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ORIGINAL RESEARCH

Growth and esterase activity of *Fusarium culmorum* grown in di(2-ethyl hexyl) phthalate in liquid fermentation

Crecimiento y actividad de esterasas de *Fusarium culmorum* crecido en di(2-etil hexil) ftalato en fermentación liquida

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ABSTRACT

Di (2-ethyl hexyl) phthalate (DEHP) is a plasticizer present in various products, mainly those made with PVC. This phthalate has attracted attention due to its important participation in the contamination of the environment. It has been reported as an endocrine-disrupting compound in mammals. *Fusarium culmorum* is a phytopathogenic fungus able to degrade DEHP, because it produces esterases, which are enzymes capable to break down ester bonds present in the structure of phthalates. In this research, growth, protein content and esterases activity by biochemical tests and polyacrylamide gel electrophoresis were characterized for *F. culmorum grown* in DEHP-supplemented (100 and 1500 mg/L) media as the only carbon source in liquid fermentation. *F. culmorum* showed higher biomass production and esterase activity in medium supplemented with 1500 mg of

DEHP/L. Zymography revealed that bands with esterase activity were observed after 24 h and 48 h in media supplemented with 1500 and 100 mg of DEHP/L, respectively. It was shown that DEHP is an inducer of esterases and that this compound was used as carbon and energy sources by *F. culmorum*. This fungus can secrete specific esterase to breakdown high concentrations of DEHP, being a promising organism for bioremediation of DEHP-polluted environments in both aquatic and terrestrial ecosystems.

Keywords: di(2-ethyl hexyl) phthalate, esterase, *Fusarium culmorum,* liquid fermentation.

RESUMEN

El di (2-etil hexil) ftalato (DEHP) es un plastificante presente en varios productos, principalmente en los fabricados con PVC. Este ftalato ha llamado la atención debido a su importante participación en la contaminación del medio ambiente. Se ha reportado como un compuesto que altera el sistema endocrino en los mamíferos. *Fusarium culmorum* es un hongo fitopatógeno capaz de degradar el DEHP, ya que produce esterasas, que son enzimas capaces de descomponer los enlaces éster presentes en la estructura de los ftalatos. En esta investigación, el crecimiento y el contenido de proteína fueron evaluadas en *F. culmorum*. La actividad de esterasa en este hongo también fue determinada empleando pruebas bioquímicas y electroforesis en gel de poliacrilamida. Los estudios antes mencionados fueron realizados en medios suplementados con DEHP (100 y 1500 mg/L) como la única fuente de carbono en fermentación líquida. *F. culmorum* mostró una mayor producción de biomasa y actividad de esterasa en medio suplementado con 1500 mg de DEHP/L. La zimografía mostró bandas con actividad esterasa después de 24 horas y 48 horas en medios adicionados con 1500 y 100 mg de DEHP/L, respectivamente. Se demostró que el DEHP es un inductor de esterasas y que este compuesto fue utilizado como fuente de carbono y energía por *F. culmorum*. Este hongo puede producir esterasa para degradar altas concentraciones de DEHP, siendo un organismo prometedor para la biorremediación de ambientes contaminados con este ftalato en ecosistemas acuáticos y terrestres.

Palabras clave: di(2-etil hexil) ftalato, esterasa, Fermentación líquida, *Fusarium culmorum.*

1. INTRODUCTION

Di (2-ethylhexyl) phthalate (DEHP) is an additive used to make plastic more flexible. It is widely used as a plasticizer for polyvinyl chloride (PVC) products, which has applications such as building materials, food packaging, cosmetics and medical devices (Peropadre *et al*., 2015). DEHP is an environmental pollutant.

DEHP can be found in the environment as a result of the slow release of phthalates of plastics and other materials containing phthalates due to the weather. Accumulation in natural water causes its wide distribution within aqueous systems such as rivers, lakes and groundwater, as well as in the ecological environment. Phthalates do not bind chemically to polymer matrices and can easily disperse into the environment during production and use, and after removal (Daiem *et al*., 2012).

