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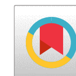

Partial characterization of esterases from *Fusarium culmorum* grown in media supplemented with di (2-ethyl hexyl phthalate) in solid-state and submerged fermentation

Caracterización parcial de esterasas de *Fusarium culmorum* crecido en presencia de di(2-etil hexil ftalato) en fermentación sólida y sumergida

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ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is a plasticizer used in the polyvinyl chloride (PVC) industry. The indiscriminate use of various products manufactured with PVC, causes this plasticizer to be considered a contaminant. *Fusarium culmorum* is a phytopathogenic fungus that has the ability to produce esterase enzymes. Esterases are of great importance because they can break the ester bonds present in the plasticizers. In this work, the activity of esterases produced by *F. culmorum* grown in media supplemented with different concentrations of DEHP (1500 and 2000 mg/L) in solid-state fermentation and submerged fermentation was characterized by biochemical tests and polyacrylamide gel electrophoresis. *F. culmorum* showed higher esterase activity in media supplemented with 1500 and 2000 mg DEHP/L in solid-state fermentation. A greater number of esterase activity bands were observed in the DEHP-supplemented media, having a molecular weight of about 20, 25, 37, 45, 55, 75 and 150 kDa, in both fermentation systems. 1500 mg of DEHP/L induced a higher production of esterases, demonstrating that high concentrations of DEHP did not inhibit the enzymatic production of the fungus.

Keywords: *F. culmorum*, di (2-ethyl hexyl) phthalate, esterases, submerged fermentation, solid fermentation.

RESUMEN

Di(2-etilhexil) ftalato (DEHF) es un plastificante utilizado en la industria del policloruro de vinilo (PVC). El uso indiscriminado de los diversos productos elaborados con PVC, ocasiona que éste plastificante sea considerado un contaminante. *Fusarium culmorum* es un hongo fitopatógeno que tiene la capacidad de producir enzimas esterasas. Las esterasas son de gran importancia debido a que pueden romper los enlaces ésteres presentes en los plastificantes. En este trabajo se caracterizó parcialmente la actividad de esterasas producidas por *F. culmorum* crecido en presencia de diferentes concentraciones de DEHF (1500 y 2000 mg/L) en fermentación sólida y fermentación sumergida, a través de ensayos bioquímicos y electroforesis en gel de poliacrilamida. *F. culmorum* presentó mayor actividad de esterasas en los medios con 1500 y 2000 mg de DEHF/L en fermentación sólida. Se observaron mayor número de bandas de actividad de esterasas en los medios adicionados con DEHF, de un peso molecular aproximado de 20, 25, 37, 45, 75 y 140 kDa, en ambos sistemas de fermentación. 1500 mg de DEHP/L indujó una mayor producción de esterasas, demostrando que concentraciones elevadas de DEHF no inhiben la producción enzimática del hongo.

Palabras clave: di (2-etil hexil) ftalato, esterasas, *F. culmorum*, fermentación sumergida, fermentación sólida.

1. INTRODUCTION

Di (2-ethylhexyl) phthalate (DEHP) is a chemical compound that is added as an additive in order to make plastics more flexible. This compound is present in various plastic products, especially in manufactured materials that are made of polyvinyl chloride, such as blood bags, food packages, and toys, which can contain up to 40% of DEHP. However, these products usually contain lower levels than this. DEHP chemical base is the dicarboxylic benzene acid with two lateral chains that can be alkyl, benzyl, phenyl, cicloalkyl or alcoxi groups (Daiem *et al*., 2012; Gao & Wen, 2015).

The widespread use of the plasticizer in various industries is the reason why it is released into the environment (Aguilar-Alvarado *et al.*, 2015). The DEHP does not form part of the molecular chain that constitutes the plastic, being liberated from plastic matrix to the environment. In this way, it can be released into the environment and to cause environmental pollution (Meng, 2015).

