

## ***Lentinula edodes* Grown on Di(2-ethylhexyl) Phthalate-Containing Media: Mycelial Growth and Enzyme Activities**

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Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer that is widely used to soften plastic products. It is an endocrine-disrupting compound, widely considered an environmental contaminant that is difficult to remove or degrade. The radial growth rate ( $u_r$ ), mycelial biomass, intracellular and extracellular activities of laccase and esterase, intracellular and extracellular contents of protein and glycogen, and the contents of soluble and insoluble glucans were evaluated in colonies of *Lentinula edodes*, grown in various concentrations of DEHP (0, 750, 1200, and 1500 mg/L) on agar. Glucose and yeast extracts were added to all media. The highest  $u_r$  was shown in medium lacking DEHP, followed by that shown in the medium containing 1500 mg of DEHP/L. The greatest protein extracellular contents were observed in medium with added DEHP. The largest extracellular glycogen contents and mycelial biomass were found in media containing 1200 and 1500 mg of DEHP/L, respectively. These results suggest that DEHP was degraded and used as a substrate by *L. edodes* during diauxic growth (glucose metabolized first, followed by DEHP), and laccases were more important than esterases in the metabolism of DEHP. *L. edodes* can be used to remove DEHP from phthalate-polluted environments.

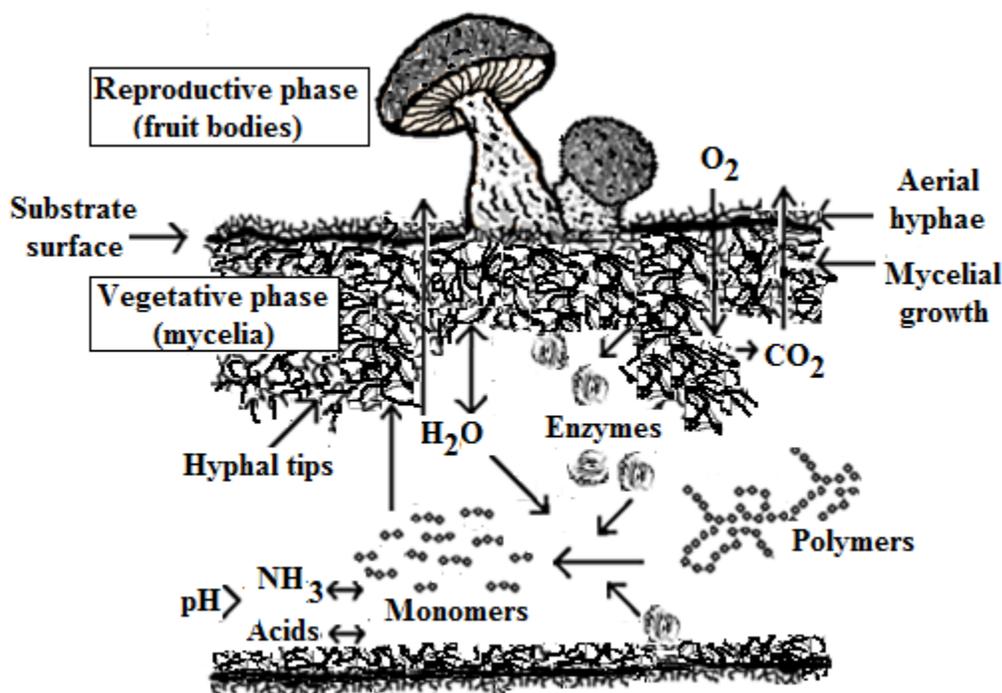
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### **INTRODUCTION**

