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Laccase Enzyme Activity during Growth and Fruiting of *Pleurotus eryngii* Under Solid State Fermentation Medium Containing Agricultural Wastes

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Abstract

The effect of different agricultural wastes and various growth periods such as mycelial growth, primordium formation, and fruit body yield on laccase activity in the edible mushroom *Pleurotus eryngii* (DC. ex Fr.) Quel. was studied. When the cap of the mature mushroom was showed, the highest laccase activity was obtained on both wheat straw and cotton stalk. Our results suggest that laccases may play a role in the morphogenesis of the mushroom. In this study, the effect of rice bran at various concentrations, which was previously added to fermentation conditions, on growth periods and laccase activity has also been investigated. It is determined that high concentration of rice bran (%10) delayed growth periods. Laccases were found to be induced by addition of rice bran in the medium but induction levels depends on the concentration. The results of the present study allow us to conclude that *P. eryngii* are good candidates for scale-up laccase production and rice bran at low concentration is a strong laccase-inducer.

Key words: *Pleurotus eryngii*, Laccase, Growth period

Tarımsal Atık İçeren Katı Substrat Fermentasyonu Ortamında *Pleurotus eryngii*'nin Büyüme ve Şapka Oluşumu Esnasındaki Lakkaz Aktivitesi

Özet

Yenilebilir mantar *Pleurotus eryngii* de misel gelişim, primordiyum oluşumu ve şapka oluşumu gibi farklı gelişim peryotlarındaki lakkaz aktivitesi üzerine farklı tarımsal atıkların etkileri çalışılmıştır. Şapka oluşumu esnasında hem buğday samanı ve hemde pamuk sapı içeren gelişim ortamında en yüksek lakkaz aktivitesi tespit edilmiştir. Bu çalışmanın sonuçları lakkaz aktivitesinin mantarın şekilsel gelişiminde rol oynadığını göstermiştir. Çalışmamızda fermentasyon ortamına farklı oranlardaki pirinç kepeği katılmasının gelişim evrelerindeki lakkaz aktivitesini nasıl etkilediği de çalışılmıştır. Yüksek oranda pirinç kepeği (%10) eklenmesinin gelişim peryotlarını geciktirdiği tespit edilmiştir. Pirinç kepeğinin konsantrasyona bağlı olmakla beraber gelişim evrelerinde lakkaz aktivitesini indüklediği tespit edilmiştir. Çalışmamızda *P. eryngii*'nin iyi bir lakkaz üreticisi olduğu ve pirinç kepeğinin düşük konsantrasyonlarda gelişim ortamına eklenmesinin lakkaz aktivitesini arttırdığını gösterdik.

Anahtar Kelimeler: *Pleurotus eryngii*, Lakkaz, Gelişim peryodu

Research/Araştırma

INTRODUCTION

Pleurotus spp. represents the third largest group of cultivated edible mushrooms in the world, grown on a variety of plant residues, and they have been found to be nutritionally and gastronomically important. They may be cultivated on a large number of substrates, according to local availability in different regions of the world (Cohen et al., 2002). The main substrate used in the production of *Pleurotus* sporocarps is wheat straw, but owing to the lignolytic enzymes they produce *Pleurotus* spp. can easily be produced on a variety of agricultural wastes. Thus, every year much low-value agricultural waste can be converted into high-value food products with a high protein content. While the major agricultural by-product utilised is wheat straw, cotton waste, peanut shell (Philippoussis et al., 2001) rice straw, sawdust and corn cobs (Poppe and Hofte 1995) can also be used. The laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) are multicopper phenol oxidases, that oxidize phenolic compounds to phenoxyl radicals. In the presence of a mediator such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) or 1-hydroxybenzotriazole, laccases are capable of oxidation of nonphenolic compounds (Eggert et al., 1996). The role of laccases in lignin and phenolic compound degradation has been evaluated in a large number of biotechnological applications such as dye degradation (Wong and Yu 1999) and bioremediation of some toxic chemical wastes (Glenn and Gold 1983; Swammy and Ramsay 1999; Mayer and Staples 2002; Yildirim et al., 2010). Laccases are also likely to be applied in the pulp and paper industries (Pratima, 1999), wastewater and soil treatments (Nelson and Elisa 2000) and also biosensor developments (Nelson et al. 2002; Kulys and Vidziunaite, 2003).

