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SHORT COMMUNICATION

Optimum pH for di(2-ethylhexyl) phthalate degradation by *Fusarium culmorum* in submerged fermentation

pH óptimo para la degradación de di(2-etilhexil) ftalato por *Fusarium culmorum* en fermentación sumergida

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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. This compound is considered as potential human health risks due to their endocrine-disrupting effects. Due to these concerns, there is a need to search of environmental remediation alternatives *Fusarium culmorum* has shown capability to degrade DEHP due its esterase production. Optimization of the pH is crucial in the fermentation process, since the cultivation conditions are essential for a successful enzyme production by the organism. In this work, the specific growth rate, maximum biomass, esterase activity and enzymatic yield parameters were determined for *F. culmorum* grown at different pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) in a medium added with DEHP (1000 mg/L) as sole carbon source in submerged fermentation. It was found

that the greatest enzymatic yield parameters were observed at pH values of 5.5 and 6.5, showing that these values were the optimum pH values for DEHP degradation by *F. culmorum*.

Keywords: Biodegradation, di(2-ethylhexyl) phthalate, esterase activity, fungal growth, *Fusarium culmorum*.

RESUMEN

Di(2-etilhexil) ftalato (DEHP) es un plastificante ampliamente utilizado en la fabricación de plásticos, y es un contaminante ambiental. Se considera que éste compuesto representa riesgos potenciales para la salud humana debido a sus efectos disruptores endocrinos. Por lo anterior, existe la necesidad de buscar alternativas para descontaminar el medio ambiente. *Fusarium culmorum* ha demostrado capacidad para degradar DEHP debido a las enzimas esterasas que produce. La optimización del pH es crucial en el proceso de fermentación, ya que las condiciones de cultivo son esenciales para la producción exitosa de enzimas por parte del organismo. En éste estudio, la tasa de crecimiento específico, la biomasa máxima, la actividad de esterasa y los parámetros de rendimiento enzimático se determinaron para *F. culmorum* cultivado a diferentes valores de pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 y 9.0) en un medio suplementado con DEHP (1000 mg/L) como única fuente de carbono en fermentación sumergida. Se encontró que los mayores valores de los parámetros de rendimiento enzimático se observaron a valores de pH de 5.5. y 6.5, siendo estos los valores de pH óptimo para la degradación de DEHP por *F. culmorum*.

Palabras clave: Actividad de esterasa, biodegradación, crecimiento fúngico, di(2-etilhexil) ftalato, *Fusarium culmorum*.

1. INTRODUCTION

Phthalic acid esters or phthalates are plastic additives that are added to the plastic manufacturing process to enhance flexibility to plastics (plasticizers) (Marturano *et al*., 2017; Hahladakis *et al*., 2018). In plastic polymers, the plasticizer molecules occupy the intermolecular spaces between the polymer chains (not covalently bound to the plastic matrix), which effectively increases the interspaces between chains and reduces the secondary intermolecular bonding forces. Therefore, plasticizers can easily be lost from polymers through migration, evaporation, or extraction phenomena and then released into the environment (Rani *et al*., 2015; Hermabessiere *et al*., 2017). Phthalates have been detected in various environments such as soil, sediment, surface water and groundwater (Net *et al*., 2015; Heo *et al*., 2020). These compounds are also considered as potential human health risks due to their endocrine-disrupting effects, which seem to be able to mimic or interfere with the binding and action of natural hormones, thus disrupting