Plasticizers are compounds that are used to make plastics more flexible. Di (2-ethylhexyl) phthalate (DEHP) is a plasticizer used in a wide range of polyvinyl chloride (PVC) products (Chai *et al.* 2008; Vats *et al.* 2013). DEHP belongs to the phthalates family, or is included with acid phthalic esters (Lee *et al.* 2004). Phthalates are unable to form covalent bonds, with the plastic matrix being easily released into the environment (Peterson and Staples 2003). These compounds have been known to bioaccumulate in fish and mammals (Bustamante-Montes *et al.* 2001; Quan *et al.* 2005; Nalli *et al.* 2006). Several studies have shown that phthalates are mutagenic, teratogenic, and carcinogenic as well (Kim *et al.* 2005; Dobrzyńska *et al.* 2012; Manikkam *et al.* 2013). Phthalates can be removed from the environment by microbial biodegradation. This involves pollutant degradation *via* enzymes present in living organisms. It has been reported that esterases play an important role in phthalates biodegradation (Kim *et al.* 2005). Fungi have two types of enzymatic systems: the hydrolytic system, which produces hydrolases (*e.g.*, esterases),

and the ligninolytic system, which degrades lignin and opens phenyl rings (*e.g.*, laccases). It has been reported that esterases are capable of catalyzing the hydrolysis of ester bonds of some compounds (*i.e.* lipids, oils, phthalates, *etc.*) breaking down such compounds into corresponding carboxylic acids (Benjamin *et al.* 2015). On the other hand, laccases can oxidase any substrate with characteristics similar to *p*-diphenol (Mayer and Staples 2002). White-rot fungi (such as the edible mushroom *Lentinula edodes*) have a very efficient enzymatic system to degrade both lignin and the esters related to the lignin units (Sánchez 2009). It has been reported that *L. edodes*, *Pleurotus ostreatus*, *Irpex lacteus*, *Polyporus brumalis*, *Merulius tremellosus*, *Trametes versicolor*, and *T. versicolor* TVMR12 were able to degrade phthalates (Hwang *et al.* 2008, 2012; Córdoba-Sosa *et al.* 2014). *L. edodes* has two phases of growth: the reproductive phase (fruit body) and the vegetative phase (mycelia) (Fig. 1). In this work, studies were performed in the vegetative phase grown on agar plates. The radial growth rate ( $u_r$ ), mycelial biomass, intracellular and extracellular activities of laccase and esterase, intracellular and extracellular contents of protein, glycogen, and soluble and insoluble glucans were evaluated in *L. edodes* grown on solid media, with various concentrations of DEHP added (0, 750, 1200, and 1500 mg/L).



**Fig. 1.** Schematic representation of the reproductive and vegetative phases of growth of the mushroom *Lentinula edodes*, showing enzymatic degradation of polymers and absorption of monomers by hyphae

## EXPERIMENTAL

### Microorganism

A strain of *Lentinula edodes* (from COLPOS-Puebla, México culture collection) was used. The strain was grown on malt extract agar at 25 °C and stored at 4 °C until use.

## Culture Media

Four solid culture media were prepared. Each media contained: a) glucose-yeast extract medium (GYE) + 0 mg of DEHP/L; b) GYE + 750 mg of DEHP/L; c) GYE + 1200 mg of DEHP/L; and d) GYE + 1500 mg of DEHP/L. The GYE medium was composed of: glucose, 10 g/L; yeast extract, 5 g/L;  $\text{KH}_2\text{PO}_4$ , 0.6 g/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g/L;  $\text{K}_2\text{HPO}_4$ , 0.4 g/L;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.25 g/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05 g/L;  $\text{MnSO}_4$ , 0.05 g/L; and  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001 g/L. The pH was adjusted to 6.5 using either 0.1 M HCl or 0.1 M NaOH. Agar (20 g/L) was added to all the media, which were subsequently autoclaved. After autoclaving, the media were cooled down to approximately 50 °C and DEHP was added to the media, which were sonicated for approximately 3 min using an ultrasonic processor (GEX 130) until the DEHP had been fully dispersed. The agar media were poured into Petri dishes. The researchers used these concentrations of DEHP because, in previous studies, similar concentrations of this compound were used and the effect of dibutyl phthalate and DEHP on the growth of filamentous fungi was clearly observed (Suárez-Segundo *et al.* 2013).

