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Short Communication

Enhanced esterase activity during the degradation of dibutyl phthalate by *Fusarium* species in liquid fermentation

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Abstract: Dibutyl phthalate (DBP) is one of the most abundantly produced and used plasticizers and is incorporated into plastic to make it more flexible and malleable. DBP has been found to be an environmental contaminant and reported as an endocrine disruptor. Therefore, it is crucial to develop ecofriendly alternatives to eliminate phthalate pollution. In the present research, the growth of *F. culmorum* and *F. oxysporum* in the presence of DBP was studied in liquid fermentation. The esterase activity, specific growth rate, and growth and enzymatic yield parameters were determined in DBP-supplemented media (1,500 or 2,000 mg/L) and in control medium (lacking DBP). These results show that in general, for both *Fusarium* species, the highest esterase activities, specific growth rates, and yield parameters were observed in media supplemented with DBP. It was observed that 1,500 and 2,000 mg of DBP/L did not inhibit *F. culmorum* or *F. oxysporum* growth and that DBP induced esterase production in both fungi. These organisms have much to offer in the mitigation of environmental pollution caused by the endocrine disruptor DBP. This study reports, for the first time, esterase production during the degradation of high concentrations (i.e., 1,500 and 2,000 mg/L) of DBP by *F. culmorum F. oxysporum*.

Keywords: Biodegradation, Dibutyl phthalate, Esterase, *Fusarium culmorum*, *Fusarium oxysporum*

Introduction

Plastic additives (e.g., plasticizers) are incorporated into plastic to change polymer properties and make plastic functional. Among plasticizers, phthalates are the largest group of additives incorporated into plastics to provide softness and flexibility and are added at concentrations between 10% and 70% of the total plastic weight (Sánchez, 2021). Esters of phthalic acid or phthalates are omnipresent organic compounds since they are released into the environment during manufacture, use, and disposal, and also during evaporation or leakage from landfill sites or containers (Boll et al., 2020; Gao & Wen, 2016; Hahladakis et al., 2018; Lesser et al., 2018; Sánchez, 2021). These substances are likely to accumulate in the food chain and contribute to health concerns such as to chronic health effects, cancer risks, and endocrine disruption (Groh et al., 2019; Hahladakis et al., 2018; Hermabessiere et al., 2017; Stojanoska et al., 2017). In particular, dibutyl phthalate (DBP) is one of the most widely produced and used phthalates. DBP is used in adhesives, personal care products, printing inks, nail polish, and paper coatings (Pérez-Andrés et al., 2017). This phthalate is listed as a priority pollutant by the U.S. Environmental Protection Agency (USEPA, 2019). Therefore, there is a need to develop ecofriendly alternatives to eliminate phthalate pollution. In nature, some microorganisms are able to use these organopollutants as carbon sources for growth, due to their enzyme production. This biodegradation process constitutes an effective, low-cost technology and an environment friendly method for the elimination of contaminants. Therefore, it is crucial to increase our understanding of enzyme production by organisms able to degrade phthalates. These microorganisms may enhance pollutant degradation in phthalate-contaminated environments. Several investigations have revealed that fungal species such as *Polyporus brumalis*, *Ganoderma lucidum*, *Trametes versicolor*, *Phlebia tremellosa*, *Neurospora sithopila*, *P. ostreatus*, *Phoma* sp., *Stropharia rugosoannulata*, *Ascocoryne* sp., *Puccinia arenariae* are phthalate-degrading organisms (Hwang et al., 2008; Kim et al., 2008; Lee et al., 2007; Kim & Song, 2009; Ahuactzin-Pérez et al., 2018a, 2018b; Carstens et al., 2020; González-Márquez et al., 2019; González-Márquez et al., 2020; Hwang et al., 2012; Liao et al., 2012; Luo et al., 2012; Sánchez, 2021; Sánchez-Sánchez & Sánchez, 2019). In particular, fungi such as *Fusarium* species have been reported to degrade phthalates due to their esterase production. *Fusarium oxysporum* was able to degrade DBP (Kim & Lee, 2005), dipentyl phthalate (Ahn et al., 2006), dipropyl phthalate (Kim et al., 2005), di(2-ethyl hexyl) phthalate (DEHP) (Kim et al., 2003), and butyl benzyl phthalate (Kim et al., 2002). In addition, *Fusarium culmorum* was able to metabolize DEHP and DBP as previously reported by our group (Ahuactzin-Pérez et al., 2016; Ahuactzin-Pérez et al., 2018c; Ríos-González et al., 2019). The proposed DBP biodegradation pathway shows that *F. culmorum* was able to metabolize this phthalate to malic and fumaric acids, which can be transformed into carbon dioxide and water (Ahuactzin-Pérez et al., 2018c). This fungus had a DBP removal efficiency of 99% (Ahuactzin-Pérez et al., 2018c).