physiological processes (Yang *et al*., 2015). Humans are exposed to phthalates through ingestion, inhalation, and dermal exposure, which is a critical concern with unknown long-term impacts (Meeker *et al*., 2009). In particular, di(2-ethyl hexyl) phthalate (DEHP) is added into high-molecular weight polymers such as polyvinyl chloride (PVC), being the most consumed phthalate in the plastic industry and it is listed as a priority hazardous substance by the China National Environmental Monitoring Center, the United States Environmental Protection Agency and the European Community (Yang *et al*., 2018). Concentrations of DEHP range from 1 to 220 µg/L have been found in surface water and from 7.5 to 2045 mg/kg in sediment in places where this phthalate is produced (Green Facts, 2008). High concentrations of DEHP (1085 mg/L) were found in a wastewater treatment plant (Olujimi *et al*., 2012) and up 1439 ng/m^3 in urban air in Europe (Guerranti *et al*., 2019). Biodegradation by microorganisms is the most effective means for phthalates remediation process. In particular, fungi are of great importance in this sense due to the highly efficient enzymatic, which release digestive enzymes by exocytosis outside of their hyphae such as esterase (Sánchez, 2020). Fungal species such as *Fusarium oxysporum* (Kim *et al*., 2003), *Neurospora crassa*, *Trichoderma harzianum* (Aguilar-Alvarado *et al*., 2015), *Polyporum brumalis* (Lee *et al*., 2007), *Fusarium culmorum* (Ahuactzin-Pérez *et al*., 2016; Ferrer-Parra *et al*., 2018; González-Márquez *et al*., 2019), *Pleurotus ostreatus* (Ahuactzin-Pérez *et al*., 2018), and *Saccharomyces cerevisiae* (Begum *et al*., 2003) among others have been reported able to degrade phthalates. In particular, *F. culmorum* was capable to degrade DEHP to butanol, hexanal, catechol and acetic acid. It was suggested that the first two compounds would transform into butanediol and the last two would enter into the Krebs cycle and would be mineralized to CO_2 and H_2O (González-Márquez *et al*., 2019). In this work, the specific growth rate, maximum biomass, esterase activity and enzymatic yield parameters were determined for *F. culmorum* grown at different pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) in a medium added with DEHP (1000 mg/L) as sole carbon source in submerged fermentation.

2. MATERIALS AND METHODS

2.1. Microorganism and culture media

F. culmorum from the culture collection of the Research Centre for Biological Sciences at Universidad Autónoma de Tlaxcala (CICB, Tlaxcala, Mexico) was used. This fungus was isolated from an industrial facility for recycling paper, where phthalates can be present as remnants of these additives used in the production of paper and cardboard (Aguilar-Alvarado *et al*., 2015). Eight different culture media were prepared at different pH values: 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The composition of the medium was as follows (in g/L): DEHP (Sigma; purity grade 99%), 1.0; $NaNO_3$, 3.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; and $FeSO_4.7H_2O$,

0.01. 100 µL of Tween 80/L were also added to the culture medium. DEHP (boiling point 385 °C) was added to the medium before autoclaving. The final pH was adjusted after autoclaving using either 0.1 M HCl or 0.1 M NaOH.

2.2. Inoculation and culture conditions for fungal growth

125 mL Erlenmeyer flasks containing 50 mL of culture medium were autoclaved at 120 ºC for 15 min, cooled to room temperature and then inoculated with three mycelial plugs (of 4 mm diameter) taken from the periphery of 7-d-old colonies of *F. culmorum* grown on malt extract agar (DIFCO). Cultures were incubated at 25 ºC 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.

2.3. Biomass production and parameters calculation

Mycelium was harvested from cultures by filtration using filter paper (pore size 20-25 µm), and the specific growth rate (µ) and yield parameters were calculated by using logistic equation as previously specified (González-Márquez *et al*., 2019). pH measurements were taken every 12 h.

2.4. Analysis of esterase activity and esterase yield parameters

Esterase activity was assessed in the supernatant obtained from the filtration of the samples using *p*-nitrophenyl butyrate (*p*NPB) as substrate as previously reported (Ferrer-Parra *et al*., 2018). One enzymatic unit of 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. The esterase specific activities were expressed as specific activity per biomass in U/gX. Yield of esterase per unit of biomass produced by the fungus ($Y_{E/X}$), maximal enzymatic activity (E_{max}), esterase productivity (P), and specific rate of enzyme production (q_p) were evaluated as reported by González-Márquez *et al*. (2019).

2.5. Data analysis

All tests were carried out in triplicates. Statistical analysis was performed using one-way ANOVA and Tukey post-test using Sigma Plot Version 12.0 (Systat Software Inc.).

3. RESULTS

3.1. Biomass production and pH profile during the fermentation

Fig. 1 shows the biomass production of *F. culmorum* in DEHP-containing medium at pH values of 5.5, 6.0, 6.5 and 7.0 (Fig. 1a), and at pH values of 7.5, 8.0, 8.5 and 9.0 (Fig. 1b). It is observed that the fungal growth was higher at acid pH values (5.0, 6.0 and 6.5) and at pH of 8.0. However, X_{max} had the highest values at pH of

6.5, 8.0, 8.5 and 9.0, which showed no significant differences (Table 1). In general, the highest μ values were observed at pH values lower than 7. The lowest μ values were observed at neutral pH (i.e. 7.0 and 7.5) and at basic pH values (i.e. 8.5 and 9.0) (Table 1). On the other hand, the pH values showed small variation during the fermentation process in all the tests (Fig. 2).

