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Degradation of di(2-ethyl hexyl) phthalate by *Fusarium culmorum*: Kinetics, enzymatic activities and biodegradation pathway based on quantum chemical modelingpathway based on quantum chemical modeling

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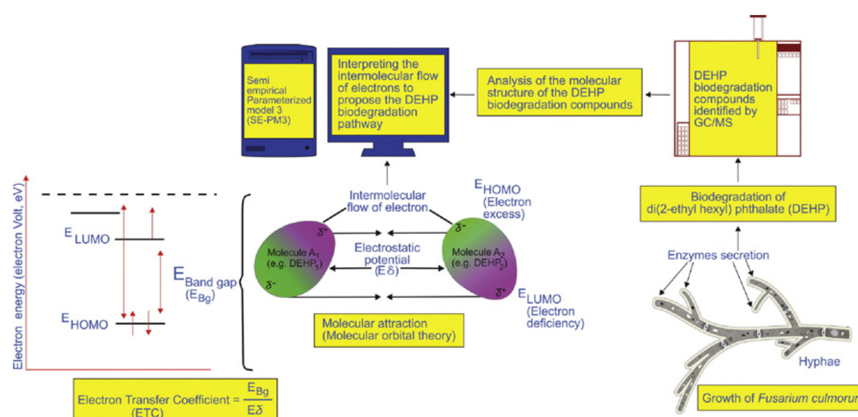
HIGHLIGHTS

- *F. culmorum* degraded 95% of DEHP (1000 mg/L) within 60 h.
- Removal efficiency of DEHP was 99.8% and 99.9% for 1000 and 500 mg/L, respectively.
- DEHP was fully metabolized by *F. culmorum*, with butanediol as the final product.
- A DEHP biodegradation pathway was proposed using on quantum chemical modeling.

GRAPHICAL ABSTRACT



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ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer widely used in the manufacture of plastics, and it is an environmental contaminant. The specific growth rate (μ), maximum biomass (X_{max}), biodegradation constant of DEHP (k), half-life ($t_{1/2}$) of DEHP biodegradation and removal efficiency of DEHP, esterase and laccase specific activities, and enzymatic yield parameters were evaluated for *Fusarium culmorum* grown on media containing glucose and different concentrations of DEHP (0, 500 and 1000 mg/L). The greatest μ and the largest X_{max} occurred in media supplemented with 1000 mg of DEHP/L. *F. culmorum* degraded 95% of the highest amount of DEHP tested (1000 mg/L) within 60 h of growth. The k and $t_{1/2}$ were 0.024 h^{-1} and 28 h, respectively, for both DEHP concentrations. The removal efficiency of DEHP was 99.8% and 99.9% for 1000 and 500 mg/L, respectively. Much higher

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specific esterase activity than specific laccase activity was observed in all media tested. The compounds of biodegradation of DEHP were identified by GC–MS. A DEHP biodegradation pathway by *F. culmorum* was proposed on the basis of the intermolecular flow of electrons of the identified intermediate compounds using quantum chemical modeling. DEHP was fully metabolized by *F. culmorum* with butanediol as the final product. This fungus offers great potential in bioremediation of environments polluted with DEHP.