In the present research, the esterase activity, biomass (X_{max}), specific growth rate (μ), maximum biomass yield ($Y_{X/S}$), and enzymatic yield parameters were determined in DBP-supplemented media (1,500 or 2,000 mg/L) and in control medium (lacking DBP).

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This study reports, for the first time, esterase production during the degradation of 1,500 and 2,000 mg of DBP/L by *F. oxysporum* and *F. culmorum*.

Materials and Methods

Strains

F. oxysporum and *F. culmorum* from the microbial collection of the Research Centre for Biological Sciences (CICB) at the Autonomous University of Tlaxcala (UAT) (Tlaxcala, Mexico) were used in this work. *F. culmorum* (GenBank accession number, HF947520) was isolated from an industrial facility for recycling paper, where phthalates can be found as additives in paper dyes, inks and adhesives for paper envelopes (Aguilar-Alvarado et al., 2015). This strain is deposited at the Collection of the Mexico's National Center for Genetic Resources (CNRG-INIFAP) (Jalisco, Mexico). Stock cultures were grown on malt extract agar at 25°C in Petri dishes for 7 days and then stored at 4°C. Cultures were periodically transferred to fresh culture medium.

Culture Media Preparation and Culture Growth Conditions

Two culture media, containing different concentrations of DBP, were prepared with the following components (in g/L): DBP (1.5 or 2.0; with 99% purity; Sigma), glucose, 10; KH_2PO_4, 0.6; $ZnSO_4.7H_2O$, 0.001; K_2HPO_4, 0.4; $FeSO_4$-$7H_2O$, 0.05; $MgSO_4$-$7H_2O$, 0.5; $MnSO_4$-H_2O, 0.05; $CuSO_4$, 0.25 and yeast extract, 5. Medium prepared as described above but lacking DBP was used as a control. DBP-supplemented media also contained 100 μL of Tween 80 per liter. The pH was adjusted to 6.5 using either 0.1 M NaOH or 0.1 M HCl, and the media were autoclaved for 15 min at 121°C. Erlenmeyer flasks (125 mL) supplemented with 50 mL of sterile culture medium were inoculated using three mycelial fragments of 10 mm diameter, which were obtained from the periphery of colonies of *F. culmorum* or *F. oxysporum* grown for 7 days on malt extract agar (DIFCO). Fungal cultures were incubated on an orbital shaker at 25°C for 7 days with shaking at 120 rpm, and samples were taken for analysis every 12 hr.

Fungal Biomass Production and Growth Parameter Calculation

Fungal biomass (X) was separated from the liquid cultures by vacuum filtration using filter paper of 20–25-μm pore size and then dried at 60°C. The specific growth rate (μ) was calculated by measuring the changes in biomass production (in dry weight) through fermentation time by using the logistic equation as follows:

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{max}}\right]X$$

where μ (h^{-1}) = specific growth rate,

X_{max} (g/L) = maximal biomass level obtained when $dX/dt = 0$ for $X > 0$.