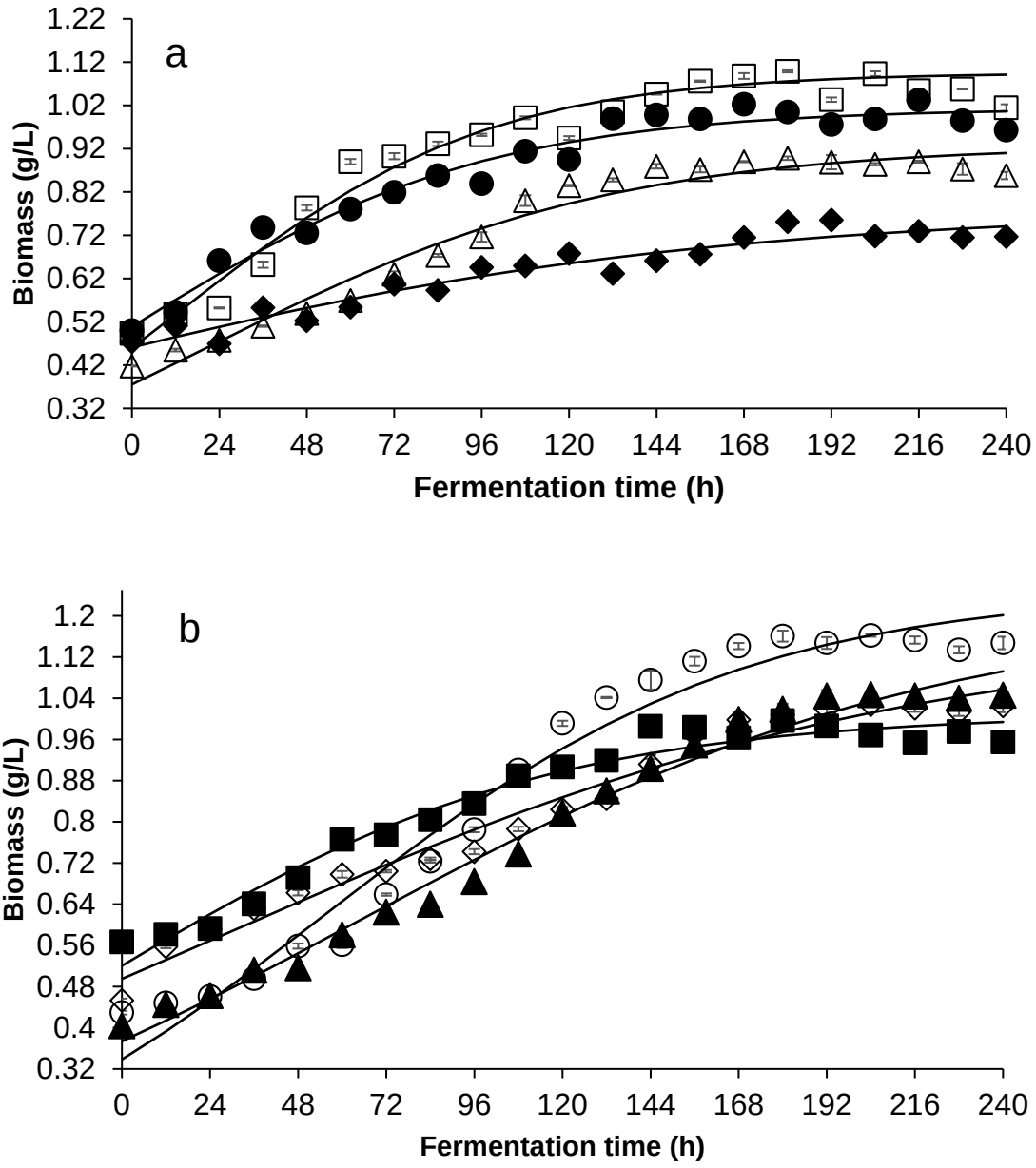

Fig. 1. Growth of *F. culmorum* in media supplemented with DEHP at different pH values in submerged fermentation. pH values **a)** 5.5 (●), 6.0 (△), 6.5 (▯), 7.0 (⧫), **b)** 7.5 (■), 8.0 (⬭), 8.5 (◊), 9.0 (▲). Biomass curves were fitted (—) using the logistic equation (González-Márquez *et al*., 2019).