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1. Introduction

Phthalates are alkyl or dialkyl esters of phthalic acid (benzene dicarboxylic acid). Their basic chemical structure is phthalic acid with two side chains (they may be branched or linear), which can be alkyl, benzyl, phenyl, cycloalkyl, or alkoxy groups (Abdel-Daiem et al., 2012). Di(2-ethylhexyl) phthalate (DEHP) is a diester of phthalic acid used as a plasticizer for polyvinyl chloride (PVC), which increases the flexibility of the plastic, enabling a wide range of products to be manufactured (Benjamin et al., 2015; Orecchio et al., 2015). DEHP is used in consumer goods, food contact materials, flooring and wall covering, and medical devices (i.e., blood storage bags, hemodialysis tubing, oxygenation tubing, and others) (Meeker et al., 2009; Chiellini et al., 2013). This compound has been reported as an endocrine-disrupting compound (Chiellini et al., 2013; Balabanič et al., 2012). It is not considered carcinogenic to humans, but its long-term effects are still unknown. Laboratory experiments (in primates and rodents) have shown that DEHP and its metabolite mono(2-ethylhexyl) phthalate produced toxic effects in liver, the reproductive tract, heart, kidneys and lungs (Chiellini et al., 2013). Phthalates in mammals are metabolized to monophthalate by lipases or esterases present in the intestines; monophthalate is degraded into oxidative metabolites. The monoesters are generally considered to be responsible for the toxic effects (Benjamin et al., 2015). Phthalates are environmental contaminants that do not readily degrade. DEHP has been detected in air, water, sediment, soil, and biota (ATSDR, 1993). The average ambient air concentration of DEHP is <0.002 μg/L in urban areas. DEHP levels in indoor air (i.e., in a newly painted room) could be higher than levels in the outdoor air (ATSDR, 1993). At DEHP production sites, concentrations in surface water range from 1 to 220 μg/L and from 7.5 to 2045 mg/kg in sediment (Green Facts, 2008). DEHP has also been identified as the main phthalate present in lagoon sludge and in activated sludge, where it accumulates to approximately 29 mg/kg and 6 mg/kg, respectively (Amir et al., 2005). Predicted environmental concentrations of DEHP in soil were between 354 mg/kg at polymer processing sites and up to 103 mg/kg at non-polymer formulation and processing sites (Green Facts, 2008). In sludge and treated sludge, 180 and 163 μg of DEHP/L were detected, respectively, in a sewage treatment plant at Espoo, Finland (Marttinen et al., 2003). The freshwater fish *Abramis brama* (bream) was contaminated with 1900 to 3120 ng/g of dry weight (Vethaak et al., 2005). DEHP was also detected in human blood in concentrations ranging between 15 and 83.2 μg/L (Inoue et al., 2005). Numerous studies have demonstrated that microorganisms play a major role in phthalate degradation in the environment (Prasad and Suresh, 2012; Pradeep et al., 2013; Ahuactzin-Pérez et al., 2014; Bouchiat et al., 2015; Meng et al., 2015). However, it is important to study the pathways of biodegradation to insure that toxic compounds are not generated as the result of microbial metabolism because some carcinogens can be generated as a result of compound biodegradation (Wackett, 2014). Several studies have reported that *Pleurotus ostreatus*, *Polyporus brumalis*, *Trametes versicolor*, and *Phlebia tremellosa* are able to biodegrade diesters of phthalic acid. However, due to the slow growth of these organisms, the compounds are degraded at very slow rates (Hwang et al., 2008; Kim et al., 2008; Kim and Song, 2009; Hwang et al., 2012; Luo et al., 2012). Some ascomycete fungi from the genus *Fusarium* have been reported to be highly efficient phthalate-degrading organisms due to their secretion of enzymes such as esterase and laccase (Kim et al., 2007; Obruca et al., 2012; Pradeep and Benjamin, 2012; Chhaya and Gupte, 2013; Aguilar-Alvarado et al., 2015; Bouchiat et al., 2015). Esterases catalyze the hydrolysis of ester bonds of various compounds (i.e., lipids, oils, and phthalates) and break them down into the corresponding carboxylic acids (Benjamin et al., 2015). Laccases oxidase any substrate similar to *p*-diphenol (Mayer and Staples, 2002). Phthalate biodegradation studies have shown the importance of esterase and laccases in the biodegradation of DEHP in liquid medium as well as in agar plates (Córdoba-Sosa et al., 2014a, 2014b).

Quantum mechanics has considerable applications on the study of biological systems, for which it is primarily used to build advanced classical force field representations of biomolecules (Merz, 2014) (i.e., quantum theory applied to model chemical structures). Molecular orbital theory extends quantum theory and states that the electrons are delocalized because they are not assigned to a particular atom or bond. Instead, the electrons move across the molecule in its orbitals. Molecular orbitals are wave functions and contain valence electrons between their atoms, which determine most of the chemical properties of molecules. This theory predicts the spatial and energetic properties of electrons to determine the molecular properties, and it is the basis for most semi-empirical approaches to quantum chemistry (Živković, 1983). The quantum method known as semi-empirical parameterized model number 3 (SE-PM3) has been used to correlate molecular quantum chemical parameters and experimental data (Odunola and Semire, 2007; González-Pérez et al., 2014). The visualization of chemical transformations helps to obtain a deeper fundamental understanding of how chemical reactions occur. It is useful to calculate quantum chemical parameters, such as the electrostatic potential of molecules, in order to understand molecular alignment and to calculate the electron transfer coefficient (ETC) (González-Pérez et al., 2014; González-Pérez, 2015). In this work, the specific growth rate (μ), maximum biomass (X_{max}), biodegradation constant of DEHP (k), half-life of DEHP biodegradation ($t_{1/2}$), esterase and laccase specific activities, and the enzymatic yield parameters were evaluated for *Fusarium culmorum* grown on media containing glucose and different concentrations of DEHP (0, 500 and 1000 mg/L). Molecular quantum chemical parameters, such as the highest occupied molecular orbital energy (E_{HOMO}), the lowest unoccupied molecular orbital energy (E_{LUMO}), the electrostatic potential (Eδ), molecular partial positive charge (δ+), molecular partial negative charge (δ−) and the energy of the band gap (E_{Bg}), were calculated to determine the ETC for each compound of the DEHP biodegradation pathway based on which biodegradation pathway of DEHP was proposed.