It has been reported that the filamentous fungi *Fusarium soliani, f. sp. Pisi, Pseidozymajejuensis sp. Nov, Aspergillus oryzae* have the capacity to produce cutinases enzymes that hydrolyze the soluble esters (Castro-Ochoa *et al*., 2010; Adame-García *et al*., 2011). In the case of the phytopathogenic fungi, this mechanism helps the fungus to degrade the cutinase from the cellular wall of plants and use the nutrients from the structure of the cellular wall, such as hemicellulose and pectin for its growth. The ability of the organisms to secrete enzymes makes them the most useful tool in nature for degrading complex compounds. Therefore, it is important to find organisms with the ability to produce esterases that can be employed for the degradation of phthalates, through the rupture of the ester bonds present in these compounds.

Aguilar-Alvarado *et al.* (2015) isolated the fungus *Fusarium culmorum* from a paper recycling industry and found that it was able to degrade high concentrations of DEHP. *F. culmorum* is a phytopathogenic fungus that has been studied for its capacity to produce esterases enzymes and to degrade DEHP.

In this work, the esterases enzymatic activity produced by *F. culmorum* grown in presence of different concentrations of DEHP (1500 and 2000 mg/L) in solid-state fermentation and submerged fermentation was evaluated. The molecular weight of the enzymes was also determined using polyacrylamide electrophoresis gel.

2. MATERIAL AND METHODS

2.1. Organism

F. culmorum from the Research Centre for Biological Sciences (CICB) culture collection at Universidad Autónoma de Tlaxcala (Tlaxcala, Mexico) was used. The strain grew in Czapek medium at 25°C and was preserved in refrigeration at 4°C.

2.2. Submerged fermentation and solid-state fermentation

Three culture media were carried out as follows; 1) 10 g glucose/L, 2) 1500 mg DEHP/L and 3) 2000 mg DEHP/L. Each culture medium contained (in g/L): $NaNO_3$, 3; KCl, 0.5; K_2HPO_4, 1.0; $FeSO_4$, 0.01. Media supplemented with DEHP were added with 400 µL of Tween 80 per liter. The pH of the medium was adjusted to 7.5, employing either NaOH 0.1M or HCl 0.1N. Flasks of 150 mL containing culture medium (50 mL) were autoclaved at 120° C for 15 minutes and then used for submerged fermentation experiments. Flasks of 250 mL containing 0.5 g of polyurethane foam (PUF) cut in cubes (0.5mm³) as inert support soaked in 15 mL of sterile culture medium were used for solid-state fermentation studies. The cubes were previously washed twice with distilled water and then placed in NaOH and HCl solutions (0.1N), for 24 hours in each solution, dried in oven at 40°C and autoclaved at 120°C for 15 minutes. In all the cases, the flasks were inoculated with three mycelium fragments of 10 mm diameter taken from the periphery of the colonies grown for 7 days. Flask were incubated during 8 days at 25°C. For submerged fermentation studies, flask were incubated at 120 rpm. In all cases, the samples were taken each 12 hours during 8 days and the studies were performed in triplicate.

2.3. Esterases Assay

In all the cultures, esterases activity was assessed in the supernatant obtained from the filtration of the samples. 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 100 µL of supernatant and 900 µL of reagent prepared with the following components: 1.76% (v/v) *p*NPB, 1.11% (v/v) acetonitrile, 0.04% (v/v) Triton X-100 and 11.11% (v/v) distilled water, dissolved in 0.01 M phosphate buffer at pH 7.5 and was incubated at 37 ºC for 5 min. 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 (Ahuactzin-Pérez *et al*., 2016). The enzymatic activities were expressed in U/L.

2.4. 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}). Enzymatic productivity ($P = E_{max}$/time) was evaluated at the time when the enzymatic activity was maximal (Ahuactzin-Pérez *et al*., 2016).

2.5. Zymographic analysis

Zymography is an electrophoretic method for measuring proteolytic activity. The polypeptide profiles of the samples with esterase activity were analyzed using 0.1% polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970). 12% and 4% acrylamide gels were used as separation and packaging gels, respectively. ProteinTM Dual Precision Xtra Plus Standards (Bio-Rad) was used as molecular marker. Samples were tested on gels of 0.75 mm in a Mini Protean electrophoresis system Tetra Cell (Bio-Rad) at 100 volts for 1.30 h. Subsequently, gels were incubated for 3 hours at room temperature in a solution containing 3 mM of α-naphthyl acetate, 1 mM Fast Red TR (Sigma) and phosphate buffer 100 mM at pH 7.5 (Karpushova *et al*., 2005). Finally, esterase activity was detected by the appearance of red-colored bands in the gels.