This equation is solved and expressed as follows;

$$X = \frac{X_{max}}{1 + Ce^{-\mu t}}$$

where $C = (X_{max} - X_0)/X_0$, $X = X_0$, the initial biomass value.

The parameters from these equations were calculated using a nonlinear least squares fitting program (Solver; Excel, Microsoft) as previously reported by us (Ahuactzin-Pérez et al., 2016).

Esterase Activity and Enzymatic Yield Parameters

The esterase activity of the supernatants of *F. culmorum* and *F. oxysporum* was evaluated from changes in the absorbance at 405 nm using a UNICO spectrophotometer (S-2150 series DAYTON, NJ, USA) with *p*-nitrophenyl butyrate (*p*NPB) as substrate. The reaction mixture contained 100 μL of supernatant and 900 μL of reagent prepared with the following components: 1.76% (vol/vol) *p*NPB, 1.1% (vol/vol) acetonitrile, 0.04% (vol/vol) Triton X-100, and 11.1% (vol/vol) distilled water, dissolved in 0.01 M phosphate buffer at pH 7.5 and was incubated at 37 °C for 5 min (Davis et al., 2000). A ρ-nitrophenol standard curve using the same reaction conditions was made by measuring the absorbance of known concentrations of ρ-nitrophenol solutions. Linear regression analysis of the standard curve was carried out to determine the concentration (μg/mL) of *p*-nitrophenol produced in each sample and then the volumetric activity (U/L) was obtained. One unit of esterase activity (U) was defined as the amount of the enzyme required to release 1 micromole of ρ-nitrophenol per minute from *p*NPB under the assay conditions. Volumetric activity was reported in U/L. The enzymatic yield parameters maximal enzymatic activity (E_{max}), yield of esterase per unit of biomass ($Y_{E/X}$), specific rate of enzyme production (q_p) and esterase productivity (P) were determined for *F. culmorum* and *F. oxysporum* as previously reported by us (Ahuactzin-Pérez et al., 2016).

Statistical Analysis

All experiments were performed in three independently repeated experiments. Statistical analyses were carried out using one-way analysis of variance followed by Tukey's post hoc test using SigmaPlot version 12.0 (Systat Software Inc, San Jose, CA, USA).

Results and Discussion

Fungal Growth Kinetic and Yield Parameters

Fig. 1 shows the biomass production by *Fusarium* species in media containing DBP (1,500 or 2,000 mg/L) and in the control medium. *F. culmorum* attained the stationary growth phase after approximately 96 hr and 132 hr in DBP-supplemented media and in the control medium, respectively (Fig. 1a). *F. oxysporum* reached the stationary growth phase after approximately 60 hr and 72 hr in DBP-supplemented media and in the control medium, respectively (Fig. 1b). Ahuactzin-Perez et al. (2018c) studied the growth of *F. culmorum* in media supplemented with lower concentrations of DBP (500 and 1,000 mg/L) than those studied in the present research. It was observed that *F. culmorum* attained the stationary phase after 84 hr and 72 hr of cultivation in media supplemented with 500 mg of DBP/L and 1,000 mg of DBP/L, respectively (Ahuactzin-Pérez et al., 2018c). Furthermore, we observed that the fungus *P. ostreatus* attained the stationary phase of growth after 408 hr and 456 hr in media containing 500 and 1,000 mg of DBP/L, respectively (Ahuactzin-Pérez et al., 2018b). Lee et al. (2007) reported that the mycelial growth of the fungus *P. brumalis* was inhibited as the DBP concentration was increased to 250, 750, and 1,250 μM.