Table 1. Growth parameters of *F. culmorum* in media supplemented with DEHP at different pH values in submerged fermentation.

Parameters	pH of the culture media							
	5.5	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**
μ (h^{-1})	0.02^{a} ±0.002	0.018^{a} ±0.001	0.023^{a} ±0.001	0.01^{b} ±0.001	0.016^{b} ±0.001	0.017^{a} ±0.001	0.01^{b} ±0.001	0.012^{b} ±0.001
X_{max} (g/L)	1.014^{b} ±0.002	0.92^{c} ±0.001	1.09^{a} ±0.002	0.77^{c} ±0.001	1.01^{b} ±0.001	1.24^{a} ±0.002	1.16^{a} ±0.001	1.21^{a} ±0.001

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

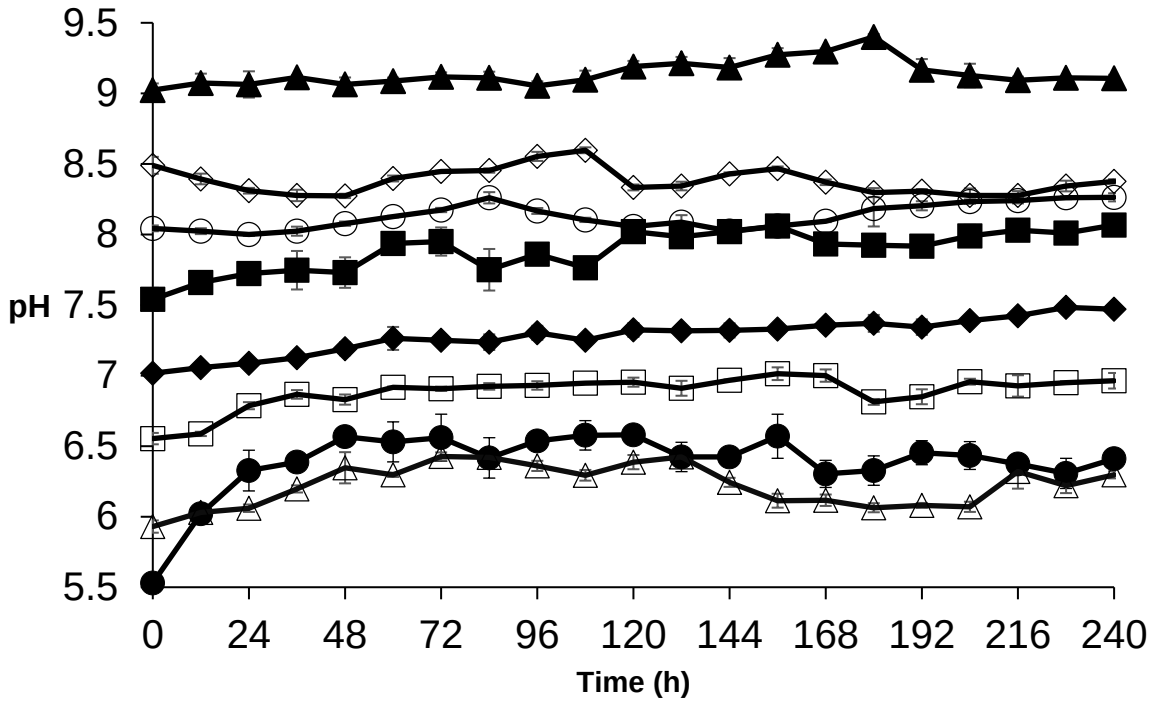

Fig. 2. pH of the cultures supplemented with DEHP during the fermentation process at pH of 5.5 (●), 6.0 (△), 6.5 (□), 7.0 (◆),7.5 (■), 8.0 (○), 8.5 (◇), 9.0 (▲).