2.6. Statistical analysis

All experiments were carried out in triplicate. Data was evaluated using one-way ANOVA and Tukey post-test using Sigma Plot Version 12.0 (Systat Software Inc.).

3. RESULTS

3.1. Esterases activity in cultures grown in submerged fermentation

Figure 1 shows esterases enzymatic activity of *F. culmorum* grown in submerged fermentation. The media added with DEHP showed more esterases activity compared to the control medium (added with glucose). The greatest esterases enzymatic activity (350.8 U/L) at 180 hours was observed when medium was supplemented with 2000 mg of DEHP/L. Table 1 shows that *F. culmorum* presented the greatest $Y_{E/X}$ and E_{max} in the medium containing 2000 mg of DEHP/L compared to the rest of the media tested. *F. culmorum* showed the greatest *P* in the media containing DEHP.

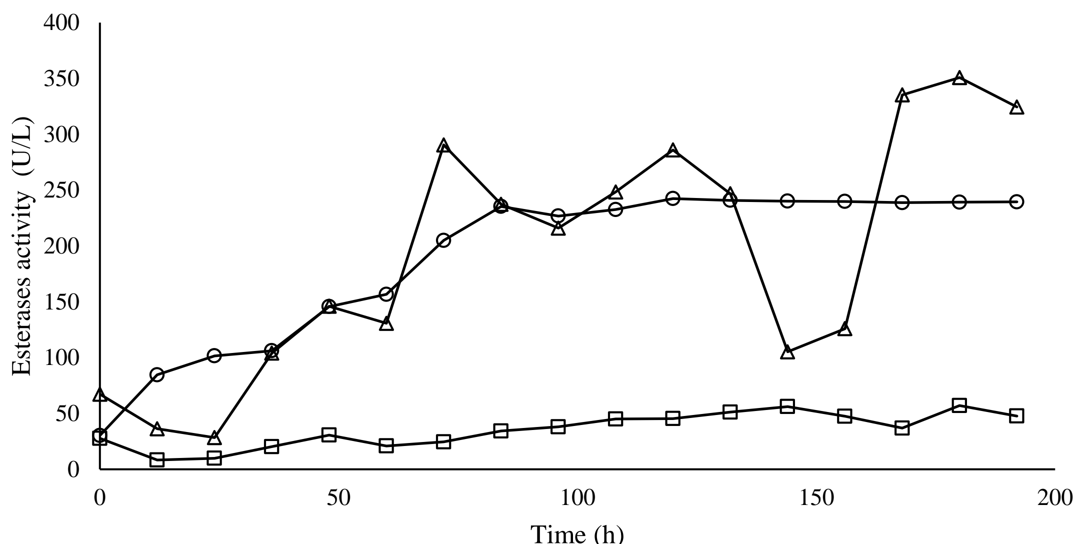

Fig. 1. Esterase activity of *F. culmorum* grown in glucose (□), 1500 (○) and 2000 (Δ) mg of DEHP/L in submerged fermentation.

Table 1. Enzymatic yield parameters of esterase of *F. culmorum* grown in media supplemented with glucose and different concentrations of DEHP in submerged fermentation.

	Culture media		
		DEHP (mg/L)	
Enzymatic yield parameters	Glucose	1500	2000
$Y_{E/X}$ (U/gX)	27.0 ± 0.4^{c*}	67.5 ± 0.8^{b}	168.6 ± 1.12^{a}
E_{max} (U/L)	57.1 ± 0.6^{b}	242.4 ± 1.15^{b}	350.8 ± 4.2^{a}
***P* (U/L/h)**	0.3 ± 0.001^{b}	2.0 ± 0.005^{a}	1.9 ± 0.003^{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.