Laccase (benzenediol:oxygen oxidoreductase, EC (1.10.3.2) is a copper (Cu) containing oxidase that can oxidase both nonphenolic and phenolic compounds. In previous papers it was reported that laccase enzyme could be used for removing phenolic contaminants, pesticide transformation, dye and textile wastewater decolorization, transformation of lignin-related compounds, dechlorination of polychlorinated phenols and guaiacols, wood pulping and pulp bleaching, enzyme immunoassay, laccase-based biosensors and for production of organic materials (Kahraman and Yesilada 2001; Srebotnik and Boisson 2005). The importance of laccase in various biotechnological areas underlines the need for expanding the spectrum of laccase producing organisms and enhancing the potential of their laccase producing ability (Arora and Gill 2001).

The objective of the present investigation was to determine the influence of RB on the growth periods and laccase activity of *P. eryngii* (DC.ex Fr.) Quel. on different agricultural wastes.

MATERIAL AND METHODS

Fungal Strain

Pleurotus eryngii (DC.ex Fr.) Quel. was obtained from Faculty of Science, Universty of Hacettepe, Ankara, Turkey. Strains of *Pleurotus eryngii* known wood-degrading fungi. Cultures were grown on malt extract agar (MEA; Merk) at 25ºC in the dark for 8 days before being transferred for specific assays.

Research/Araştırma

Spawn Preparation

One kg wheat grain was used for spawn production. The grain was cooked for 40 min and washed in tap-water. Grain was drained and supplemented with 2 g lime and 8 g gypsum and mixed manually. Then, 120 g grain, cooked and supplemented, was placed in erlenmayer flask (250 ml), closed and steriliazed in autoclave at 121 ºC, for 15 min. After cooling, each erlenmayer was inoculated with two agar disks of 6 mm diam., containing mycelium (actively growing mycelial growth on MEA plates), and incubated at 25 ºC in full darkness for two weeks.

Solid State Fermentation

Wheat straw (WS) and Cotton stalk (CS) were used as a main material in this study for cultivation of *P. eryngii*. This agricultural lignocellulosic waste are usually burned or left in the field to rot in Southeastern Anatolia of Turkey. One kg of material from each trial was placed in plastic buckets and kept for 48 h until compost reached a humidity of 70-75 %. The compost was then mixed with rice bran (RB) at a ratio of 5 and 10% (w/w) and then in order to obtain the desired pH values (5.5-6.5), for one kg material, 35 g of lime and 35 g of gypsum was added to compost (Yildiz and Karakaplan, 2003). Each compost medium was mixed manually. Polypropylene bags (height 18 cm, diameter 15 cm) were filled with 200 g of substrate, and closed and sterilized in autoclave at 121 ºC for 15 min. After cooling the substrates to room temperature, they were inoculated by spreading spawn on the surface of the substrate with a weight percentage of about 4 % of the wet weight of compost and incubated at 25±1 °C in the dark for 2 to 3 weeks or until the mycelium had completely colonized the substrate. After the substrate was fully colonised the bags were opened, which was then incubated at 20 ± 2 °C and a light intensity of 600 lux m^{-2} for 12 h day^{-1} by Juorescent lamps. After primordium formation, the CO_2 level was maintained around 1000 ppm by aeration.

Sampling and Preparation of Crude Enzyme Extract

The samples, taken from the bags periodically at different growth periods (GP), mycelial growth (MG), primordium formation (PF), and fruit body yield (FBY) periods, consisted of 3 g of substrate colonized with mycelium. Crude extract was obtained by adding distilled water to the samples from each freshly harvested culture (5:1, w/w) stirring for 20 min, followed by filtration and centrifugation (for 5 minutes at 5000 rpm at 4°C). All of the steps for crude extraction were performed at room temperature. The supernatant was stored at -20 °C until needed. For all experiments, measurements were carried out in triplicate parallel cultures. The values are reported as the mean with an experimental variation less than 10%.