F. culmorum showed the greatest μ value in the medium containing 2,000 mg of DBP/L, followed by the medium supplemented with 1,500 mg of DBP/L and then the control medium. The X_{max} value for *F. culmorum* was higher in the media supplemented with DBP than in the control medium. The $Y_{X/S}$ values did not show significant differences between the culture media (Table 1). *F. oxysporum* had the highest μ value in

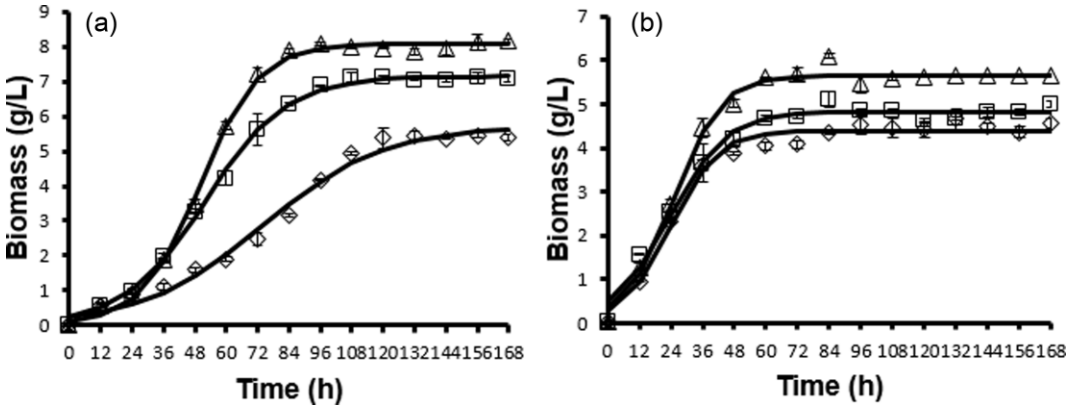

Fig. 1 Biomass production of *F. culmorum* (**a**) and *F. oxysporum* (**b**) grown in medium lacking DBP (diamond) and in media supplemented with 1,500 (square) and 2,000 (triangle) mg of DBP/L. Biomass curves were fitted (—) using the logistic equation.

Table 1. Growth Parameters of *F. culmorum* and *F. oxysporum* Grown in Glucose Medium and in DBP-Supplemented Media in Liquid Fermentation Conditions

	Culture media		
Parameters	**Control**	**DBP (1,500 mg/L)**	**DBP (2,000 mg/L)**
F. culmorum			
μ (h^{-1})	0.04^{c}	0.06^{b}	0.08^{a}
	(0.001)	(0.004)	(0.006)
X_{max} (g/L)	5.7^{b}	7.1^{a}	8.1^{a}
	(0.04)	(0.03)	(0.23)
$Y_{X/S}$ (gX/gS)	0.6^{a}	0.7^{a}	0.8^{a}
	(0.001)	(0.001)	(0.001)
F. oxysporum			
μ (h^{-1})	0.11^{a}	0.09^{b}	0.11^{a}
	(0.001)	(0.001)	(0.001)
X_{max} (g/L)	4.4^{b}	4.8^{b}	5.7^{a}
	(0.05)	(0.01)	(0.1)
$Y_{X/S}$ (gX/gS)	0.4^{a}	0.5^{a}	0.6^{a}
	(0.001)	(0.02)	(0.04)

Note: Values are expressed as mean (standard deviation in parentheses) ($n = 3$). Means within the same column not sharing common superscript letters differ significantly at 5% level.