## Radial Growth Rate and Mycelial Biomass

Petri dishes were inoculated in the center (using a plug 4 mm in diameter) and incubated at 25 °C for 12 days. The radius of the mycelial growth was measured daily from the 2<sup>nd</sup> to 12<sup>th</sup> days of incubation using a Vernier (digital mitutoyo, Taiwan). The radial growth rate ( $u_r$ ) was calculated as previously reported (Sánchez and Viniegra-González 1996; Suárez-Segundo *et al.* 2013). The mycelial biomass was evaluated in 12-day-old colonies. The mycelium was filtered from the culture medium using a boiling water bath, then placed in a pre-weighed watch glass. The specimens were weighed, oven-dried at 60 °C for 24 h, and then weighed again (Suárez-Segundo *et al.* 2013).

## Intracellular and Extracellular Laccase and Esterase Assays

The mycelia were removed from the surface of the 12-d-old colonies, placed in a tissue macerator, and then lysed using 3 mL of deionized water. The mycelia lysed were centrifuged at 15,000 RPM for 10 min at -2 °C. The supernatant corresponded to the intracellular enzymatic extract (IEE), and the sediment corresponded to the broken cells. The extracellular enzymatic extract (EEE) was obtained by rinsing the surface of the 12-d-old colonies (from which the mycelia had been removed) with 20 mL of deionized water. Intracellular and extracellular laccase activities were determined in IEE and in EEE, respectively, by measuring the changes in the absorbance at 468 nm (using a Jenway 6405 UV/Vis spectrophotometer, NJ, USA). During this test, 2,6-dimethoxyphenol (DMP, Sigma, Mexico) was used as a substrate. The assay mixture contained 900 µL of 2 mM DMP in 0.1 M acetate buffer (pH 4.5) and 100 µL of enzymatic extract, which were incubated at 40 °C for 1 min. Intracellular and extracellular esterases activities were determined by changes in the absorbance at 405 nm (using a Jenway 6405 UV/Vis spectrophotometer), using *p*-nitrophenyl butyrate (*p*NPB) as a substrate. The reaction mixture contained 10 µL of *p*NPB solution (1.76% (v/v) of *p*NPB in acetonitrile), 790 µL of 50 mM acetate buffer (pH 7.0), 0.04% Triton X-100, and 100 µL of either IEE or EEE, which were incubated at 37 °C for 5 min (Alves-Macedo and Fontes-Pio 2005; Dutta *et al.* 2009). One enzymatic unit of laccase activity or esterase activity (U) is defined as the amount of enzyme needed to yield an increase of 1 unit of absorbance per min in the reaction mixture. The enzymatic activities were expressed in U/L of enzymatic extract.

### Intracellular and Extracellular Contents of Protein, Glycogen, and Glucans

Intracellular and extracellular contents of protein and glycogen (mg/g of DW biomass) were evaluated accordingly in the IEE and EEE. In both cases, the protein content was measured using the Bradford method (Bradford 1976), utilizing bovine serum albumin as the standard. The glycogen was considered to be the difference between the total sugars minus the reducing sugars. Total and reducing sugars were determined as previously reported (Sánchez *et al.* 2004). The glucans content (mg/g of DW biomass) was evaluated in the sediment (broken cells). Sediment was rinsed with water and a 76% (v/v) ethanol solution (three times with each) to remove the membrane and cytoplasmic components. The glucans from the cell wall were separated into two fractions: soluble (S-glucans) and insoluble (R-glucans) in alkali (Sánchez *et al.* 2004).

### Statistical Analysis

All the experiments were carried out in triplicate. Data were evaluated using one-way ANOVA and Tukey post-test using The Graph Pad Prism® program (San Diego, CA, USA) (Córdoba-Sosa *et al.* 2014).

## RESULTS AND DISCUSSION

*Lentinula edodes* had the greatest  $u_r$  and the largest amount of mycelial biomass in the medium lacking DEHP, as well as in the medium containing 1500 mg of DEHP/L (Table 1). Suárez-Segundo *et al.* (2013) reported that the  $u_r$  values of *Pleurotus ostreatus* (ATCC-32783) grown on media with glucose, 500, and 1000 mg of DEHP/L were 0.29, 0.23, and 0.060 mm/d, respectively.

