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Growth of *Pleurotus ostreatus* ATCC 3526 in different concentrations of di (2-ethylhexyl) phthalate in submerged fermentation

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Abstract: (Di (2-ethylhexyl) phthalate (DEHP) is a plasticizer used in the manufacture of plastics which impart flexibility to polyvinyl chloride resins. This is an endocrine disrupting compound that could lead to cancer. It has been reported that *Pleurotus ostreatus* is a fungus capable of growing using DEHP as energy source. Specific growth rate (μ), maximum biomass (X_{max}), laccase and esterase activities, pH profiles and enzymatic kinetic parameters were evaluated in *Pleurotus ostreatus* grown in DEHP in submerged fermentation. Flasks of 125 ml containing 0, 750, 1200 and 1500 mg of DEHP/l were used. All media were added with 10 g of glucose/l. Flasks containing 50 ml culture medium were inoculated and incubated at 25 ^{0}C for 16 days on a rotary shaker (120 rpm). X_{max} and μ were evaluated using the logistic equation. Biomass (X) was determined by dry weight method. Laccase and esterase activities were evaluated using 2, 6-dimethoxyphenol and *p*-nitrophenyl butyrate as substrates, respectively. Enzymatic kinetic parameters were evaluated based on maximal enzymatic activity (E_{max}). Results showed that the highest X_{max} was observed in media containing 1500 mg of DEHP/l and the esterase activity was much higher than the laccase activity at the beginning of the stationary phase in medium containing 1500 mg of DEHP/l. These results suggest that

there was no catabolite repression (glucose effect) and that DEHP was used as carbon and energy source by this fungus.

Key words: Di (2-ethylhexyl) phthalate, *Pleurotus ostreatus*, esterase activity, laccase activity, submerged fermentation.

INTRODUCTION

Di (2-ethylhexyl) phthalate (DEHP) belongs to the family of the phthalates or acid phthalic esters. More than 60 kinds of phthalates are produced nowadays[1]. These compounds are used each year as plasticizers in flexible polyvinyl chloride (PVC) products, resins, cellulosic, polyvinyl acetate and polyurethanes[3]. The annual worldwide production of phthalates exceeds 5 million tons[2]. DEHP is a high production volume chemical used in the manufacture of a wide variety of consumer food packaging, some children's products, and some polyvinyl chloride (PVC) medical devices. The European government banned the use of DEHP in toys and children's products that might be placed in the mouth (http://www.marchem.com/materials/plastisols/phthalatefree.html). It has been reported that phthalates are mutagenic, teratogenic and carcinogenic[4,5].

Phthalates are important environmental contaminants and are difficult to degrade easily. Elimination of DEHP by microorganisms is considered to be one of the major routes of environmental degradation. Hwang *et al.*[6] studied the degradation of 100 mg/l of butylbenzyl phthalate (BBP) by *P. ostreatus*. They found that the degradation of this compound was higher when the BBP was dispersed in an optimum liquid medium (yeast-malt extract-glucose) than in a minimal medium. They also reported that the esterases are more important than laccases in the degradation of this compound. On the other hand, *P. ostreatus* is the second most cultivated edible mushroom worldwide. This mushroom has a very important enzymatic machinery that is able to produce laccases and manganese peroxidases[7,8].

In this research, specific growth rate (μ), laccase and esterase activities, pH profiles and enzymatic kinetic parameters were evaluated in *P. ostreatus* grown in media containing 0, 750, 1200 and 1500 mg of DEHP/l in submerged fermentation.

METHODS

Microorganism: A strain of *P. ostreatus* from the American Type Culture Collection (ATCC 3526) (Manassas, Virginia, U.S.A.) was used. The strain was grown on malt extract agar (MEA) at 25 ^{0}C and stored at 4 ^{0}C until used.

Culture media: Four liquid media were used, these media had; 1) 50 ml of glucose-yeast extract medium (GY) + 0 g of DEHP/l, 2) 50 ml of GY + 750 g of DEHP/l, 3) 50 ml of GY + 1200 g of DEHP/l and 4) 50 ml of GY + 1500 g of DEHP/l. The YG medium had (in g/L): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4$-$7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4$-$5H_2O$, 0.25; $FeSO_4$-$7H_2O$, 0.05; $MnSO_4$, 0.05 and $ZnSO_4$-$7H_2O$, 0.001. The pH was adjusted at 6.5 using either 0.1 M HCl or 0.1 M NaOH. DEHP was added to the autoclaved media.