Exposure to chemicals such as phthalates can occur through a variety of sources, such as food, air, dust, and use of personal care products, phthalates end up in the human body through ingestion, inhalation or dermal absorption. Particularly, DEHP is considerate an endocrine-disrupting compound in mammals (Wittassek *et al*., 2010).

Different microorganisms have been used to study due to their capacity to produce enzymes able to degrade degrading phthalates. It has been reported that some species of bacteria (Zhao *et al*., 2016; Yang et al., 2018) and different species of fungi (Pradeep & Benjamin, 2012; Aguilar-Alvarado *et al*., 2015; Ahuactzin-Pérez *et al*., 2018) have been able to degrade these compounds. Filamentous fungi, such as *Fusarium* have been widely studied (Kim *et al*., 2003). *Fusarium culmorum* is a phytopathogenic fungus able to produce esterases in the presence of high concentrations of DEHP (Ahuactzin-Pérez *et al.,* 2016). In this research, growth, esterase activity, and molecular weight of esterase of *Fusarium culmorum* were evaluated in media added with DEHP (100 and 1500 mg/L) in liquid fermentation.

2. MATERIALS Y METHODS

2.1. Microorganism

The strain of *Fusarium Culmorum* used belongs to the culture collection of the Research Centre for Biological Sciences (CICB) at Universidad Autónoma de Tlaxcala. This strain was isolated from the mixture of waste from a paper recycling industry, where DEHP is used as an adhesive in paper envelopes and paper emulsifiers (Aguilar-Alvarado *et al.,* 2015). This strain grew in malt extract agar at 25 °c and was preserved at 4 ° C.

2.2. Culture media

Two culture media were prepared containing DEHP (100 and 1500 mg/L) and a mineral medium. The composition of the mineral 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 to 6.5 using either 0.1 M HCl or 0.1 M NaOH. In each flask, 50 ml of culture medium were placed and sterilized at 120 ° C for 25 minutes, cooled and then inoculated with approximately 10^7 spores. The cultures were incubated at 25 °C for 7 days in a rotary incubator at 120 rpm.

2.3. Biomass production and pH measurement

Biomass and supernatant were separated by filtration of the culrures, and the biomass was determined by difference in dry weight between the filter paper and the biomass. The pH of the supernatant was measured using a digital potentiometer. The samples were taken each 112 hours during 7 days and the studies were performed in triplicate.

2.4. Protein production

Protein production was quantified in the supernatant of the samples using the method of Bradford (1976). In a test tube, 700 µL of distilled water, 200 µL of Bradford reagent and 100 µL of the supernatant were added. The total protein was evaluated using a spectrophotometer at 595 nm; the tests were done in triplicate.

2.5. Enzymatic activity

Specific esterase activity was evaluated in the supernatant. A substrate was used, containing; 1.108 mL of acetonitrile, 19.5 µL of p-nitrophenyl butyrate (PNPB), 11,08 mL distilled water, 4.44 µL Triton X-100 and was diluted with phosphate buffer (PH 7.5) in order to obtain 100 mL of substrate. A test tube containing 900 µL of substrate and 100 µL of the supernatant was used. The enzymatic activity was evaluated using a spectrophotometer at 405 nm. The studies were performed in triplicate.

2.6. Zymographic

Electrophoresis was carried out at 120 volts for 4 hours, using an 18% separation polyacrylamide gel and a 4% packing gel. ProteinTM Dual Precision Xtra Plus Standards was used as a molecular marker. The gels were incubated for 3 h in a solution containing 3 mM of α-naphthyl acetate, 1 mM of fast red and phosphate buffer 100 mM (pH 7.5). Esterase activity was detected as bands marked in the gels of a reddish color.

3. RESULTS

3.1. Biomass production

Biomass production by *Fusarium Culmorum* is shown in Fig. 1. The highest biomass production was obtained in the media supplemented with 1500 mg DEHP/L. The highest biomass production was observed at 168 h in both media.