3.2. Esterases activity in cultures grown in solid-state fermentation

The DEHP supplemented media showed greater enzymatic activity compared to the control medium (Fig. 2). The medium containing 1500 mg DEHP/L showed an activity of 448.1 U/L and the medium containing 2000 mg/L showed esterases enzymatic activity of 420.2 U/L. Table 2 shows that *F. culmorum* presented greater E_{max} y P in the media containing DEHP than the control medium. The greatest $Y_{E/X}$ was observed in the medium containing 1500 mg DEHP/L.

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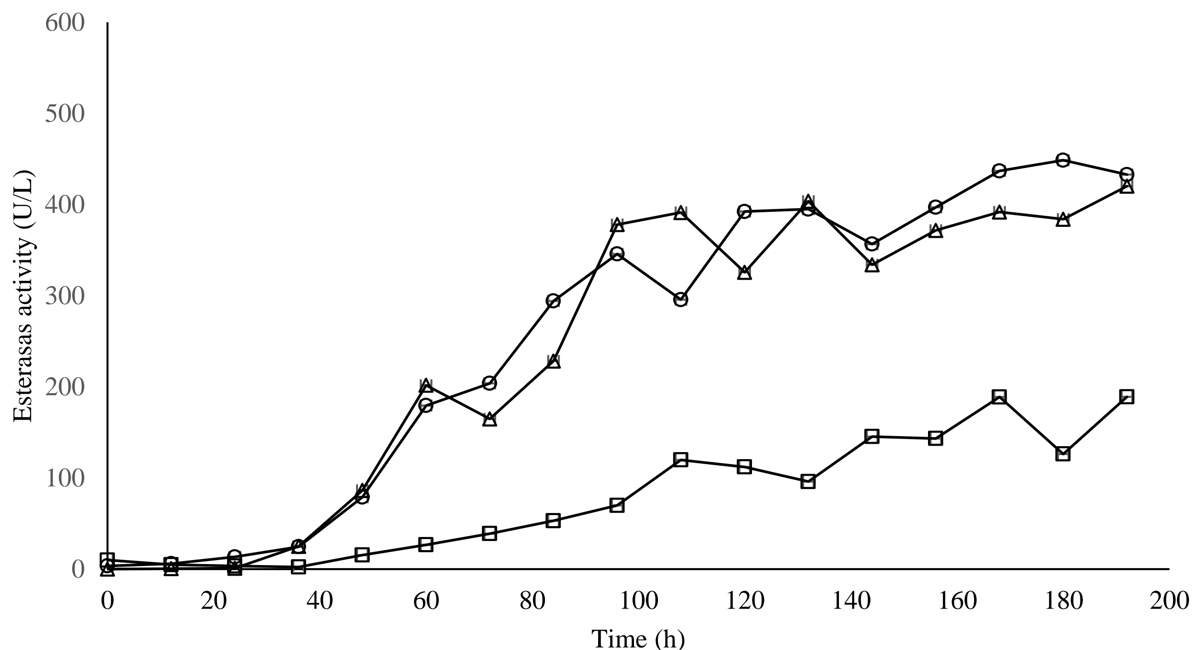

Fig. 2. Esterase activity of *F. culmorum* grown in Glucose (□), 1500 (○) and 2000 (Δ) mg DEHP/L in solid-state fermentation.

Table 2. Enzymatic yield parameters of esterase of *F. culmorum* grown in media supplemented with glucose and different concentrations of DEHP in solid-state fermentation

Enzymatic yield parameters	**Culture media**		
		DEHP (mg/L)	
	Glucose	1500	2000
$Y_{E/X}$ (U/gX)	$36.4 \pm 0.5^{c*}$	50.3 ± 0.7^{a}	42.8 ± 0.6^{b}
E_{max} (U/L)	189.0 ± 1.0^{b}	448.6 ± 2.9^{a}	420.1 ± 2.9^{a}
***P* (U/L/h)**	1.0 ± 0.001^{b}	2.5 ± 0.005^{a}	2.2 ± 0.004^{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.

3.3. Zymographic analysis in cultures grown in submerged fermentation

Figure 3 shows the zymogram of the medium that contained glucose as carbon source. A band with esterase activity was observed after 12 h with a molecular weight of 45 kDa approximately, which was observed during the fermentation. Bands with esterases activity were observed at 12, 84 and 192 h, showing a molecular weight of 37, 45 and 50 kDa, respectively.