Specific growth rate: Flasks of 125 ml containing 50 ml of the different culture media were inoculated with three mycelial plugs of 10 mm of diameter and incubated at 25°C for 23 days on a rotary shaker at 120 rpm. The biomass (*X*) was obtained by filtration of the samples using filter paper (Whatman No. 4),

and it was determined as difference of dry weight (g/l) [$X = X(t)$] using the Velhurst-Pearl or logistic equation (Equation 1). The X was measured daily until that the stationary phase of growth of the fungus started (16 days of incubation).

$dX/dt=\mu[1-X/X_{max}]X$ or $X[X_{max}/1+Ce^{-\mu t}]$ Equation 1

Where, $X = X_0$ (the initial biomass value), $C = (X_{max} - X_0)/X_0$, μ is the maximal specific growth rate and X_{max} is the maximal (or equilibrium) biomass level achieved when $dX/dt = 0$ for $X > 0$. Thus, in logistic growth, the growth rate decreases as biomass increases.

Evaluations of kinetic parameters of the logistic equation were carried out using a non-linear least square-fitting program (Solver; Excel, Microsoft)[9-11]. The value of μ was evaluated from the third to the 16 d of growth in order to avoid the variation adjustment problem.

Laccase and esterase activities: The supernatant obtained from the filtration of the samples corresponded to the enzymatic extract (EE). Laccase activity was determined in each EE by changes in the absorbance at 468 nm (using a Jenway 6405UV/Vis spectrophotometer), using 2, 6-dimethoxyphenol (DMP, SIGMA) as substrate.

The assay mixture contained 900 µL of 2 mM DMP in 0.1 M acetate buffer pH 4.5 and 100 µl EE, which were incubated at 40 ºC for 1 min. Esterases activity was determined by changes in the absorbance at 405 nm (using a Jenway 6405UV/Vis spectrophotometer), using *p*-nitrophenyl butyrate (*p*NPB) as substrate. The reaction mixture contained 10 µl of a *p*NPB solution [1.76 % (v/v) of *p*NPB in acetonitrile], 790 µl of 50 mM acetates buffer pH 7.0, 0.04% Tritón X-100 and 100 µl of the EE, which were incubated[12,13] at 37 ^{0}C for 5 min[12,13]. One enzymatic unit of laccase activity or esterase activity (U) is defined as the amount of enzyme which gives an increase of 1 unit of absorbance per min in the reaction mixture.

The enzymatic activities were expressed in U/l of EE.

Enzymatic kinetic parameters: The enzymatic kinetic parameters were evaluated in those cultures grown in liquid medium. Yield of laccases per unit of biomass produced ($Y_{E/X}$) was estimated as the relation between maximal enzymatic activity (E_{max}) and X_{max} (see specific growth rate in the methodology section). Enzymatic productivity (P = U/l h) was evaluated using the time of E_{max}. The specific rate of enzymatic production was calculated[9,10,14] by the equation; $qP= (\mu) (Y_{E/X})$

STATISTICAL ANALYSIS

All the experiments were carried out by triplicated. Data were evaluated using one-way ANOVA and Tukey post-test using The Graph Pad Prism® program.

RESULTS

P. ostreatus reached the stationary growth phase in medium without DEHP after 15 days in the media containing 750, 1200 and 1500 mg of DEHP/l at day 12 (Fig. 1).

The highest μ was obtained in medium containing 750 mg of DEHP/l, followed by the media containing 1200 mg of DEHP/l, 1500 mg of DEHP/l and the medium lacking DEHP (Equation 1, Fig. 1, and Table 1). The highest X_{max} was obtained in the culture media with addition of 1500 and 1200 mg of DEHP/l, and the lowest X_{max} was showed in the medium containing 750 mg of DEHP/l (equation 1, Table 1).

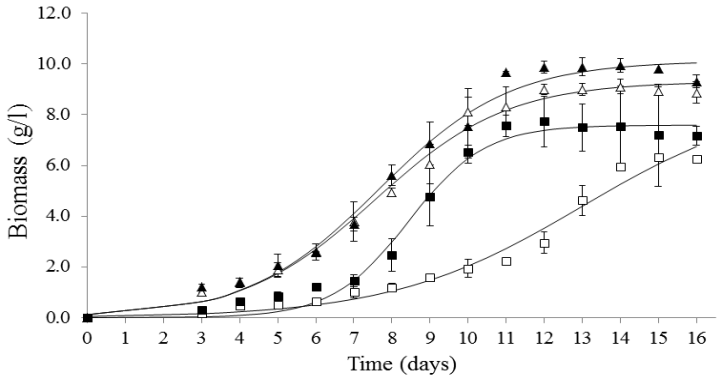

Figure 1: Specific growth rate of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (△) and 1500 (▲) mg of DEHP/l in submerged fermentation. The experimental data of μ were adjusted using the equation 1.