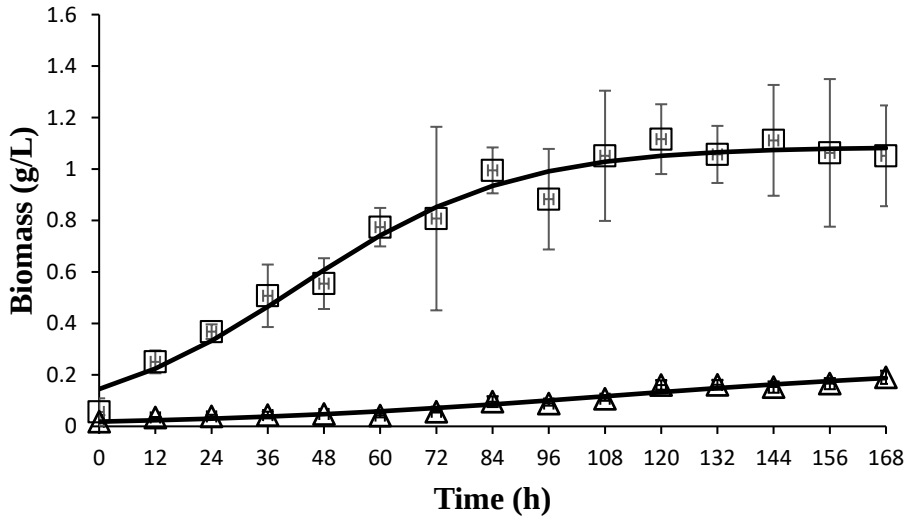

Fig. 1. Biomass production by *Fusarium culmorum* grown on media supplemented with 100 (Δ) and 1500 (□) mg of DEHP/L in liquid fermentation.

3.2. Protein production

Fig. 2 shows the protein content of *Fusarium culmorum.* Ian increased in protein content was observed in the media supplemented with 1500 mg DEHP/L after 72 h, with a maximum of 0.00126 mg/mL after 48 h. Higher protein content was observed after 84 h in that media supplemented with 100 mg of DEHP/L, showing a maximum of 0.001 mg/mL after 108 h.

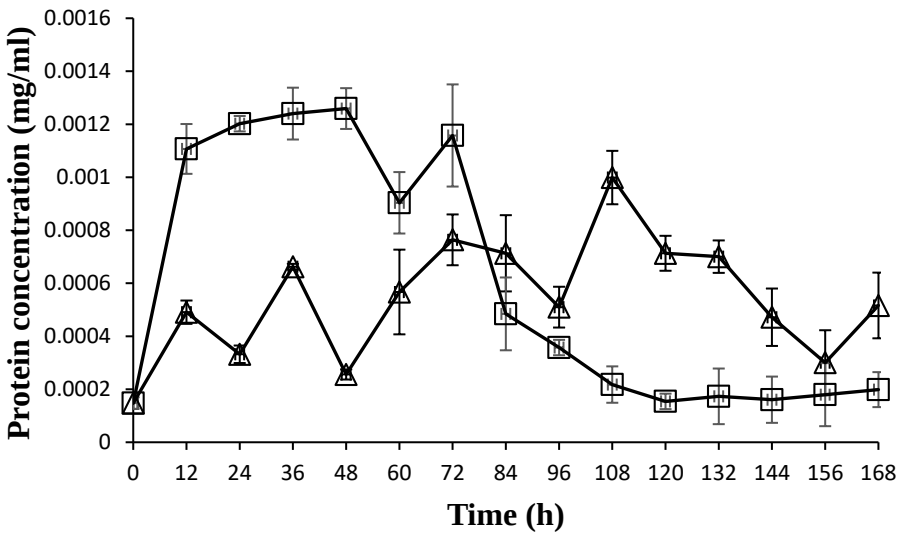

Fig. 2. Protein production by *Fusarium culmorum* grown in media supplemented with 100 (Δ) and 1500 (□) mg of DEHP/L in liquid fermentation.

3.3 Esterase activity and zymography

Esterase activity was very similar in both media after 96 h of incubation (Fig. 3). An increase in esterase activity was observed in the media supplemented with 100 mg DEHP/L after 108 h. Enzymatic activity was also observed in the zymograms. In both zymograms (Fig. 4 and 5) no bands were observed during the first 48h. Intensity of the bands was similar in both zymograms after 60 h. A slight increase in the intensity of the bands was observed after 108 h in the zymogram of 100 mg of DEHP/L after 108 h. Esterases showed an approximate molecular weight of 16, 18, 20, 23, 25, 50, 70, 75, 150 kDa.