2. Material and methods

2.1. Microorganism

A strain of *F. culmorum* from the culture collection from the Research Centre for Biological Sciences (CICB) at Universidad Autónoma de Tlaxcala, Mexico, was used. This strain was isolated from the recycling of mixed pulper wastes in a recycled paper industry in which phthalates can be found as emulsifiers in paper dyes, inks and adhesives for paper envelopes (Aguilar-Alvarado et al., 2015). The strain was grown on malt extract agar (Difco) at 20 °C and stored at 4 °C until it was used.

2.2. Culture media and culture conditions

Three liquid culture media were prepared: 1) glucose yeast extract (GYE), 2) GYE + 500 mg DEHP/L, and 3) GYE + 1000 mg DEHP/L. Each medium contained (in g/L) 10 glucose, 5 yeast extract,

$0.6KH_2PO_4$, $0.5MgSO_4$-$7H_2O$, $0.4K_2HPO_4$, $0.25CuSO_4$-$5H_2O$, $0.05FeSO_4$-$7H_2O$, $0.05MnSO_4$, and $0.001ZnSO_4$-$7H_2O$. The pH was adjusted to 6.5 using either 0.1 M HCl or 0.1 M NaOH. All media also contained 400 μ of Tween 80 per liter. Flasks containing 50 mL of culture medium were autoclaved at 120 °C for 15 min, cooled to room temperature and then inoculated with three mycelial plugs (of 10 mm diameter) taken from the periphery of 7-day-old colonies of *F. culmorum* grown on potato dextrose agar (DIFCO).

Cultures were incubated at 28 °C for 10 days on a rotary shaker at 120 rpm. Analyses were carried out on samples taken at 12-h intervals and performed in triplicate. These concentrations of DEHP have been used previously (Suárez-Segundo et al., 2013).

2.3. Biomass production, growth kinetic parameters and pH measurements

Biomass (X) was obtained by filtration of the samples through filter paper (pore size 20–25 μm), and the specific growth rate was determined from changes in dry weight (g/L) [$X = X(t)$] using the Velhurst-Pearl or logistic equation (Eq. (1)),

$$dX/dt = \mu[1 - X/X_{max}]X or X\left[X_{max}/1 + Ce^{-\mu t}\right] \quad (1)$$

where $X = X_0$ (the initial biomass value), $C = (X_{max} - X_0) / X_0$, μ is the specific growth rate, and X_{max} is the maximal (or equilibrium) biomass level achieved when $dX/dt = 0$ for $X > 0$. Evaluations of the kinetic parameters of the logistic equation were carried out using a non-linear least squares fitting program (Solver; Excel, Microsoft). The specific growth rate (μ) was determined by fitting the biomass data across time using the logistic equation. The biomass yield ($Y_{X/S}$) was estimated as the coefficient of the linear regression of biomass concentration versus substrate concentration in grams of biomass/g of substrate consumed (Córdoba-Sosa et al., 2014a, 2014b). The pH was measured every 12 h in the supernatant of the cultures using a digital potentiometer (Hanna Instruments, México).

2.4. Laccase and esterase specific activities

Enzyme activity was assessed in the supernatant (ST) obtained from the filtration of the samples. Laccase activity was determined in each ST as the change in absorbance at 468 nm using a Jenway 6405 UV/Vis spectrophotometer (NJ, USA), using 2,6-dimethoxyphenol (DMP, SIGMA) as substrate. The assay mixture contained 950 μL of 2 mM DMP in 0.1 M acetate buffer (pH 4.5) and 50 μ of ST and was incubated at 40 °C for 1 min (Díaz et al., 2013).

Esterase activity was determined from changes in the absorbance at 405 nm using a Jenway 6405UV/Vis spectrophotometer (NJ, USA) with *p*-nitrophenyl butyrate (*p*NPB) as substrate. The reaction mixture contained 10 μ of a *p*NPB solution [1.76% (*v*/*v*) of *p*NPB in acetonitrile], 790 μ of 50 mM acetate buffer (pH 7.0), 0.04% Triton X-100 (*v*/*v*) and 100 μ of ST and was incubated at 37 °C for 5 min (Alves-Macedo and Fontes-Pio, 2005). One enzymatic unit of laccase or esterase activity (U) was defined as the amount of enzyme that produces an increase of 1 unit of absorbance per min in the reaction mixture (Córdoba-Sosa et al., 2014a, 2014b). The enzymatic activities were expressed as specific activity per biomass in U/gX.

2.5. Enzymatic yield parameters

Yield of enzyme per unit of biomass produced by the fungus ($Y_{E/X}$) was estimated as the relation between maximal enzymatic activity obtained during the exponential growth (E_{max}) and X_{max} (see Section 2.3. Biomass production and growth kinetic parameters in the methodology section). Enzymatic productivity ($P = E_{max}$ / time) was evaluated at the time when the enzymatic activity was maximal. The specific rate of enzyme production was calculated from the equation: $q_p = (\mu)(Y_{E/X})$ (Díaz et al., 2011; Córdoba-Sosa et al., 2014a, 2014b).