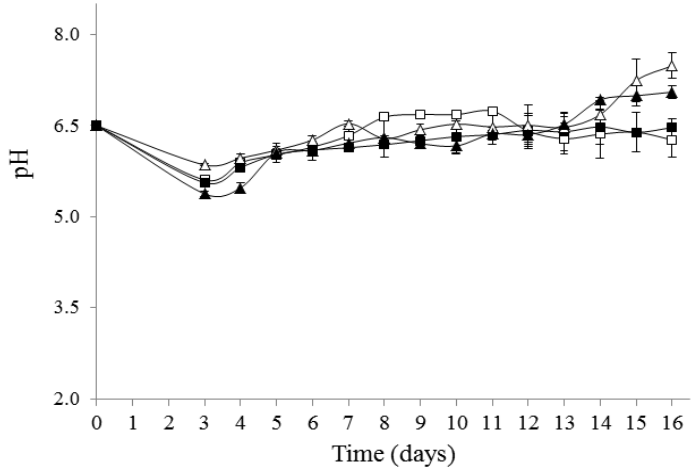

Figure 2: Profile of pH of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (△) and 1500 (▲) mg of DEHP/l in submerged fermentation.

Table 1: Parameters of growth of *P. ostreatus* grown in different concentrations of DEHP in submerged fermentation.

Parameter	Culture media			
	Without DEHP	DEHP (mg/l)		
		750	1200	1500
μ (1/h)	0.016^{b} (0.001)	0.041^{a} (0.007)	0.024^{b} (0.003)	0.024^{b} (0.003)
X_{max} (g/l)	8.78^{bc} (0.27)	7.59^{c} (0.64)	9.31^{abc} (0.017)	10.12^{ab} (0.17)

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

From all the culture media, the medium containing 1500 mg of DEHP/l had the lowest pH value (after 3 d of growth) (5.4 approx.). The media containing 1200 and 1500 g of DEHP/l showed a similar pH profile.

The pH profiles of the medium added with 750 mg of DEHP/l and medium lacking DEHP were similar (Fig. 2). The pH of the media containing 1200 and 1500 mg of DEHP/l showed higher pH than the rest of the culture media at the end of the fermentation.

The highest activity of laccase was showed during the stationary phase of growth (13 d of fermentation) in the media containing 1500 mg of DEHP/l (Fig. 3a). The highest activity of esterase also was observed at the end of stationary phase (15 d of fermentation) in media containing 1500 mg of DEHP/l (Fig. 3b). In general, the lowest activities of laccase and esterase were observed in the medium lacking DEHP and in the medium containing 750 mg of DEHP/l (Figs. 3a, b). In general, the activity of esterase was observed in all the media containing DEHP, however, the activity of laccase was mostly observed in media containing 1500 and 1200 mg of DEHP/l (Figs. 3a, b). In general, the medium lacking DEHP showed the lowest activities of laccase and esterase (Figs. 3a, b). The production of esterase at the beginning of the stationary phase of growth was similar in the media containing 0, 750 and 1200 g of DEHP/l (Fig. 3a). The highest laccase and esterase kinetic parameters were observed in 1500 mg of DEHP/l, followed by the rest of the culture media DEHP/l (Tables 2, 3).

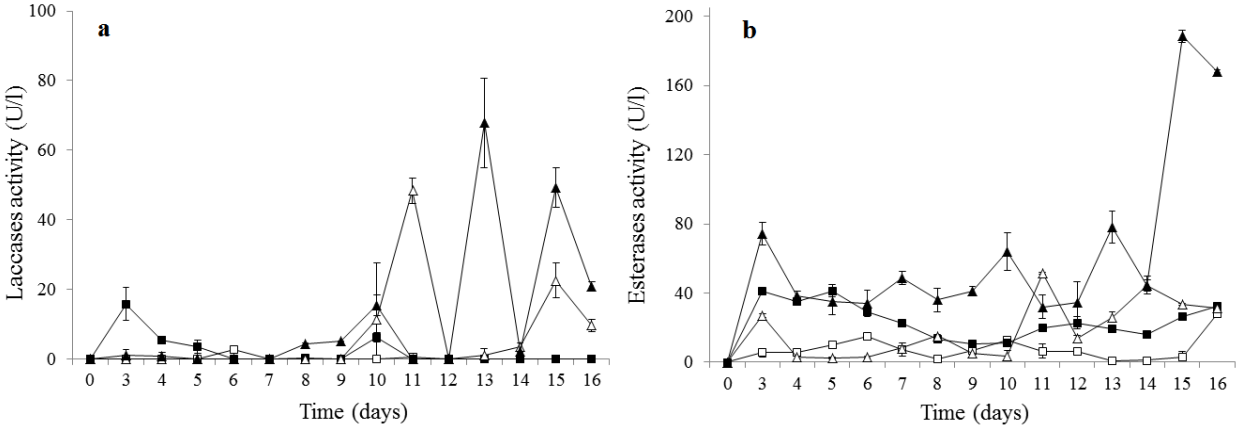

Figure 3: Laccases (a) and esterases (b) activities of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (△) and 1500 (▲) mg of DEHP/l in submerged fermentation.