both the medium supplemented with 2,000 mg of DBP/L and the control medium. The lowest μ was observed in medium containing 1,500 mg of DBP/L. The greatest X_{max} value for *F. oxysporum* was in medium containing 2,000 mg of DBP/L, followed by the medium containing 1,500 mg of DBP/L and the control medium. The $Y_{X/S}$ values did not show a significant difference between the DBP-supplemented media and the control medium (Table 1). Kumar & Maitra (2016) reported that *Methylobacillus* sp. was able to grow on DBP-supplemented medium, and had a μ value of 0.07 h^{-1}, which was similar to the values shown by *F. culmorum* in media supplemented with DBP (0.06 h^{-1} and 0.08 h^{-1} for 1,500 and 2,000 mg of DBP/L, respectively) (Table 1). Furthermore, *P. ostreatus* showed μ, X_{max}, and $Y_{X/S}$ values of 0.015 h^{-1}, 7.3 g/L, and 0.67 gX/gS, respectively, when grown in medium containing 1,000 mg of DBP/L (Ahuactzin-Pérez et al., 2018b). Ahuactzin-Pérez et al. (2018c) found that *F. culmorum* had μ, X_{max}, and $Y_{X/S}$ values of 0.01 h^{-1}, 5 g/L, and 0.49 gX/gS, respectively, when grown in DBP-supplemented medium (1,000 mg/L). In the present research, *F. culmorum* showed μ, X_{max}, and $Y_{X/S}$ values of 0.08 h^{-1}, 8.1 g/L, and 0.8 gX/gS, respectively, in media supplemented with 2,000 mg of DBP/L. These results show that *F. culmorum* was able to use high concentrations of DBP and reach the stationary phase in a shorter period of time compared to our previous studies in which lower concentrations of DBP were used (1,000 mg/L) (Ahuactzin-Pérez et al., 2018c).

Esterase Activity and Esterase Yield Parameters

In general, *F. culmorum* had higher esterase activity in the medium containing 2,000 mg of DBP/L than in the medium supplemented with 1,500 mg of DBP/L or in the control medium (Fig. 2a). In the medium supplemented with 2,000 mg of DBP/L, esterase activity increased during the first 48 hr, at which time the maximum activity (208.5 U/L) was observed. It is suggested that the high esterase activity detected at 48 hr could be due to the de-esterification processes in DBP molecules at the beginning of its biodegradation. Esterase activity then decreased after 60 hr but showed slight increases after 72 hr and 120 hr of growth. In general, the medium containing 1,500 mg of DBP/L and the control medium had esterase activity that increased gradually during the course of fermentation and showed the maximum activity at the end of cultivation. *F. oxysporum* showed high enzymatic activity during the first 108 hr of cultivation in medium supplemented with 2,000 mg of DBP/L. Similar enzymatic activity was observed during the first 108 hr in the medium supplemented with 1,500 mg of DBP/L and in the control medium; however, enzymatic activity increased, reaching a maximum value (292.5 U/L) after 168 hr in the medium containing 1,500 mg of DBP/L, which was the highest esterase activity demonstrated by *F. oxysporum* (Fig. 2b).

From all the media tested, *F. culmorum* had the greatest E_{max}, $Y_{E/S}$, P_{RO} and q_p in the medium containing 2,000 mg of DBP/L. In fact, E_{max}, $Y_{E/S}$, P_{RO}, and q_p were approximately threefold, twofold, sixfold, and fourfold higher, respectively, in medium supplemented with 2,000 mg of DBP/L than in the control medium. The lowest E_{max} and q_p for *F. culmorum* were observed in the control medium (Table 2). As shown in Table 2, *F. oxysporum* had the highest E_{max} and $Y_{E/S}$ in medium supplemented with 1,500 mg of DBP/L, and this fungus showed the lowest E_{max} in the control medium. The highest P_{RO} and q_p for *F. oxysporum* were observed in the medium containing 2,000 and 1,500 mg of DBP/L, respectively. *F. culmorum* and *F. oxysporum* showed similar E_{max}; however, *F. culmorum* had an approximately twofold higher P_{RO} than that of *F. oxysporum* in medium supplemented with 2,000 mg of DBP/L.