2.6. Determination of glucose consumption

All media contained glucose, as the only reducing sugar present, and were measured using the dinitrosalicylic acid reagent (DNS, SIGMA) (Miller, 1959). The assay mixture contained 950 μ of sterile distilled water, 2 mL of DNS and 50 μ of ST. The mixture was boiled for 5 min and then placed in an ice bath for 5 min to stop the reaction. Reducing sugars were determined by changes in the absorbance at 575 nm (using a Jenway 6405UV/Vis spectrophotometer, NJ, USA). A glucose standard curve was made by measuring the absorbance of known concentrations of glucose solutions at 575 nm.

2.7. Gas chromatography/mass spectrometry analysis

Samples (1 mL of each ST) were placed at 95 °C for 2 min in a water bath (to denature protein). Intermediate products of biodegradation of DEHP were analyzed by GC/MS, using a GC/MSD Agilent 890A gas chromatograph coupled to a mass spectrometer (5975C VL MSD, USA) equipped with a triple-axis detector and a capillary Agilent column (HP-5MS, USA) with 5% phenyl methyl siloxane (325 °C, 30 m, 320 μm, 0.25 μm). A 2-μl aliquot of the ST from cultures was injected with an injection source (GC ALS) at a port temperature of 300 °C with the purge valve on (split mode), using a split ratio of 20:1 and split flow of 21.14 mL/min. The following chromatographic conditions were used: initial oven temperature of 90 °C, held for 5 min, ramped up at 20 °C/min to 290 °C, and then held for 15 min using a syringe size of 10 μ in split mode at 20:1 at 300 °C in the injector. Helium was used as the carrier gas at 1.057 m/min. Mass spectra were collected in the scan mode within a *m*/*z* 25–550 range using a threshold of 150 and gain factor of 1. Ionization was performed by electron impact at 1870.588 eV with calibration by auto tuning and Mass Spec FC-43 as the internal standard. The ion source temperature and quadrupole temperature were 230 °C and 150 °C, respectively. Data were analyzed with the MSD ChemStation software (Agilent Technologies), and compounds were identified using the Nist MS 2.0 library. Compound matches were greater than 90%.

2.8. Biodegradation and removal efficiency of DEHP

Biodegradation of DEHP was determined by measuring the disappearance of DEHP in the cultures. It has been reported that the biodegradation percentage of DEHP (% *BD*) in a sample at time interval t (h) can be calculated as follows:

$$\%BD_t = \frac{C_t}{C_0} \times 100 \quad (2)$$

where C_0 = Initial concentration of DEHP in the test solution (mg/L). C_t = Concentration of DEHP in the test solution at time t.

A linear regression was carried out using the fermentation time versus the natural logarithm (ln) of the concentration of DEHP. The slope of such regression was considered the first-order biodegradation constant of DEHP (k) expressed in h^{-1} (Kim et al., 2007). The half-life ($t_{1/2}$) of DEHP, resulting from biodegradation, corresponded to the time interval of the concentration of DEHP to decrease to half of its initial value (Benjamin et al., 2015). These parameters were calculated as follows:

$$dC/dt = -kC$$

where C is the concentration of DEHP in mg/L, t = time (h) and k = degradation constant.

$$\text{At initial time } t_o,\ \ln C = -kt + \ln C_0 \quad (3)$$

$$t_{1/2} = 0.693/k \quad (4)$$

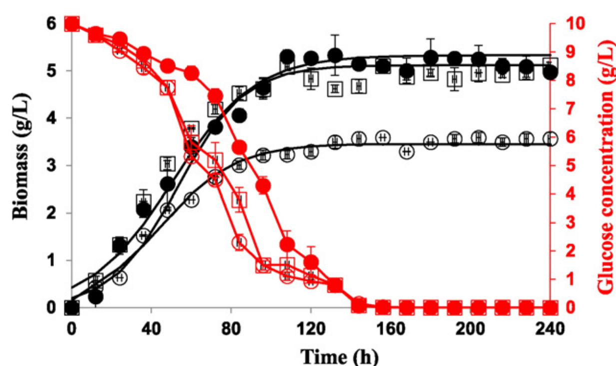

Fig. 1. Glucose consumption and biomass production by *F. culmorum* on GYE medium, (○), 500 (□) and 1000 (●) mg of DEHP/L in submerged fermentation. Biomass curves were fitted (—) using the logistic equation (Eq. (1)).

