the surface area of the wastes. As a consequence of the pulverization process, the particle size of LCW dropped from 0.053 to 0.250 mm in mesh size. Afterwards, varied amounts (0.15, 0.30, and 0.50 g) of PWS, PB, PASS, and PWHS were added separately into 250-mL flasks containing 50 mL of SBM. The same volume of homogenates (2 mL) was inoculated into the flasks following the autoclaving

mentioned above. The agitated cultures were incubated at 30 °C in an orbital shaking incubator for 5 days at 150 rpm. SBM was added to the sterilized flasks with the aim of moisturizing the LCW and enriching the fungal growth in SsF and SF studies. The initial pH of SBM was adjusted to pH 5.0 prior to sterilization by adding 5 N HCl. Generally, white rot fungi require an acidic pH for a high amount of laccase production (7). The pH 5.0 is a suitable value for the fungal proliferation and laccase production by *T. trogii* and *T. versicolor*. The laccase activities of the samples obtained from SsF and SF were monitored once every 2 days and every day, respectively. The maximum laccase activities under these conditions of *T. trogii* and *T. versicolor* are listed in the Table.

2.5. Laccase activity assay

Five hundred microliters of culture fluids taken from the static cultures every 2 days and from the agitated cultures every day under aseptic conditions were used to determine the laccase activities. Laccase activities were determined spectrophotometrically (Shimadzu UV-1601, UV/Visible) by monitoring the oxidation of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to its cation radical ($ABTS^{\bullet+}$) at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) for 1 min. The reaction medium contained 100 mM sodium acetate buffer (pH 5.0), 0.5 mM ABTS, and a suitable amount of enzyme. One unit of enzyme activity is defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute at 30 °C (34). Data obtained from spectrophotometric measurements were evaluated statistically with SPSS 15.0 for Windows. After this statistical evaluation, the laccase activities in the culture fluids were expressed as U/L. All activity values are the mean of at least 3 replicates.

3. Results and discussion

Solid materials used for cultivation of the fungi act as either inert or noninert materials. Inert materials only act as an attachment place for the fungi, while noninert materials not only function as an attachment place, but also supply some nutrients to the fungi. Because of these roles, noninert materials are called support substrate (35). Because lignocellulose-based agricultural wastes contain lignin, cellulose, and hemicelluloses, which are rich in sugar, these wastes can be utilized as support substrate. While this approach can contribute to the production of industrially valuable products such as laccase enzyme, it can also help to remove economic loss and environmental pollution.

Laccase production is significantly affected by many different factors, such as the species of organism, the type of fermentation processes, and the physical properties and composition of the growth media. Because

Table. Maximum laccase activities (U/L) obtained from SsF and SF cultures of *T. trogii* and *T. versicolor* at 30 °C.

LCW	Maximum laccase activities (U/L) in SsF	
	T. trogii	*T. versicolor*
SR	2201 (day 10)	107 (day 8)
WS	2206 (day 10)	62 (day 10)
HS	384 (day 10)	68 (day 8)
ASS	1723 (day 10)	107 (day 8)
CC	198 (day 10)	387 (day 10)
WHS	244 (day 10)	215 (day 10)

Pulverized LCW	Maximum laccase activities (U/L) in SF					
	T. trogii			*T. versicolor*		
	0.15 g	0.30 g	0.50 g	0.15 g	0.30 g	0.50 g
PWS	(day 4) 281	(day 4) 256	(day 5) 286	(day 2) 579	(day 5) 838	(day 2) 812
PB	(day 2) 115	(day 5) 330	(day 5) 344	(day 3) 1054	(day 2) 1157	(day 2) 1216
PASS	(day 5) 364	(day 5) 386	(day 5) 350	(day 5) 727	(day 3) 787	(day 5) 1007
PWHS	(day 5) 70	(day 5) 77	(day 5) 175	(day 2) 528	(day 3) 722	(day 3) 848

SR: sunflower receptacle, WS: walnut shell, HS: hazelnut shell, ASS: apricot seed shell, CC: corncob, WHS: wheat straw, PWS: pulverized walnut shell, PB: pulverized bulrush, PASS; pulverized apricot seed shell, PWHS: pulverized wheat straw.

considerable amounts of low-cost enzymes are required in biotechnological applications, one of the most appropriate approaches for ensuring the efficient production of ligninolytic enzymes is the utilization of LCW. Thus, in the current study, the effects of various LCWs on laccase production with the help of 2 different white rot fungi in SsF and SF were investigated.