Numerous studies have demonstrated that esterase is responsible for phthalate degradation (Hernández-Sánchez et al., 2019;

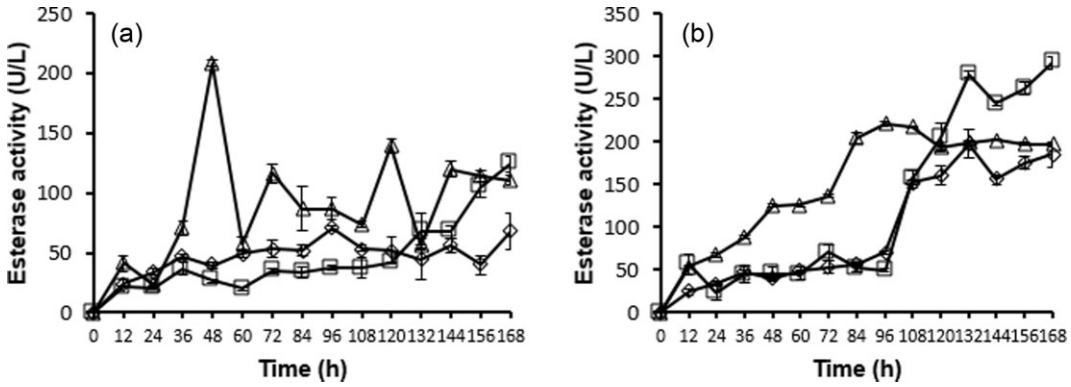

Fig. 2 Esterase activity of *F. culmorum* (**a**) and *F. oxysporum* (**b**) grown in medium lacking DBP (diamond) and in media supplemented with 1,500 (square) and 2,000 (triangle) mg of DBP/L.

Table 2. Enzymatic Yield Parameters of *F. culmorum and F. oxysporum* Grown in Glucose Medium and in DBP-Supplemented Media in Liquid Fermentation Conditions

	Culture media		
Parameters	**Control**	**DBP (1,500 mg/L)**	**DBP (2,000 mg/L)**
F. culmorum			
E_{max} **(U/L)**	70.5^{c}	124.2^{b}	208.5^{a}
	(45)	(24)	(19)
$Y_{E/X}$ **(U/gX)**	12.4^{b}	17.5^{b}	25.7^{a}
	(0.5)	(0.24)	(0.8)
P_{RO} **(U/L/h)**	0.7^{b}	0.7^{b}	4.3^{a}
	(0.003)	(0.002)	(0.001)
q_p **(U/h/gX)**	0.5^{c}	1.1^{b}	2.1^{a}
	(0.001)	(0.002)	(0.001)
F. oxysporum			
E_{max} **(U/L)**	197.9^{c}	292.5^{a}	221.3^{b}
	(29)	(12)	(18)
$Y_{E/X}$ **(U/gX)**	45.0^{b}	61.0^{a}	39.0^{b}
	(1.4)	(2.5)	(2.2)
P_{RO} **(U/L/h)**	1.5^{b}	1.7^{b}	2.3^{a}
	(0.05)	(0.05)	(0.1)
q_p **(U/h/gX)**	4.9^{b}	5.5^{a}	4.3^{b}
	(0.6)	(0.02)	(0.05)

Note: Values are expressed as mean (standard deviation in parentheses) ($n = 3$). Means within the same column not sharing common superscript letters differ significantly at 5% level.

Sánchez, 2021). In particular, the involvement of esterase as a key enzyme in DBP biodegradation has been reported in bacteria such as *Delftia* sp. (Patil et al., 2006), *Sphingobium* (Sungkeeree et al., 2016), *Acinetobacter* sp. (Fang et al., 2017), *Bacillus megaterium* (Feng et al., 2018), *Bacillus* (Huang et al., 2020; Xu et al., 2020), *Halomonas* sp. (Wright et al., 2020), and *Mycobacterium* sp. (Lu et al., 2020) among others (Hu et al., 2021); in fungi such as *F. oxysporum* (Kim & Lee, 2005), *F. culmorum* (Ferrer-Parra et al., 2018), and *P. ostreatus* (Córdoba-Sosa et al., 2014); and the yeast *Candida cylindracea* (Kim & Lee, 2005). In particular, *F. culmorum* was able to degrade DBP to fumaric and malic acids (Ahuactzin-Pérez et al., 2018c), and *P. ostreatus* was also able to degrade DBP to acetyl acetate and benzene (Ahuactzin-Pérez et al., 2018b); however, very little is known about esterase production by fungi during the course of DBP biodegradation.