The removal efficiency of DEHP was calculated as follows:

$$\%\text{Removal efficiency} = 1-(C_t/C_0) \times 100 \quad (5)$$

2.9. 2.9. Quantum chemical modeling

A DEHP biodegradation pathway was proposed on the basis of the compounds identified by GC/MS as potential intermediates from DEHP and the quantum chemical parameters. The quantum chemical parameters of E_{HOMO} (electron Volts; eV), E_{LUMO} (eV), $\delta+$ (electron charge/Å; ec/Å), $\delta-$ (ec/Å) E_{Bg} (eV), $E\delta$ (ec/Å) and the ETC (eVÅ/ec) of each compound were calculated using the SE-PM3 (Fig. 1) (Odunola and Semire, 2007; González-Pérez et al., 2014).

$$E_{Bg} = E_{HOMO} - E_{LUMO} \quad (6)$$

$$E\delta = |(\delta-)-(\delta-+)| \\ ETC = E_{Bg}/E\delta \quad (7)$$

Calculations were performed on a Microsoft hardware HP ATA (ST500DM002 IDB14SCSI.6.1.7600.16385) workstation using a molecular simulator Hyper Chem (HC) 10 for Windows (Hyper Chem Hypercube, MultiON for Windows, serial #12-800-1501800080, Mexico city, Mexico). The Polak Ribiere technique was used to optimize the three-dimensional molecular geometry for model compounds. All calculations were carried out using the specific parameters of the simulations previously reported (González-Pérez et al., 2014).

2.10. Statistical analysis

All 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., 2014a, 2014b).

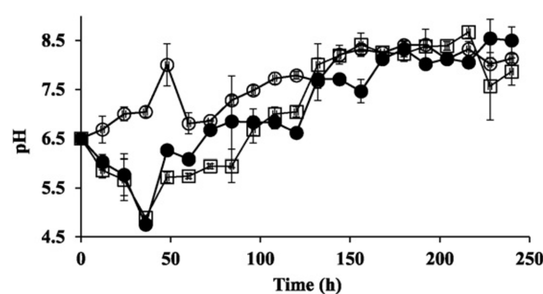

Fig. 2. pH of *F. culmorum* grown on GYE medium (○), 500 (□) and 1000 (●) mg of DEHP/L in submerged fermentation.

3. Results and discussion

3.1. Glucose consumption, biomass production and growth kinetic parameters

Fig. 1 shows biomass production and glucose consumption by *F. culmorum*. Slightly higher glucose consumption was observed in the GYE medium than in media containing DEHP. Within 84 h, 80% of the glucose was consumed in the GYE medium, but only 60% and 50% of glucose was used in media containing 500 and 1000 mg of DEHP/L, respectively. Glucose was completely consumed by *F. culmorum* after 156 h of growth in all of the media tested. This result suggests that *F. culmorum* used glucose and DEHP simultaneously as carbon and energy sources in those media containing both substrates. The X_{max} was higher in those media containing both glucose and DEHP (Table 1). The greatest μ were observed in the media supplemented with 500 or 1000 mg of DEHP/L. The lowest X_{max} and μ values were observed in the GYE medium (Table 1). The largest $Y_{X/S}$ was obtained in medium containing 1000 mg of DEHP/L, which shows that the DEHP was used as a carbon source. *F. culmorum* reached the stationary phase after approximately 96 h (4 days) of growth in all of the media tested. Córdoba-Sosa et al. (2014b) reported that the *P. ostreatus* grown in media containing 750, 1200 and 1500 mg of DEHP/L in submerged fermentation attained the stationary growth phase after 15 days of growth. Aguilar-Alvarado et al. (2015) studied the growth of several filamentous fungi on DEHP in agar and found that *F. culmorum* produced more biomass than other fungi in media containing 1500 mg of DEHP/L.

3.2. pH of the cultures.

The pH value of the cultures decreased during the first 36 h of fermentation to a minimum of 4.7 in media with DEHP added (Fig. 2). The pH of the cultures then increased and reached 7.8 and 8.5 after 240 h of growth in media that contained 500 and 1000 mg of DEHP/L, respectively. However, the pH of GYE cultures increased to 8.0 during the first 50 h of growth, decreased to 6.8 and then increased to 8.1 by the end of the cultivation (after 240 h) (Fig. 2). Córdoba-Sosa et al. (2014b) reported similar changes in media pH when *P. ostreatus* was grown in 1200 and 1500 mg of DEHP/L in submerged fermentation. They suggested that the increase in the pH during the exponential

Table 1
Growth kinetic parameters of *F. culmorum* grown in submerged fermentation.

Growth kinetic parameters	Culture media		
		DEHP (mg/L)	
	GYE	500	1000
X_{max} (g/L)	3.4 ± 0.1[c*]	4.9 ± 0.06[b]	5.1 ± 0.10[a]
μ (h^{-1})	0.05 ± 0.001[a]	0.05 ± 0.006[a]	0.08 ± 0.026[a]
$Y_{X/S}$ (gX/gS)	0.34 ± 0.01[c]	0.49 ± 0.01[b]	0.51 ± 0.01[a]

Values are expressed as mean ± SD (n = 3); means within the same column not sharing common superscript letters (a–c) differ significantly at 5% level. Kinetic parameters (X_{max} and μ) of the logistic equation were evaluated using a non-linear least squares fitting program. The biomass yield ($Y_{X/S}$) was estimated as the coefficient of the linear regression of biomass concentration versus substrate concentration (see Section 2.3).