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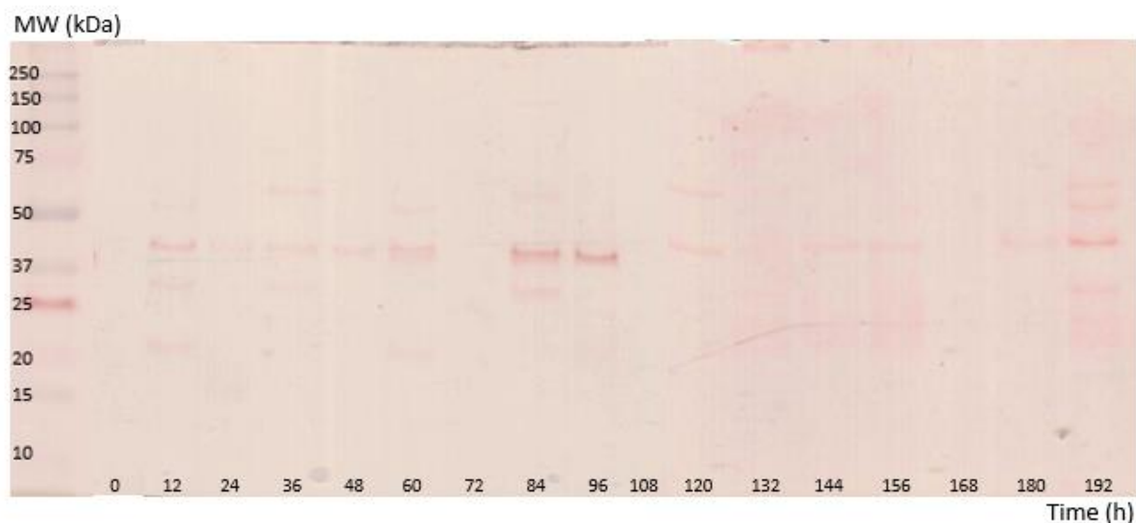

Fig. 3. Esterases zymogram of *F. culmorum* grown in medium containing glucose in submerged fermentation.

The esterases zymogram of the medium containing 1500 mg of DEHP/L (Fig. 4) showed 7 bands after 24 h with a molecular weight of approximately 20, 23, 25, 37, 45, 75, and 140 kDa, which was observed during the fermentation period. After 120 h increased the intensity of the bands with esterase activity

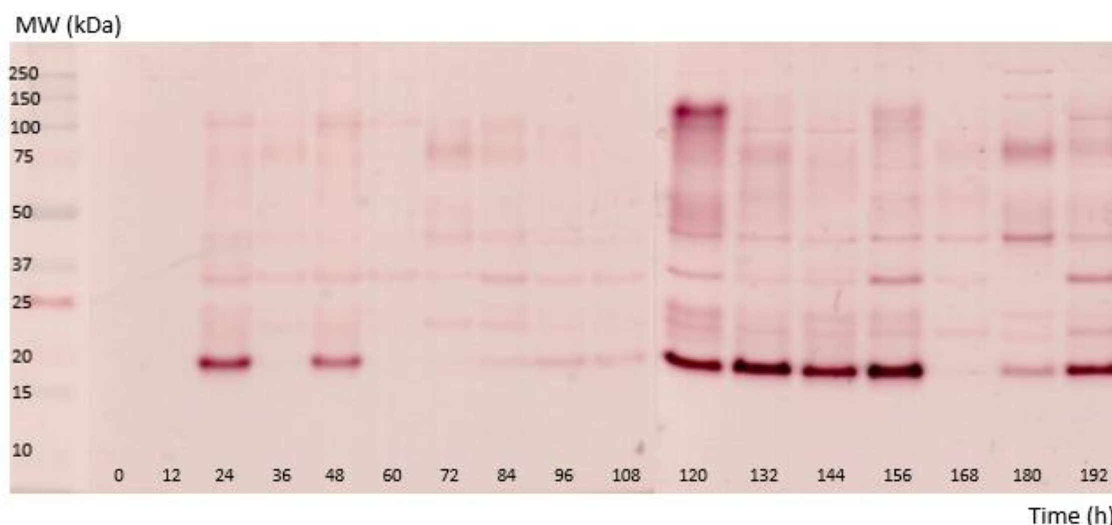

Fig. 4. Esterases zymogram of *F. culmorum* grown in medium supplemented with 1500 mg of DEHP/L in submerged fermentation.