3.1. The effect of various LCWs on laccase production in SsF

Since LCWs are an abundantly available renewable carbon and energy source, many researchers have utilized them for laccase production by using different fungi under various fermentation methods. When compared to other LCWs, WHS, one of the most abundant LCWs in the world, has been more often investigated for laccase production under solid-state fermentations. Dinis et al. (6) tested WHS for laccase production of *Bjerkandera adusta*, *Trametes versicolor*, *Phlebia rufa*, and *Ganoderma applanatum* under solid-state fermentation and the maximum laccase activities were determined to be 0.01, 0.11, 0.15, and 0.35 U/mL, respectively. Sharma and Arora (36) similarly used WHS as a growth medium for *Phlebia floridensis* for laccase production under solid-state fermentation, and the highest laccase activity obtained under this condition was approximately 0.72 U/mL. Kachlishvili et al. (26) also utilized WHS by adding additional nitrogen sources for various lignocellulolytic enzyme production by *Funalia trogii* IBB 146, *Lentinus edodes* IBB 363, *Pleurotus dryinus* IBB 903, and *P. tuberregium* IBB 624 in solid-state fermentation. However, they reported that WHS and yeast extract, without an additional nitrogen source, ensured high-level formation of all lignocellulosic enzymes including laccase by the 4 different fungi tested. In a study performed by Philippoussis et al. (25), laccase secretion was determined during solid-state fermentation of wheat straw, reed grass, and bean stalk residues by using *Lentinula edodes* strains 121 and 122. The results of the study showed that laccase production is highly affected even by the different laccase-producing strains as well as the nature and composition of the lignocellulosic substrate.

Although there are a limited number of studies on laccase production in SsF, this fermentation method is fairly similar to the natural environment of the white rot fungi (15). In order to determine the effect of these factors on laccase production, 2 effective laccase producers were incubated on SBM containing various amounts of different LCWs in SsF under batch culture conditions. Figures 1A and 1B show that both *T. trogii* and *T. versicolor* could produce low amounts of laccase at the beginning of incubation. As they were adapted and colonized to the media containing the LCWs (SR, WS, HS, ASS, CC, and WHS), the enzyme activities generally increased, particularly after 6, 8, and 10 days of incubation. The laccase activities of *T. trogii* and *T. versicolor* on the day

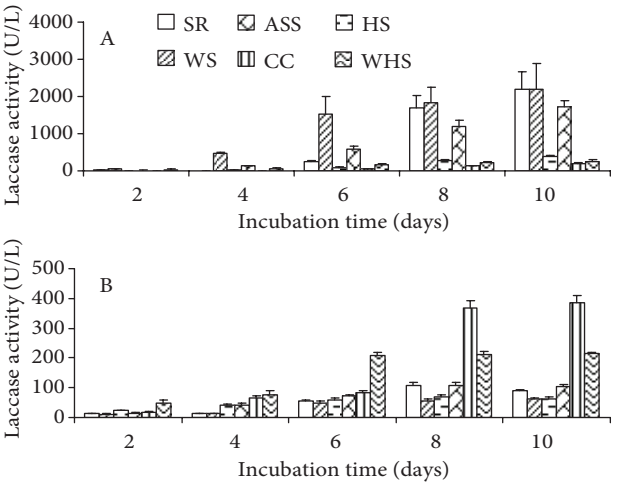

Figure 1. Laccase production of *T. trogii* (A) and *T. versicolor* (B) incubated on SBM containing different LCW at 30 °C for 10 days. SR: sunflower receptacle, WS: walnut shell, HS: hazelnut shell, ASS: apricot seed shell, CC: corncob, WHS: wheat straw.