Laccase Activity Assays

Laccase (E.C. 1.10.3.2) activity was determined spectrophotometrically by monitoring the increase in absorbance at 420 nm. One unit was defined as the amount of enzyme that oxidized 1 μmol of ABTS (2,2_-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) per minute (Birhanli and Yesilada, 2006).

Statistical Analyses

SPSS v13.0 statistical software was used for statistical analysis (SPSS Inc., Chicago, IL, USA). The

experimental design was completely randomized with 3 replications. Data was statistically analyzed for standard error. Means were calculated and Duncan's new multiple range test was used to compare the laccase activity.

RESULTS AND DISCUSSION

Effect of RB on Growth Periods

Periods for growth of *P. eryngii* are given in Fig 1. White cottony mycelial mat on the surface of substrate was observed in all sets of the different rates of RB tested. The periods for complete mycelial growth recorded were variable with respect to the concentration of RB used. The most rapid micelial growth took place within 20 and 25 days in the WS or CS respectively. The primordial formation in terms of pin heads was observed in all experimental sets.

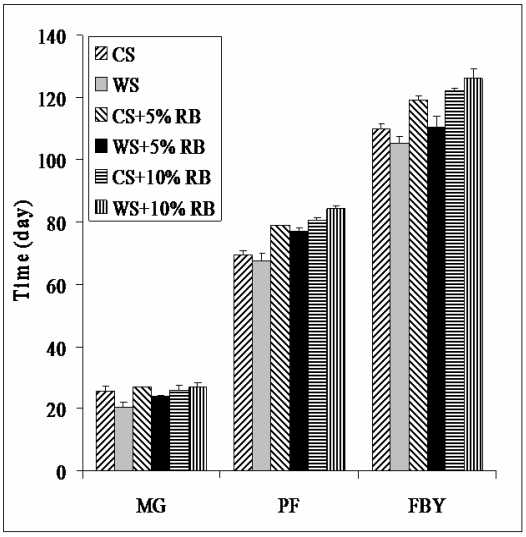

Figure 1. The effect of RB on growth periods of *P. eryngii*, MG; Miycelial growth, PF; Primordium formation, FBY; Fruit body yield.

The duration of primordial formation was found to be variable for different groups. The formation of primordia was observed within a lesser period of 67 and 69 days with the WS or CS respectively. The fruit body yield was observed within a lesser period of 105 and 110 days with the WS or CS, whereas the combination WS+5 %RB or 10 %RB took more time of 110, 126 days respectively. Similarly the combination CS+5 %RB or 10 %RB took more time of 119, 122 days respectively. The higher doses of nitrogen rich supplements resulted in temperature increase (thermogenesis) sufficient to kill the mycelia (Lalley and JanBen, 1993). The slower spawn running at higher concentrations of additives in this study may be due to the excess nitrogen, which is known to inhibit mushroom growth (Demirci, 1998; Baysal et al., 2003). Gupta and Vijay (1991) also reported that supplementation above 2% resulted in undue heating of compost.

Effect of Growth Periods on Laccase Activity

For these white rot fungi, it is very likely that the enzymes are produced for lignocellulosic substrate degradation in order to provide nutrients for the growing organism. In this experiment, laccase activities were followed up within two agricultural wastes (cotton stalks and wheat straw) inoculated with *Pleurotus eryngii* (DC. ex Fr.) Quel. strain. In all two substrate, when laccase activities were comparably low during MG and PF periods, the activity increased at FBY period. Within WS the highest laccase activity was obtained on WS+5% RB (125.65 U/L) at FBY period. Within CS the highest laccase activity was obtained on CS+5% RB (205.83 U/L) at FBY period. Comparison of laccase activity at differnt growth periods within the two substrates is given in

Research/Araştırma

Table 1. The effect of RB on laccase activity in different growth period of *P. Eryngii*.