Figure 5 shows an esterases zymogram of *F. culmorum* grown in medium containing 2000 mg of DEHP/L. A band with esterases activity of 45 kDa approx. was observed after 24 h. This band activity was showed during the fermentation period. Three bands of the growth

activity were observed at different molecular weights (23, 37, 45, 70, kDa approximately) after 36 h. The zymogram showed bands of 23, 45 and 150 kDa approximately between 108 h and 192 h.

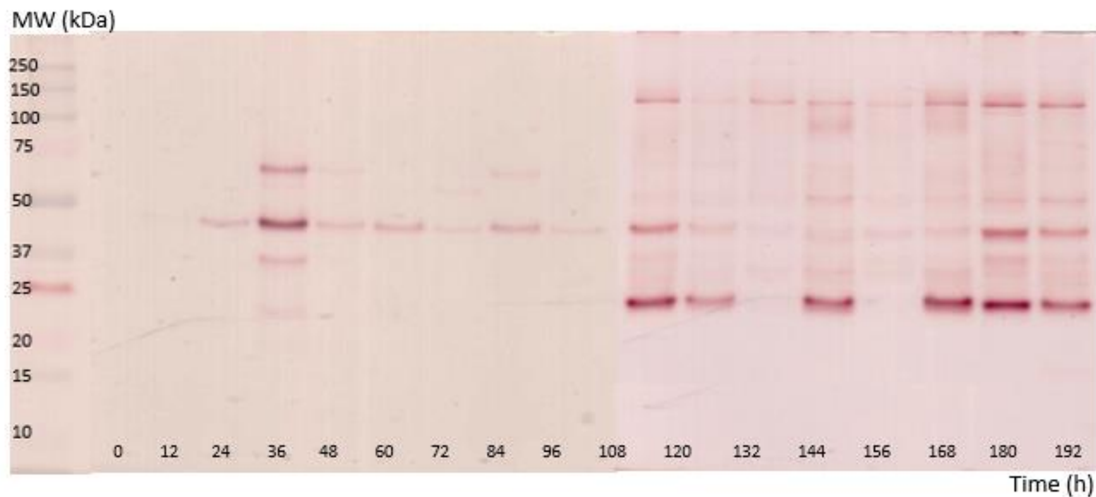

Fig. 5. Esterases zymogram of *F. culmorum* grown in medium supplemented with 2000 mg of DEHP/L in submerged fermentation.

3.4. Zymography analysis in cultures grown in solid-state fermentation

Figure 6 shows the zymogram with esterase activity of *F. culmorum* grown in a medium containing glucose as a carbon source. A band with esterase activity was observed with a molecular weight of approximately 55kDa after 24, 60, 108, 120, 144, 180 and 182 h of incubation. Two esterase activity bands were observed with a molecular weight of 150 kDa approx. between 60 and 72 h of incubation. The greatest activity of esterases was showed at 108 h of incubation.