3.2. Esterase activities and esterase yield parameters

In general, esterase production increased during the exponential phase of growth, reaching the greatest esterase activity at pH value of 5.5 after 204 h (Figure 3a). Among the basic pH tested, a pH value of 8.0 showed the highest esterase activity after 96 h (Fig. 3b). Little esterase activity was observed at pH values of 6.0, 7.0, 8.5 and 9.0 (Fig. 3). It was observed that the lowest esterase activity was shown at pH value of 8.5 and 9.0 at the end of the fermentation (Fig. 3b). E_{max}, $Y_{E/X}$, P and q_p were higher at pH values of 5.5 than in the rest the media tested (Table 2). However, E_{max}, $Y_{E/X}$, P and q_p show no statistically significant difference between pH values of 5.5 and 6.5 (Table 2).

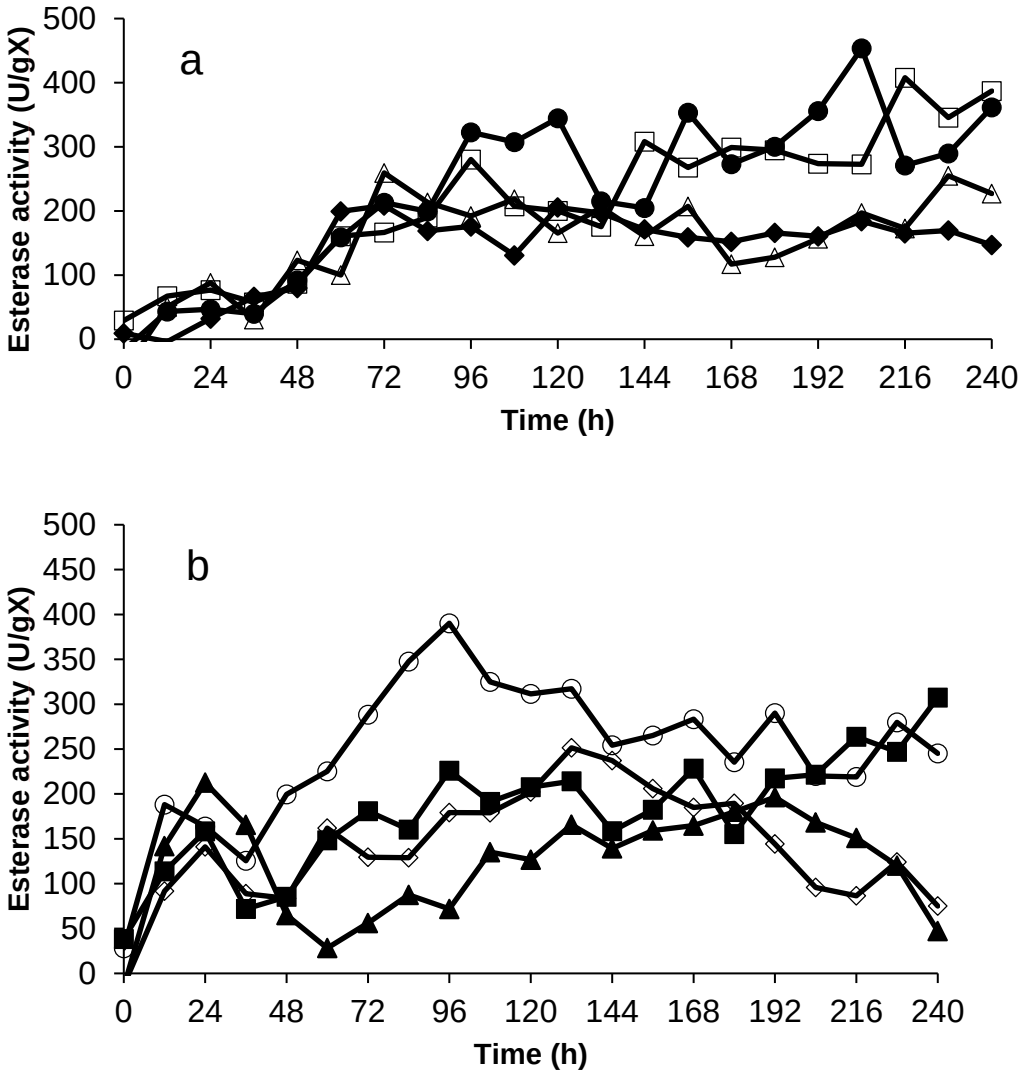

Fig. 3. Specific activity of esterase of *F. culmorum* grown at different pH values in medium added with DEHP in liquid fermentation. pH values of; **a)** 5.5 (●), 6.0 (Δ), 6.5 (□), 7.0 (♦), **b)** 7.5 (■), 8.0 (○), 8.5 (◊), 9.0 (▲).