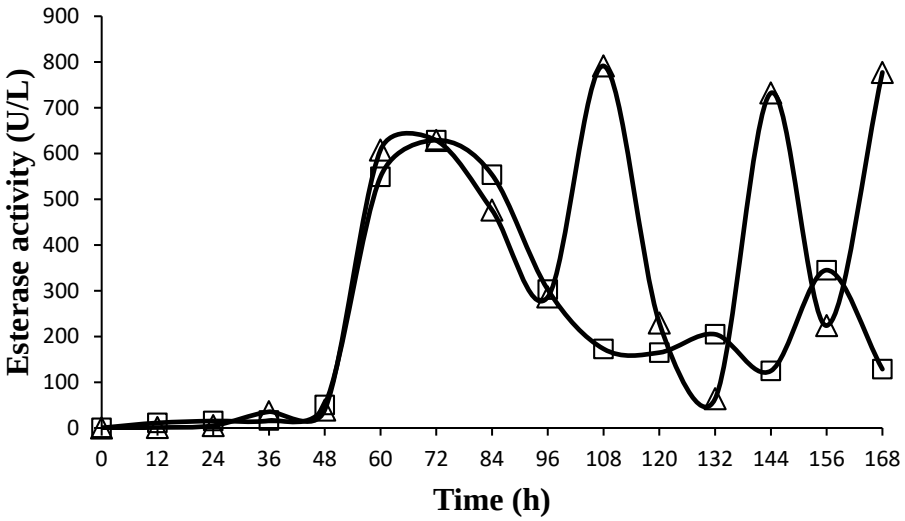

Fig. 3. Esterase activity of *Fusarium culmorum* grown on media supplemented with 100 (Δ) and 1500 (□) mg of DEHP/L in liquid fermentation.

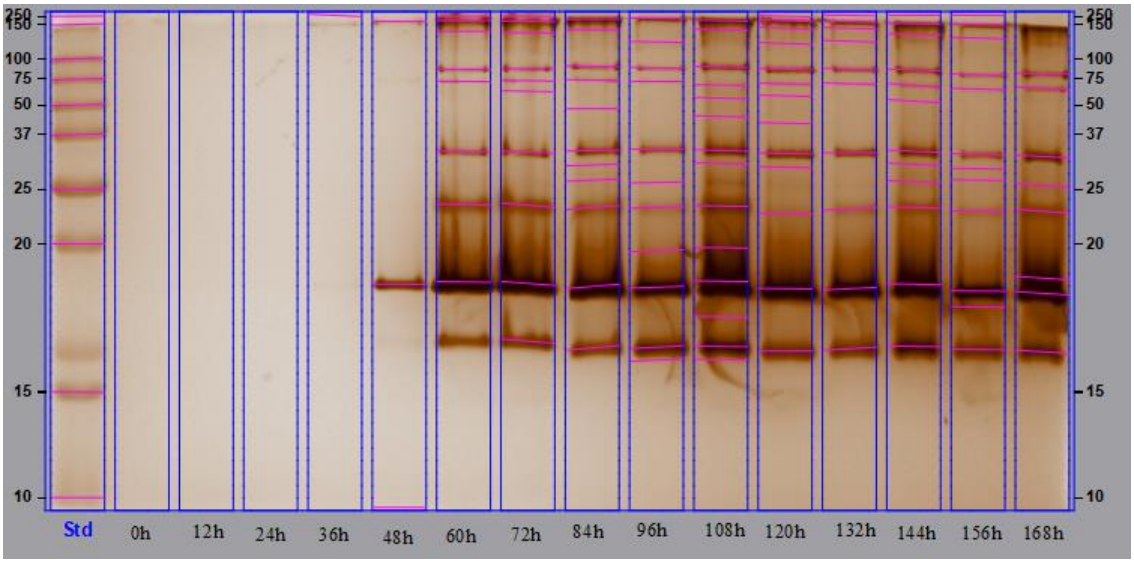

Fig. 4. Zymogram of *Fusarium culmorum* grown in media supplemented with 100 mg of DEHP/L in liquid fermentation.