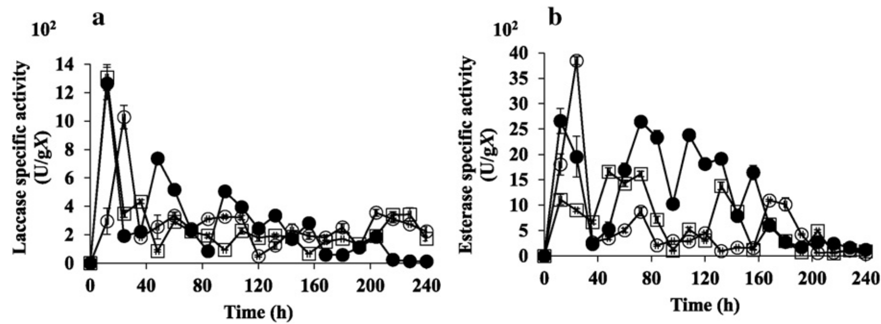

Fig. 3. Specific activities of laccase (a) and esterase (b) of *F. culmorum* grown in GYE (○), 500 (□) and 1000 (●) mg of DEHP/L in submerged fermentation.

phase (to 7.6) could be due to the degradation of DEHP, which released basic breakdown products into the media.

3.2. Laccase and esterase specific activities and enzymatic yield parameters

In general, higher laccase and esterase specific activities were observed during the exponential phase of growth in media containing DEHP (Fig. 3). Specific esterase activity was much higher than specific laccase activity in all of the media tested. This result showed that DEHP induced esterases in those media supplemented with DEHP (Fig. 3). In this study, the highest enzymatic yield parameters were observed in media containing 1000 mg of DEHP/L (Table 2). Córdoba-Sosa et al. (2014a) studied DEHP biodegradation (750, 1200 and 1500 mg of DEHP/L) by *P. ostreatus* and found that the highest laccase and esterase yield parameters were observed in the highest DEHP concentration tested (1500 mg/L). They reported that high concentrations of DEHP increased the yield of enzymes enhancing the metabolic activity of *P. ostreatus* (e.g., higher respiratory activity).

3.3. Biodegradation and removal efficiency of DEHP

Fig. 4 shows the percentage of DEHP biodegraded during the cultivations (Eq. (2)) and DEHP concentration (log-transformed) plotted versus time, which was used to determine the first-order biodegradation constant of DEHP (Eq. (3)). *F. culmorum* had degraded 99% (initial concentration 1000 mg/L) after 144 h of incubation. When 500 mg of DEHP/L was provided, 93% and nearly 98% of DEHP was degraded within 84 h and 144 h of incubation, respectively (Fig. 4a). Wen et al. (2014) reported that the strain *Arthrobacter* sp. could degrade 20 mg of DEHP/L and that its degradation rates reached approximately 52% after 72 h of incubation. The percentage of DEHP biotransformation by *Sphigomonas* sp. and *Corynebacterium* sp. was 11.5 and 11.2%, respectively (initial concentration 5 mg/L) at 168 h (Chang et al., 2004). Kim et al. (2007) reported that a purified cutinase of *F. oxysporum* decomposed almost 70% of the dihexyl phthalate (DHP) (initial concentration 10 mg/L) within 4.5 h, whereas a commercial yeast esterase (from *Candida cylindracea*) degraded approximately 10% of the initial concentration of DHP after 3 days of treatment. Kim et al. (2003) also studied DEHP degradation (500 mg/L) using purified fungal cutinase (from *F. oxysporum*) and a commercial esterase (from *C. cylindracea*) and found that 70% of the initial DEHP was decomposed by the fungal cutinase within 2.5 h, but only approximately 15% of the DEHP was degraded by yeast esterase within 3 days. In the present study, 95% of the initial concentration of DEHP (1000 mg/L) was degraded by *F. culmorum* in 2.5 days (Fig. 4a). We suggest that the esterase activity produced by *F. culmorum* corresponds to that of fungal cutinase. Further studies are needed to confirm if the esterase of *F. culmorum* specifically corresponds to a cutinase. The k was 0.0242 h^{-1} and 0.0241 h^{-1} for 1000 and 500 mg of DEHP/L, respectively (Fig. 4b). The k was similar in both concentrations of DEHP, and no inhibition of fungal growth was observed in either concentration. Gavala et al. (2003) reported that the constants of degradation for DEHP in batch and continuous experiments during the mesophilic anaerobic digestion of sludge were 0.0035 day^{-1} and 0.0359 day^{-1}, respectively, using up to 150 mg of DEHP/L. They reported that high-molecular-weight phthalates such as DEHP are non-biodegradable under methanogenic conditions. In the present study, $t_{1/2}$ was 28.64 h and 28.75 h for 1000 and 500 mg of DEHP/L, respectively (Eq. (4)). It has been reported that the $t_{1/2}$ of DEHP biodegradation in nature was 29 days, 45 days and 14 days in the composting activated sludge, in lagoon sludge and in sediment biodegradation, respectively (Benjamin et al., 2015). Juneson et al. (2001) reported that $t_{1/2}$ of DEHP biodegradation was 2–5 days in a sequencing batch reactor inoculated with a consortium of *Brevibacterium iodinum*, *Rhodococcus luteus* and *Bacillus brevis* (DEHP concentrations ranged from 2000 to 7000 mg/kg). It has been reported that the $t_{1/2}$ of DEHP by abiotic degradation (via photolysis plus hydrolysis) was between 390 and 1600 days under sunlight irradiation (Benjamin et al., 2015). Feng et al. (2002) reported that $t_{1/2}$ of