Table 2: Laccase kinetic parameters of *P. ostreatus* grown in different concentrations of DEHP in submerged fermentation.

Parameter	Culture media			
	DEHP (mg/l)			
	0	750	1200	1500
E_{max} (U/l)	2.8^{c} (0.346)	15.8^{c} (0.95)	48.35^{b} (3.522)	67.85^{a} (12.896)
$Y_{E/X}$ (U/g)	0.318^{b} (0.032)	2.003^{b} (0.052)	5.181^{a} (0.384)	6.69^{a} (1.299)
qP (U/g/h)	0.005^{d} (0.0002)	0.084^{c} (0.015)	0.124^{b} (0.005)	0.160^{a} (0.016)
P (U/l/h)	0.02^{b} (0.002)	0.022^{a} (0.013)	0.183^{a} (0.013)	0.218^{a} (0.041)

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

Table 3: Esterase kinetic parameters of *P. ostreatus* grown in different concentrations of DEHP in submerged fermentation.

Parameter	Culture media			
	DEHP (mg/l)			
	0	750	1200	1500
E_{max} (U/l)	27.845^{d} (0.622)	41.293^{c} (3.342)	51.402^{b} (0.641)	188.414^{a} (3.313)
$Y_{E/X}$ (U/g)	3.169^{c} (0.157)	5.243^{b} (0.690)	5.508^{b} (0.077)	18.569^{a} (0.435)
qP (U/g/h)	0.053^{c} (0.006)	0.2228^{b} (0.066)	0.133^{cb} (0.012)	0.451^{a} (0.039)
P (U/l/h)	0.073^{d}(0.002)	0.344^{c} (0.028)	0.195^{b} (0.002)	0.523^{a} (0.009)

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

CONCLUSIONS

These results showed that the DEHP is used by this fungus to grow, since the media containing high amount of DEHP showed the lowest μ and the highest X_{max} (Fig. 1, Table 1). It is known that from that 100% of carbon source added to a culture media, 50% is used by microorganisms for biomass production and 50% for structure formation. In this study 10 g glucose/l were added to all the cultures (see materials and methods), since a diauxic growth might occurs in some microorganism that grow on complex compounds[6]. The X_{max} produced in medium lacking DEHP was approx. 5 g/ l (amount that corresponded to 50% of 10 g/l of glucose that was added to all the media (Table 1). These results showed that the DEHP was used as carbon and energy sources, since the biomass production was enhanced as the concentration of DEHP increased (Fig. 1, Table 1).

The pH increased during the fermentation in the media containing 1200 and 1500 mg of DEHP/l. This could be due to the degradation of this compound, releasing basic breakdown products of DEHP (http://umbdd.ethz.ch/index.html).

Hwang *et al.*[6] studied the addition of 100 mg/l of BBP to yeast-malt extract-glucose culture medium and reported that the esterases activity was induced by BBP itself and that these enzymes were more important than the laccases in the BBP degradation by *P. ostreatus* in submerged fermentation. Similarly, we found that *P. ostreatus* had higher activity of esterase than activity of laccase in submerged fermentation containing different concentrations of DEHP. However, Van der Vlught-Bergmans *et al.*[15] studied the growth of the white rot fungus *Phlebia tremellosa*, in submerged fermentation containing benzylbutyl phtalate and diethyl phtalate in concentration of 30% and 80%, respectively, and found that the fungus increased the laccase production after 9 days of growth. On the other hand, the maximal enzymatic activity, enzymatic productivity and rate of the enzymatic production depend on the amount of phthalate added to the liquid medium (Tables 2 and 3). The kinetic parameters were higher in the medium containing 1500 mg of DEHP/l than in the rest of the media. It shows that high concentrations of DEHP increased the yield of enzyme and enhanced the metabolism of the strain.

These results show that the type of enzyme produced during the DEHP degradation depends, at least in part, on the DEHP concentration. The production of laccase and esterase, and the results of the growth of *P. ostreatus* in the medium containing 1500 mg of DEHP/l suggest that there was no catabolite repression (glucose effect) and that DEHP was used as carbon and energy source.

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