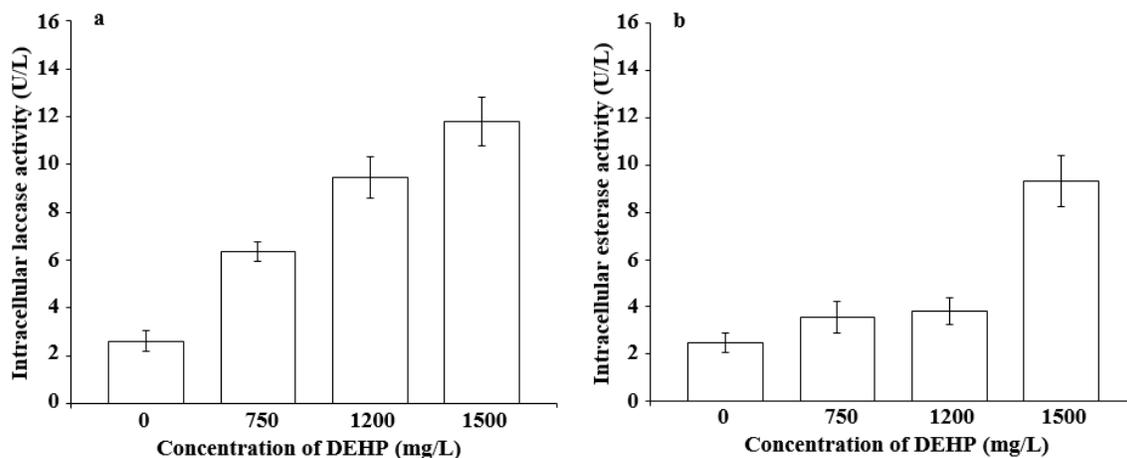
**Table 1.**  $u_r$ , Mycelial Biomass, and Glucans Content of *L. edodes* Grown in DEHP on Agar Plates

Parameter	Concentration of DEHP (mg/L)			
	0	750	1200	1500
$u_r$ (mm/d)	0.989 <sup>a</sup> (0.012)	0.126 <sup>c</sup> (0.015)	0.123 <sup>c</sup> (0.001)	0.166 <sup>b</sup> (0.002)
Mycelial biomass (mg/cm <sup>2</sup> )	0.020 <sup>b</sup> (0.016)	0.018 <sup>b</sup> (0.003)	0.021 <sup>b</sup> (0.001)	0.037 <sup>a</sup> (0.006)
Soluble glucans (mg/g of DW biomass)	64.457 <sup>a</sup> (20.187)	14.123 <sup>d</sup> (3.296)	48.494 <sup>b</sup> (8.060)	25.784 <sup>c</sup> (9.290)
Insoluble glucans (mg/g of DW biomass)	0.347 <sup>d</sup> (0.466)	231.619 <sup>b</sup> (30.836)	355.628 <sup>a</sup> (8.060)	156.309 <sup>c</sup> (3.096)

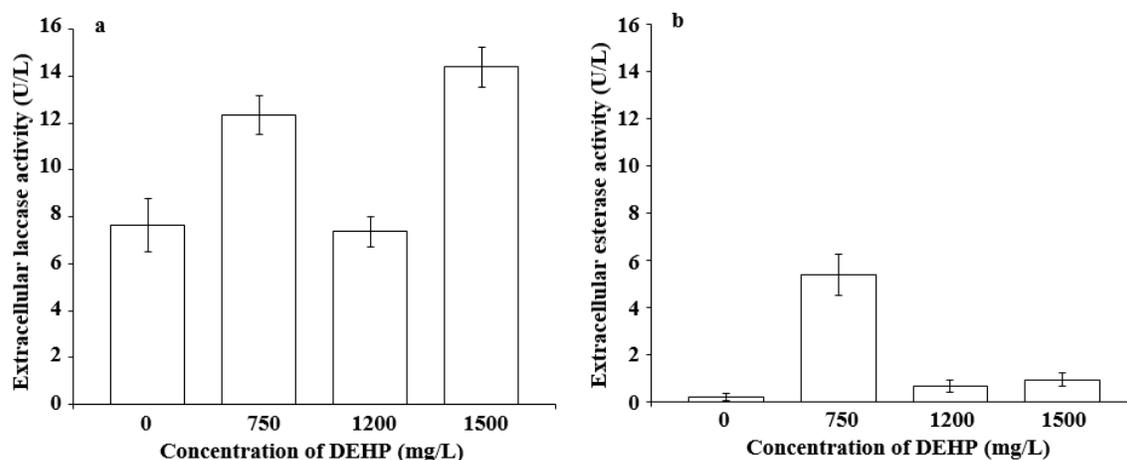
Means with the same letter within a row are not significantly different. Numbers in parenthesis correspond to standard deviation of three separate experiments.