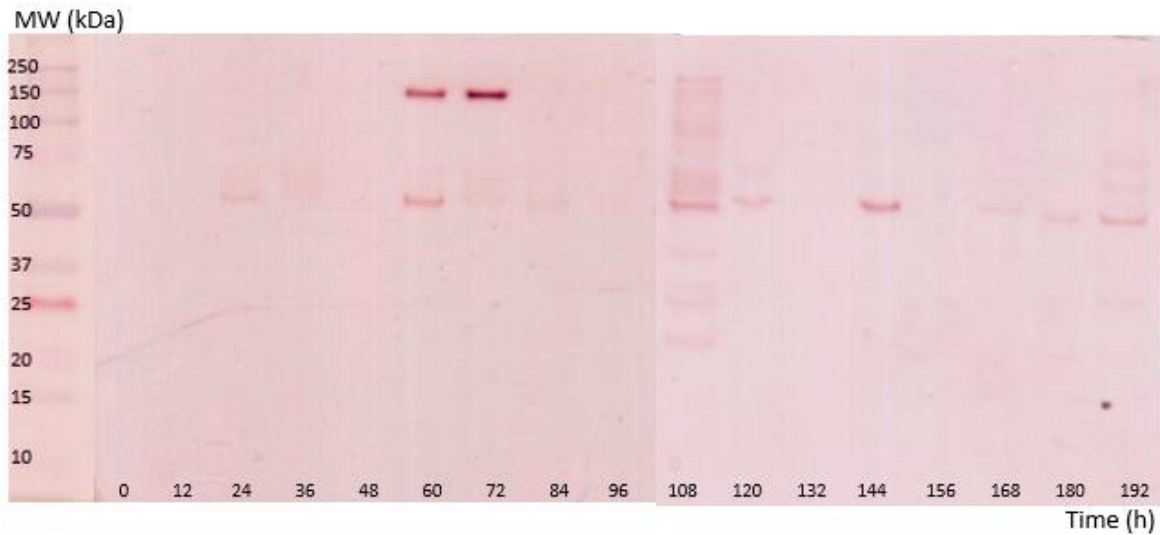

Fig. 6. Esterases zymogram of *F. culmorum* grown in medium containing glucose in solid-state fermentation.

The esterases zymogram of *F. culmorum* grown in medium supplemented with 1500 mg of DEHP/L showed 2 bands with esterase activity with a molecular weight of approximately 45 and 70 kDa, respectively, between 60 h and 156 h of incubation (Fig. 7). Four different bands were observed with different molecular weights (20, 25, 45 y 55 kDa approximately) after 60 h of incubation. A band with esterase activity was observed at 156 h with a molecular weight of 150kDa approximately.

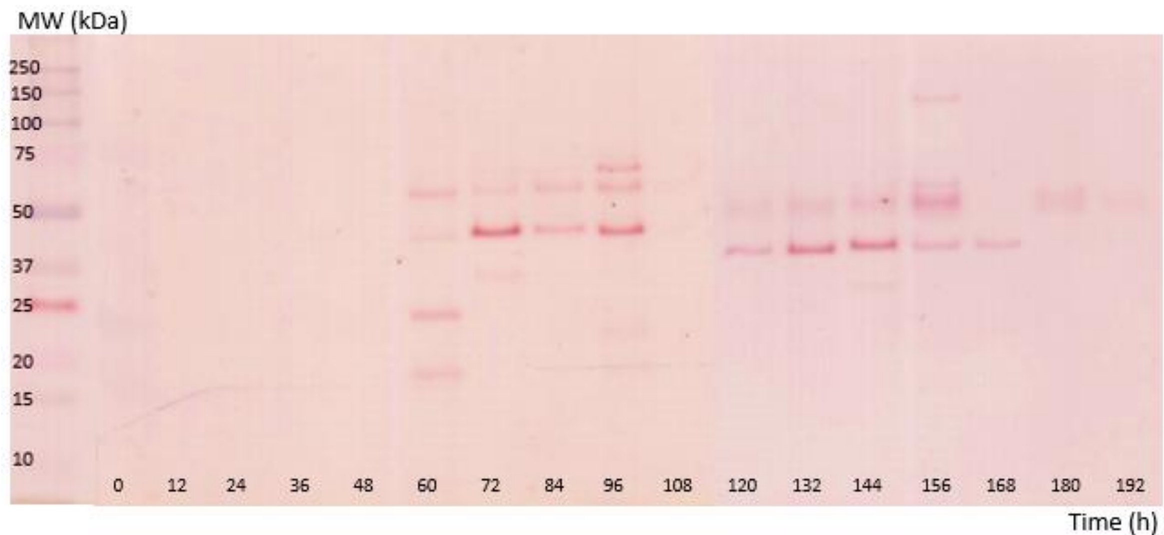

Fig. 7. Esterases zymogram of *F. culmorum* grown in medium supplemented with 1500 mg of DEHP/L in solid-state fermentation.