	Laccase activity (U/L)					
	Groups					
GP*	**CS**	**WS**	**CS+5% RB**	**WS+5% RB**	**CS+10% RB**	**WS+10% RB**
MG	$3,76\pm0,62z^{c}$	$4,01\pm1,15z^{c}$	$11,84\pm2,43y^{c}$	$11,22\pm3,95y^{c}$	$5,01\pm1,85z^{c}$	$13,92\pm1,99x^{c}$
PF	$47,81\pm0,97z^{b}$	$62,36\pm3,53y^{b}$	$66,99\pm0,44y^{b}$	$83,40\pm4,26x^{b}$	$53,55\pm2,57z^{b}$	$41,03\pm2,93t^{b}$
FBY	$149,49\pm1,37y^{a}$	$103,19\pm8,77z^{a}$	$205,83\pm4,25x^{a}$	$125,65\pm2,55z^{a}$	$139,54\pm15,03y^{a}$	$81,75\pm1,73t^{a}$

GP*: Growth period; MG; Miycelial growth, PF; Primordium formation, FBY; Fruit body yield, The growth period differences in the laccase activity were analyzed by using analysis of variance (ANOVA) for post-hoc test (Duncan's multiple-range test). Different letters in same column are significantly different from each other ($^{abc}P < 0.05$), Different letters in same line are significantly different from each other ($^{xyzt}P < 0.05$).

Research/Araştırma

Table 1. Similarly, a lot of researcher repotred that high enzymatic activities were observed in lignocellulosic substrates during growth phases in the white-rotting basidiomycetes *Lentinus tigrinus* (laccase and peroxidase) (Lechner and Papinutti, 2006), *Lentinula edodes* (laccase and peroxidase) (Mata and Savoie, 1998), *Grifola frondosa* (laccase) (Xing et al., 2006), *Pleurotus flabellatus* (laccase) (Rajarathnam et al., 1987) and with drastic reductions in enzyme activities during the period of fruiting body formation. Similarly, the laccase gene is strongly expressed during that part of the mushroom developmental cycle involving fruit body morphogenesis (Chen et al., 2004). In contrast some other researchers reported that, in cultures of the fungus *Agaricus bisporus*, laccase and peroxidase activities increase in the substrate from vegetative growth to early stages of fruiting body development and drop strongly during fruiting body maturation (Bonen et al., 1994).

Effect of RB on Laccase Activity

The effect of RB on laccase activity at differnt growth periods within the two substrates is given in Table 1. Laccase activity was measured as 103.19, 125.65 and 81.75 U/L within WS, WS+5% RB and WS+%10 RB at FBY period respectively. Within CS, 149.49, 205.83 and 139.54 U/L at FBY period respectively. At PF period, laccase activity was determined as 62.36, 83.40 and 41.03 U/L at WS, WS+5% RB and WS+%10 RB respectively. Laccase activity was 47.81, 66.99 and 53.55 U/L within CS, CS+5% RB and CS+%10 RB at PF period respectively. This results indicate that high concentration of RB inhibit laccase activity, low concentration induced the activity. Claye et al. reported that RB contains total carbohydrate 82% (w/w) approximately and the main composition (31%) was hemicellulose (Claye et al., 1996). The rice bran was also used as a sole carbon by Mod *et al.* (1978). When the levels of this RB decreases, laccase synthesis was induced by phenolic compounds containing in RB, leading to increasing of laccase production. This induction mechanism may help fungus to degrade lignin or aromatic compounds in RB to supply further nutrients especially carbon and nitrogen. The similar pattern in production of laccase was also found with several white- and brown rot fungi cultivated on *Eucalyptus grandis* wood chips (Machuca and Ferraz, 2001). The fungal response in enzyme activity support the previous work as the deprivation of nitrogen and carbon sources is considered as a major factor in triggering ligninolytic system of white rot fungi. (Leatham and Kirk, 1983; Mester et al., 1997).

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