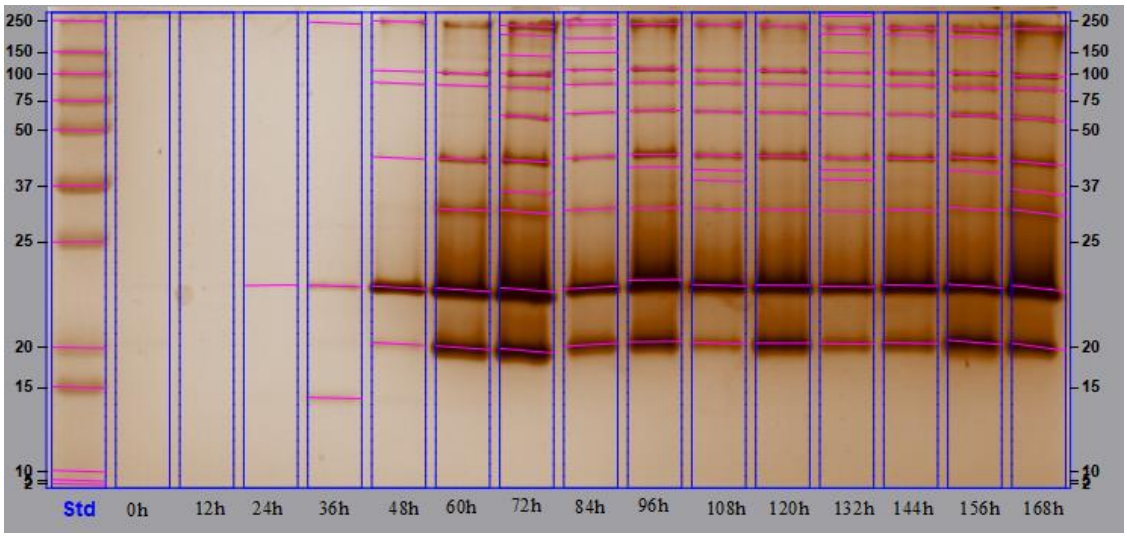

Fig. 5. Zymogram of *Fusarium culmorum* grown in media supplemented with 1500 mg of DEHP/L.

4. DISCUSSION

In the present work, two media were tested with different concentrations of DEHP (100 and 1500 mg/L). *F. culmorum* showed a higher biomass production in the medium supplemented with 1500 mg of DEHP/L. Aguilar-Alvarado *et al*. (2015) also reported that the greatest biomass production by *F. culmorum* was observed in the media supplemented with 1500 mg DEHP/L. Ahuatzin-Pérez *et al*. (2016) reported that *F. culmorum* had the maximum biomass production media supplemented with 1000 mg of DEHP/L, showing that this fungus used DEHP as a carbon source. In the present research, no stationary phase is observed in that medium added with 100 of DEHP/L, however, the stationary phase is observed after approx. 96 h in the medium supplemented with 1500 mg of DEHP/L. Cordoba-Sosa *et al*. (2014) studied DEHP biodegradation (750, 1200 and 1500 mg of DEHP/L) by *P. ostreatus* and found that the highest esterase activity was observed in the highest DEHP concentration tested (1500 mg/L). The maximum value of esterase activity in the media supplemented with 1500 mg DEHP/L was 629.95 U/L, being higher than that showed in previous studies (Ferrer-Parra *et al*., 2018). Ferrer-Parra *et al*. (2018) reported that *F. culmorum* had a esterase activity of 242 U/L and 350 U/L in media supplemented with 1500 mg and 2000 mg of DEHP/L, respectively. In the present research, the molecular weight of the bands of esterase were observed in similar range (approximately 16 to 150 kDa) to those reported by Ferrer-Parra *et al*. (2018). These studies showed that DEHP is an inducer of esterases and that this compound is used as carbon and energy sources by *F. culmorum*. This fungus can secrete specific esterase to breakdown

high concentrations of DEHP, being a promising organism for bioremediation of DEHP-polluted environments in both aquatic and terrestrial ecosystems.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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