In the present study, the  $u_r$  of *L. edodes* grown in medium with 1500 mg of DEHP/L was more than two times higher than the  $u_r$  shown by *P. ostreatus* in medium with 1000 mg of DEHP/L. The results could be due to the addition of glucose to the DEHP-containing media in the present work. *L. edodes* showed the highest insoluble glucans content when grown in media with DEHP added. Sánchez *et al.* (2004) reported that the fruit bodies of *P. pulmonarius* had higher insoluble and soluble glucans contents than the mycelia of the colony, as the hyphal ageing in *P. pulmonarius* involves glucan storage.

In general, the highest intracellular and extracellular laccase and esterase activities were observed in those media added with DEHP (Figs. 2 and 3). The greatest laccase and esterase intracellular activities for *L. edodes* were observed in the medium with 1500 mg of DEHP/L (Fig. 2). The increase of the intracellular laccase and esterase activities were positively correlated with the concentration of DEHP added to the culture media (Fig. 2). The highest laccase and esterase extracellular activities were observed in the media containing 1500 and 750 mg of DEHP/L, respectively (Fig. 3). In general, the lowest intracellular and extracellular laccase and esterase activities were shown in the medium lacking DEHP (Figs. 2 and 3).

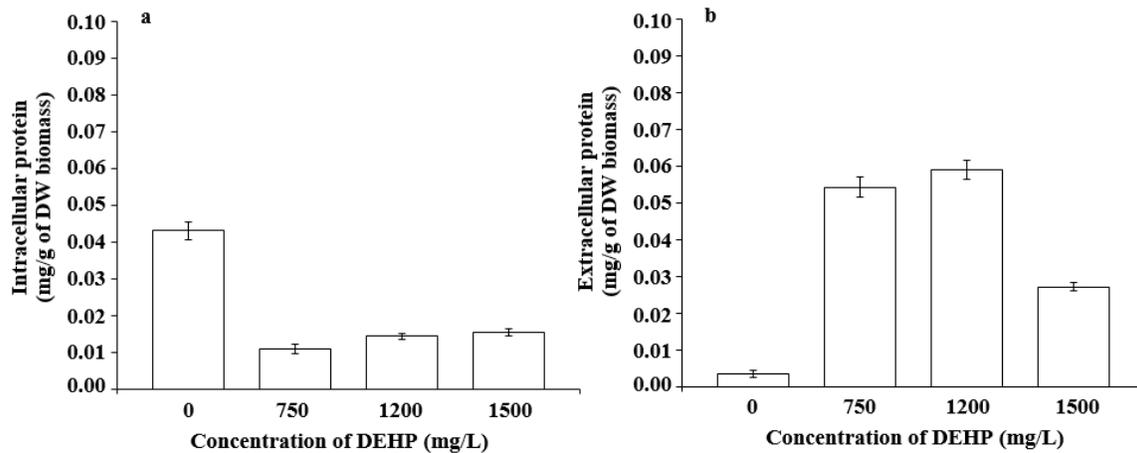


**Fig. 2.** Intracellular activities of (a) laccase and (b) esterase of *L. edodes* grown in DEHP on agar plates

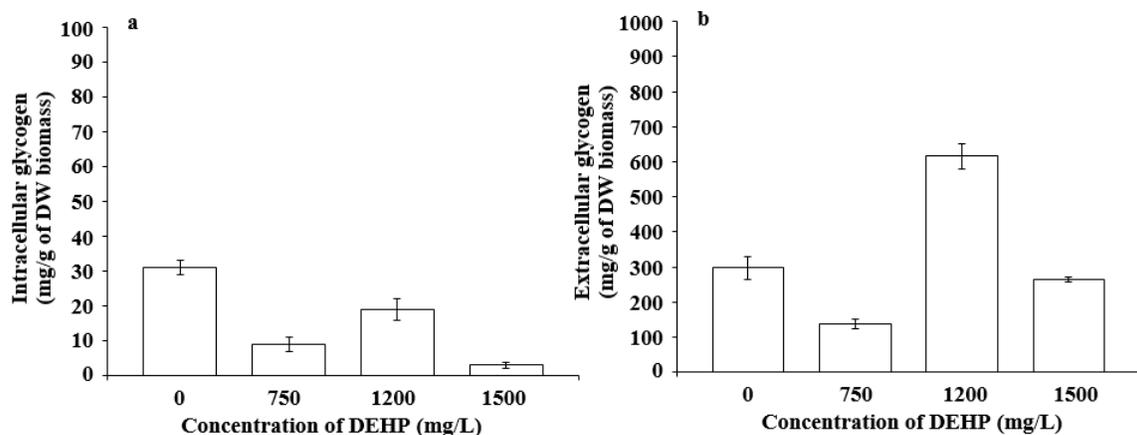


**Fig. 3.** Extracellular activities of (a) laccase and (b) esterase of *L. edodes* grown in DEHP on agar plates

Hwang *et al.* (2012) reported that laccase activity was lower than esterase activity for *P. ostreatus* grown in a medium with 100 mg of butylbenzyl phthalate, glucose, and yeast extract in submerged fermentation. Similarly, *P. ostreatus* (ATCC 3526) had much higher esterase activity than laccase activity at the beginning of the stationary phase in media containing 1500 mg of DEHP/L in flasks (Córdoba-Sosa *et al.* 2014). *Fusarium culmorum* and *Trichoderma harzianum* had higher esterase activity than laccase activity in media with 500, 750, 1000, 1200, and 1500 mg of DEHP/L added on agar (Aguilar-Alvarado *et al.