10 in CC-containing media were 152 and 22 times higher, respectively, than they were on day 2. However, in a study by Rodríguez Couto et al. (15), no laccase activity was observed in *Phanerochaete chrysosporium* cultured on CC under semisolid-state conditions. The laccase activities were detectable only in veratryl alcohol or manganese oxide-supplemented CC-containing media, and the highest laccase activities were detected as 295 U/L and 275 U/L, respectively. Similar laccase activity values were obtained in the current study from CC-containing media without any inducers (Table). According to the results, SsF provided remarkable amounts of laccase production by *T. versicolor* and especially *T. trogii* after a short incubation time. The maximum laccase activities of *T. trogii*, detected on day 10 of incubation, were 2206 and 2201 U/L in WS- and SR-containing media, respectively (Figure 1A). The highest enzyme activities of *T. versicolor*, also obtained on day 10 of incubation, were 387 and 215 U/L in CC- and WHS-containing media, respectively (Figure 1B). Rodríguez Couto et al. (30) reported that when wheat straw and barley straw were used, the highest laccase activities of *T. versicolor* were determined to be 490 U/L on day 17 and 591 U/L on day 18, respectively. While some LCWs such as WHS and CC were used previously for laccase production in SsF by other researchers, this is the first study that uses SR and ASS.

3.2. The effect of various LCW on laccase production in SF

Submerged, solid-state, and semisolid-state fermentation methods could be used to produce various enzymes (37,38). In this study, various LCWs in pulverized forms were tested for laccase production under SF, which is an enzyme production method different from solid or

semisolid-state fermentations. Different studies have been conducted previously with the aim of stimulating maximum laccase production by using several LCWs, such as rice bran with *Coriolus versicolor* (0.22 U/mL) (32) or *Phanerochaete chrysosporium* (0.24 U/mL) (39), in SF. However, the current study is also aimed at investigating the effects of different amounts of various LCWs in pulverized forms on laccase production. Therefore, the fungi were incubated in various growth media containing different quantities (0.15, 0.30, and 0.50 g) of LCWs (PWS, PB, PASS, and PWHS). The results showed that the maximum laccase activities of the fungi were generally obtained from 0.50 g of LCW-containing media on day 5 of incubation. Furthermore, in contrast to the laccase activities in SsF, in SF the laccase activities obtained from *T. versicolor* were higher than those of *T. trogii*. Although the chemical composition of lignocellulosic wastes was not analyzed in this study, different LCWs affected the laccase production of these fungi differently. Similarly, Elisashvili et al. (19) reported that the nature of lignocellulosic material and the method of cultivation are important factors for biosynthetic potential of fungi. While the highest laccase activities were respectively reported as 88.62 and 193.12 U/L for *Phanerochaete chrysosporium* and *Pleurotus ostreatus* in a liquid medium containing banana waste (40), it was reported as 639 U/L for *Trametes versicolor* in a barley bran medium (31). In the present study, all the highest laccase activity results for *T. trogii* were obtained from different amounts of PASS-containing media. The maximal laccase activities in 0.15, 0.30, and 0.50 g of PASS-containing media were 364, 386, and 350 U/L, respectively (Figures 2A–2C). It was determined that all amounts of PB were the most effective LCWs among those tested for laccase production by *T. versicolor*. The highest enzyme activities obtained from 0.15, 0.30, and 0.50 g of PB media were 1054, 1157, and 1216 U/L, respectively (Figures 3A–3C). Erden et al. (33) reported that the maximum laccase activity obtained from submerged cultures of *T. versicolor* cultivated in powdered hazelnut cobs was 47.09 U/L. This value was much lower than the values of laccase activity obtained in the present study. For SF, all the maximum laccase activities obtained from the fungi grown in various amounts of pulverized LCW are listed in the Table.