pH of the Cultures During Fermentation

Fig. 3a shows the pH profile of the cultures of *F. culmorum* during growth. The pH dropped during the first 36 hr of growth to a minimum of 4.9, 5.5, and 5.8 for the media supplemented with 2,000 mg of DBP/L, 1,500 mg of DBP/L, and the control medium, respectively. The pH of *F. culmorum* cultures increased, reaching 7, 7.4, and 7.8 in the control medium and in media supplemented with 2,000 mg of DBP/L and 1,500 mg of DBP/L, respectively, at the end of fermentation. The pH profile of the cultures of *F. oxysporum* during fermentation is shown in Fig. 3b. The pH decreased to a minimum of 4.9 after 24 hr of cultivation in all the tested media and then increased to 7.8, 8, and 8.2 in the control medium and in the media supplemented with 1,500 mg of DBP/L and 2,000 mg of DBP/L, respectively, at the end of cultivation. The pH profile of the cultures showed a similar pattern during fermentation for both fungi. An acidic pH was observed at the beginning of fermentation, indicating organic acid production as a result of the DBP degradation process, as previously reported by

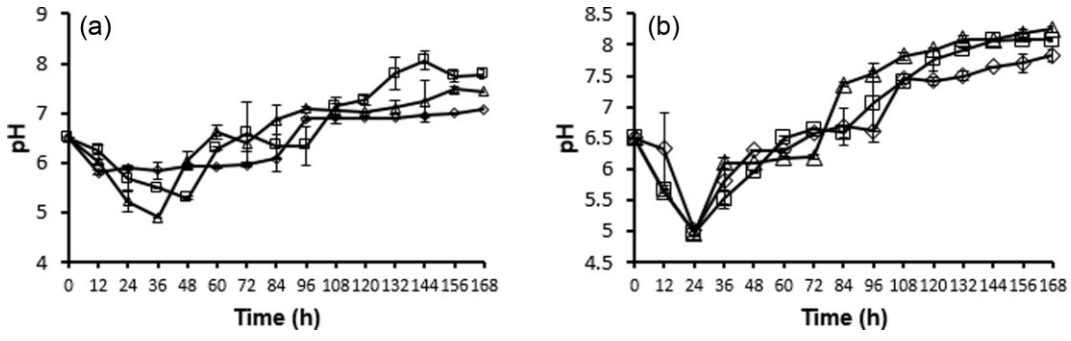

Fig. 3 pH profile of *F. culmorum* (**a**) and *F. oxysporum* (**b**) grown in medium lacking DBP (diamond) and in media supplemented with 1,500 (square) and 2,000 (triangle) mg of DBP/L.

us (Ahuactzin-Pérez et al., 2018c). In general, for both fungi, the pH increased after 48 hr, reaching a range of approximately 7–8.2, which is within the pH range reported for optimal esterase activity (7.5–8.9) in DBP biodegradation (Akita et al., 2001; Huang et al., 2019; Huang et al., 2020; Lu et al., 2020; Sarkar et al., 2020; Zhang et al., 2014).

These results show that in general, for both *Fusarium* species, the highest esterase activities, specific growth rates, and growth kinetic and enzymatic yield parameters were observed in media supplemented with DBP. It was observed that 1,500 and 2,000 mg of DBP/L did not inhibit *F. culmorum* or *F. oxysporum* growth and that DBP induced esterase production in both fungi. These organisms have much to offer in the mitigation of environmental pollution caused by the endocrine disruptor DBP.

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Conflict of Interest

The authors declare no conflict of interest.

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