Table 2
Enzymatic yield parameters of laccase and esterase of *F. culmorum* grown in submerged fermentation.

	Culture media		
		DEHP (mg/L)	
Enzymatic yield parameters	GYE	500	1000
Laccase			
E_{max} (U/L)	1272.9 ± 0.8^{c*}	1739.1 ± 172.9^{b}	2345.4 ± 28.7^{a}
$Y_{E/X}$ (U/gX)	374.4 ± 11.1^{b}	355.4 ± 41.4^{c}	456.9 ± ^{a}9.3
P (U/L/h)	6.2 ± 0.0^{c}	7.7 ± 0.5^{b}	24.4 ± 0.3^{a}
q_p (U/gX/h)	19.8 ± 0.5^{b}	16.5 ± 2.3^{c}	36.4 ± 11.7^{a}
Esterase			
E_{max} (U/L)	3754.3 ± 205.4^{c}	6772.7 ± 238.9^{b}	12,645.0 ± 216.8^{a}
$Y_{E/X}$ (U/gX)	1106.0 ± 92.5^{c}	1319.2 ± 35.1^{b}	2582.0 ± 96.0^{a}
P (U/L/h)	21.8 ± 0.4^{c}	94.1 ± 3.3^{b}	117.1 ± 2.0^{a}
q_p (U/gX/h)	58.6 ± 4.2^{c}	61.5 ± 7.4^{b}	207.7 ± 72.1^{a}

Values are expressed as mean ± SD (n = 3); means within the same column not sharing common superscript letters (a–c) differ significantly at 5% level. $Y_{E/X}$ was estimated as the relation between E_{max} and X_{max} (logistic equation). $P = E_{max}$ / time was evaluated at the time when the enzymatic activity was maximal. $q_p = (\mu)(Y_{E/X})$ (see Section 2.5).

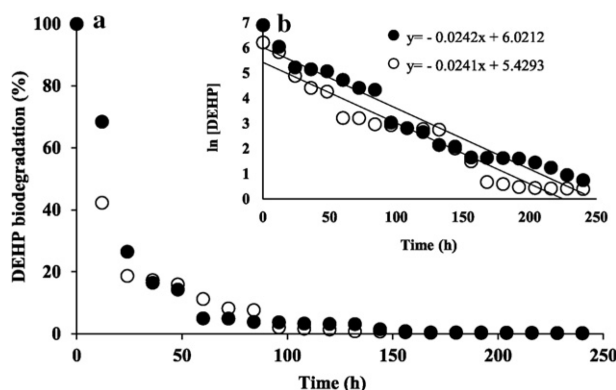

Fig. 4. Biodegradation percentage of DEHP by *F. culmorum* (a) and DEHP concentration (log-transformed) plotted across time used to determine k (b). Media with 500 (o) and 1000 (●) mg of DEHP/L added.

DEHP biodegradation by *Pseudomonas fluorescens* was 10.07 days, and the constant of biodegradation was 0.068 day^{-1} when the initial concentration of DEHP was less than 50 mg/L. Higher DEHP concentrations showed growth-inhibitory effects. In this study, the removal efficiency of DEHP was 99.8% and 99.9% for 1000 and 500 mg of this compound, respectively (Eq. (5)). Dargnat et al. (2009) reported that the removal efficiency of DEHP from wastewater was approximately 78% in a wastewater treatment plant. Removal efficiency for DEHP was 20% in an aeration basin (Birkett and Lester, 2003). The DEHP removal efficiency from the water phase at the sewage treatment plant was approximately 94% (Marttinen et al., 2003). Gao and Wen (2016) reported that the efficiency in the removal of phthalate esters was greater for the combination of wastewater treatment technologies than in individual treatment steps, increasing the efficiency of phthalate ester removal from 65%–71% to 95%–97%. In the present study, it was shown that greater removal efficiencies of DEHP were achieved using *F. culmorum*.