Table 2. Enzymatic yield parameters of *F. culmorum* in media supplemented with DEHP at different pH values in submerged fermentation.

Parameters	pH of the culture media							
	5.5	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**
E_{max}**(U/L)**	448.4^{a} ±49	222.5^{c} ±35	429.8^{a} ±45	139.3^{d} ±22	293.5^{b} ±37	332.8^{b} ±38	216.2^{c} ±25	205.1^{c} ±24
$Y_{E/X}$**(U/gX)**	444^{a} ±68	241.8^{b} ±32	394.3^{a} ±41	181^{c} ±21	290.6^{b} ±34	268.4^{b} ±33	186.4^{c} ±23	169.5^{c} ±18
P_{RO}**(U/L*h)**	2.2^{a} ±0.002	1.0^{c} ±0.001	1.9^{a} ±0.001	1.2^{c} ±0.002	1.2^{c} ±0.001	1.7^{b} ±0.002	1.5^{b} ±0.001	1.1^{c} ±0.001
q_p**(U/gX*h)**	8.9^{a} ±0.006	4.4^{b} ±0.004	9.1^{a} ±0.006	1.8^{c} ±0.002	4.6^{b} ±0.003	4.6^{b} ±0.003	1.9^{c} ±0.001	2.0^{c} ±0.001

Values are expressed as mean ± SD (n=3); means within the same column not sharing common superscript letters (a-d) differ significantly at 5% level

4. DISCUSSION

Several studies have shown that the pH is as important factor in the fermentation process to enhance the enzyme production. Dinarvand *et al*. (2017) reported that the pH and temperature value of the culture medium were the most significant variables in the inulinase and invertase production. It was found that a pH value of 6.5 was the optimum for the production of such enzymes by *Aspergillus niger* (Dinarvand *et al*., 2017). In addition, Deng *et al*. (2020) found that an optimum pH value of 7.0 enhanced the β-galactosidase production in a *Lactobacillus leichmannii* batch culture. Similarly, the protease production by *Bacillus subtilis* increased at an optimum pH value of 7.0 (Abusham *et al*., 2009). In addition, Maan *et al*. (2016) reported that temperature, incubation period, and pH had significant influence on xylanase and cellulase production by *Coprinus cinerea* under solid state fermentation. It was found that pH strongly affected the chitosanase production by *Thrichoderma* strains, which presented optimum activity at pH values of 5.0 and 5.5 (da Silva *et al*., 2012). Saxena & Singh (2011) reported an optimum pH of 6.0 for a thermostable amylase production by *Bacillus* sp. in solid state fermentation. In the present study, it was observed that acidic pH levels (i.e. 5.0, 5.5. and 6.5) and a basic pH level (i.e. 8.0) enhanced the growth of *F. culmorum*. However, pH values of 5.5 and 6.5 were clearly most favorable for esterase production. It has been reported that esterase enzymes are involved in DEHP degradation (Pradeep *et al*., 2015; Ferrer-Parra *et al*., 2018; Fan *et al*., 2018; González-Márquez *et al*., 2019). González-Márquez *et al*. (2019) reported that *F. culmorum* produced eight esterase isoforms in medium supplemented with DEHP as sole carbon source. It was suggested that five enzymes (25.7, 29.5, 31.8, 97.6

and 144.5 kDa) were involved in the primary biodegradation of DEHP and the rest of them (45.9, 66.6 and 202.9 kDa) might be involved in the final steps for DEHP metabolism. Zhang *et al*. (2020) found that the optimal initial pH for DEHP degradation by *Gordonia terrae* was 6.0. The strain showed good performance under acidic conditions, with a degradation percentage above 80.0% when the initial pH was 5.0, while the degrading ability was not very good under alkaline conditions (pH, 9.0). Furthermore, a halotolerant bacterial consortium (*Gordonia* sp., *Rhodococcus* sp. and *Achromobacter* sp.) was capable of degrading DEHP (1000 mg/l) at an optimal pH of 6.0 (Li *et al*., 2018). In addition, Surhio *et al*. (2017) reported an optimum pH of 7.0 for degradation of several phthalates by *Bacillus thuringiensis*. It has been demonstrated that pH is crucial to fully metabolized phthalates. Further studies on other variables affecting the DEHP biodegradation process such as temperature should be carried out in order to increase our knowledge on the optimal condition for enzyme production by this fungus.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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