In conclusion, most researchers have investigated the effect of LCW on laccase production by using solid-state fermentations. In this study, various LCW were tested for their potential as additional sources of laccase production by *T. trogii* and *T. versicolor* in SsF and SF. According to our literature survey, this is the first report using LCWs such as SR, ASS, and PB for laccase production by *T. trogii* and *T. versicolor* in SsF and SF. High levels of laccase enzymes were obtained from these fungi grown on various LCWs. Large amounts of LCW can be evaluated for laccase

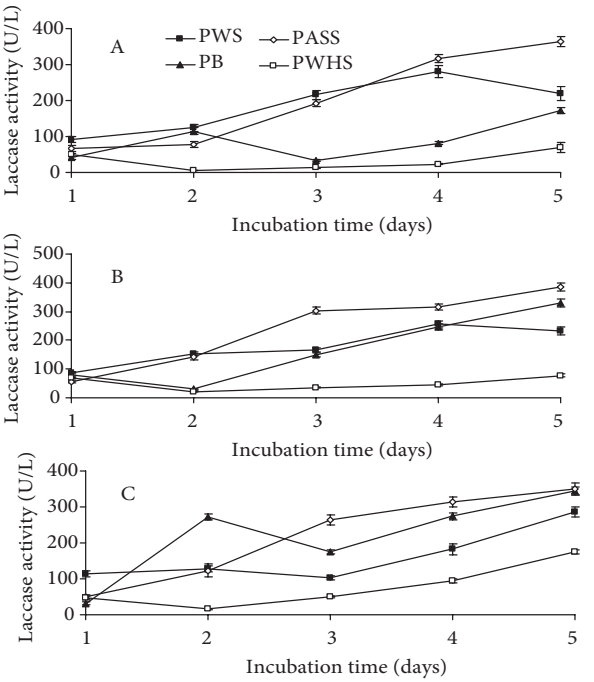

Figure 2. Laccase production of *T. trogii* incubated in SBM containing 0.15 g (A), 0.30 g (B), and 0.50 g (C) of LCW at 30 °C for 5 days. PWS: pulverized walnut shell, PB: pulverized bulrush, PASS: pulverized apricot seed shell, PWHS: pulverized wheat straw.

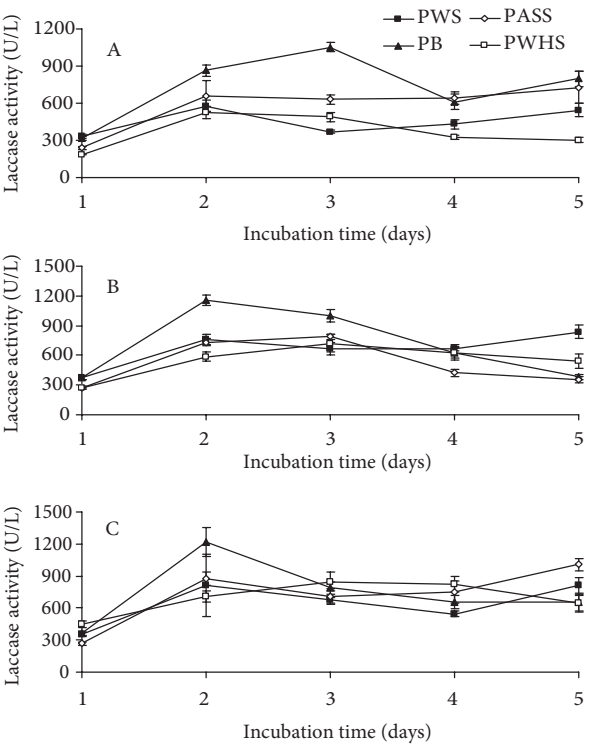

Figure 3. Laccase production of *T. versicolor* incubated in SBM containing 0.15 g (A), 0.30 g (B), and 0.50 g (C) of LCW at 30 °C for 5 days. PWS: pulverized walnut shell, PB: pulverized bulrush, PASS; pulverized apricot seed shell, PWHS: pulverized wheat straw.

production and removed from the environment without causing any pollution or energy loss. Moreover, the laccase enzymes obtained from these processes can be used for many beneficial purposes in various applications.

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