3.4. Proposed DEHP biodegradation pathway

Calculation of the molecular quantum parameters was useful for proposing the DEHP biodegradation pathway. Compounds identified by GC/MS (Figs. S1 and S2) are shown by dashed lines, and the rest of the compounds were proposed using quantum chemical modeling (Fig. 5).

The DEHP biodegradation pathway appears to proceed by enzymatic ester hydrolysis of DEHP with the formation of mono(2-ethylhexyl) phthalate (MEHP). DEHP is metabolized to produce MEHP, and the reaction moves from an excited state to a less excited state because the ETC value

Fig. 5. DEHP biodegradation pathway by *F. culmorum* proposed using quantum chemical modeling. The dashed line shows compounds identified by GC/MS.

Eqs. (6) and (7) for DEHP was much higher than the ETC for MEHP. This means that the electrons can easily move from DEHP to MEHP. MEHF was probably metabolized into 6-ethyl-4-oxodec-2-enoic acid (EOEA) through the McLafferty rearrangement in which cyclobutadiene is released after an aromatic ring-opening reaction. MEHP had an ETC value higher than the ETC value of EOEA, which shows that this pathway step can also move from an excited state to a less excited state. EOEA would be hydrogenated with the formation of 2,3-dihydroxybutanedioic acid (DHBA). A combined action of esterase and hydroxylase is suggested for this step. The electrons cannot easily move from EOEA to DHBA because the ETC value for EOEA is lower than that for DHBA, which shows that the hydrogenation process occurs from a less excited state to an excited state. DHBA would then be metabolized to 2,3-dihydroxybutanedial (DHB) by the action of an aldehyde-forming reductase (aldose reductase). This molecular transition would be carried out from an excited state to a less excited state because the ETC value of DHBA is higher than the ETC value of DHB. DHB would be reduced to 1,2,3,4-butanetetrol (BTT) by a reductase from a less excited state to an excited state (ETC value of DHB is lower than that ETC value of BTT). The electrons cannot easily move from DHB to BTT. Finally, BTT is metabolized to 2,3-butanediol (BD) by a hydrogenation/reduction reaction from a less excited state to an excited state because the ETC value of BTT is lower than the ETC value of BD. In this pathway step, the electrons cannot easily move from BTT to BD (Fig. S3). An enzyme should be used when a reaction occurs from a less excited state to an excited state. If a reaction occurs from an excited state to a less excited state, an enzyme may or may not be required. Fig. S4 shows quantum chemical parameters used for the calculation of ETC.

Fermentation of glucose (as well as xylose) by *Klebsiella oxytoca* (ATCC 8724) may produce 2,3-butanediol as the main product (Voloch et al., 1986). 2,3-Butanediol is the product of anaerobic fermentation of glucose. The overall stoichiometry of the reaction is 2 pyruvate + NADH → $2CO_2$ + 2,3-butanediol. Alternatively, acetoin could be oxidized to 2,3-butanedione (diacetyl) in the presence of O_2, indicating that the final product depends on the microorganism used in the fermentation process. Liang et al. (2008) reported that biodegradation of phthalates involved primary biodegradation from phthalate diethyl esters to phthalate monoethyl esters then to phthalic acid, followed by a biodegradation from phthalic acid to CO_2 and/or CH_4. In natural environments, phthalate biodegradation is always carried out by a consortium of microorganisms, and a single organism is unlikely to be able to completely metabolize phthalates (Staples et al., 1997; Gu et al., 2005). Several studies have shown that anaerobic degradation of phthalates is generally much slower than aerobic degradation and that a high concentration of phthalates and/or their metabolites inhibits their biodegradation (Staples et al., 1997; Yuan et al., 2002; Liang et al., 2008).

4. Conclusions

F. culmorum degraded 95% of the highest amount of DEHP tested (1000 mg/L) within 60 h of growth. The k and $t_{1/2}$ were 0.024 h^{-1} and 28 h, respectively, for both DEHP concentrations. Removal efficiency of DEHP was 99.8% and 99.9% for 1000 and 500 mg/L, respectively. Much higher esterase activity than laccase activity was observed in all media tested. DEHP was fully metabolized by *F. culmorum* with butanediol as the final product. This is a novel DEHP biodegradation pathway proposed for *F. culmorum*. The use of quantum chemical modeling provided an excellent prediction of the DEHP biodegradation pathway by *F. culmorum*. This fungus degraded DEHP to non-toxic compounds, showing that degradation products are harmless to the environment and to living creatures. A deeper understanding of the fungus biodegradation mechanisms of DEHP will facilitate the development of new methods to enhance the bioremediation of DEHP-contaminated sites. This fungus offers great potential in bioremediation of environments polluted with DEHP because it can use high concentrations of DEHP as a carbon and energy source.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.05.169.

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