* 2015). In the present study, *L. edodes* showed higher laccase activity than esterase activity for all concentrations of DEHP. Ko *et al.* (2005) studied the laccase production from spent mushroom compost and found that *P. ostreatus* showed higher laccase activity than *L. edodes* in spent compost. These results show that the enzyme produced by fungal cultures depends on the fungal physiology, substrate, and culture system. In the present research, medium-lacking DEHP had higher intracellular protein contents than media containing DEHP (Fig. 4a). Media with DEHP showed higher extracellular protein contents than that medium lacking this compound (Fig. 4b).



**Fig. 4.** (a) Intracellular and (b) extracellular protein contents of *L. edodes* grown in DEHP on agar plates



**Fig. 5.** (a) Intracellular and (b) extracellular glycogen contents of *L. edodes* grown in DEHP on agar plates

Sánchez *et al.* (2004) reported that hyphae from the periphery of the colony of *P. pulmonarius* grown on potato extract agar (PEA) had higher protein and glycogen contents than hyphae from fruit bodies (which fructify on PDA media). It is typical of actively growing hyphae, since the hyphae grow at their tips (periphery of the colony), using protein and glycogen to grow. In this research, the greatest extracellular glycogen contents were observed in the medium with 1200 mg of DEHP/L (Fig. 5). Crow (2013) found that the ascomycetes *Aspergillus niger* and *Penicillium chrysogenum* were able to grow and sporulate on plates containing DEHP and that this compound had no negative effect on the fungal growth.

## CONCLUSIONS

1. *L. edodes* had the highest insoluble glucan content in media with DEHP added, showing that the mycelia grew rapidly in these media. The glucans were stored in the cell wall as a result of the hyphal maturation.
2. It is suggested that DEHP was metabolized and used as an additional carbon source by *L. edodes* during diauxic growth (glucose metabolized first, followed by DEHP).
3. Laccases were more important than esterases in the metabolism of DEHP in an agar medium.
4. *L. edodes* can be used to remove DEHP from phthalate-polluted environments.

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