Figure 8 shows esterases zymogram of *F. culmorum* grown in medium supplemented with 2000 mg of DEHP/L. 8 bands were observed with esterase activity (20, 23, 25, 37, 45, 55, 75, 100 kDa approximately) between 12 and 192 h of incubation. A band of 250 kDa approximately was observed after 36, 48, 72, 120, 156 and 168 h of incubation.

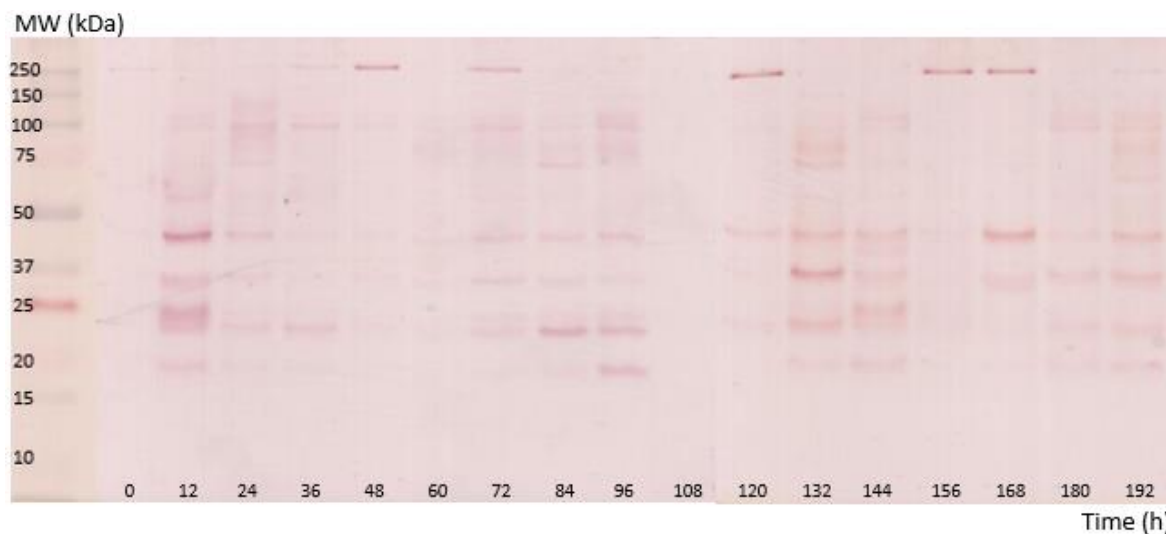

Fig. 8. Esterases zymogram of *F. culmorum* grown in medium supplemented with 2000 mg of DEHP/L in solid-state fermentation.

4. DISCUSSION

In the present study, two fermentation systems were used for the growth of *F. culmorum* (submerged fermentation and solid-state fermentation). *F. culmorum* showed higher enzymatic activity in solid-state fermentation than in submerged fermentation. Viniegra-Gonzalez et al. (2003) reported that the growth and enzymatic activity of filamentous fungi increased in solid-state fermentation conditions. Macedo & Pio (2005) reported that glucose repressed esterases enzymatic activity in *Fusarium oxysporum* grown in medium containing glucose as sole carbon and energy source. The results obtained in the present research showed that the presence of glucose as a carbon and energy source, did not affect the esterases activity of *F. culmorum*.

Luo *et al*. (2012) used a esterase enzyme produced by *Fusarum* sp in the dimethyl terephthalate degradation, which showed a molecular weight of 45 kDa approx. Other bands with esterase activity were also observed with different molecular weights, possibly isoforms, of these proteins.

It has been reported that *F. culmorum* used DEHP (500 and 1000 mg/L) as carbon and energy source and showed more esterase enzymatic activity at a higher DEHP concentration (Ahuactzin-Pérez *et al*., 2016). In the present research, *F. culmorum* showed higher enzymatic activity in those media supplemented with high DEHP concentrations

(2000 mg/L in submerged fermentation and 1500 mg/L in solid-state fermentation). Furthermore, the growth of this organism was not inhibited by such DEHP concentrations.

These results showed that esterases of *F. culmorum* were induced in media supplemented with DEHP. The addition of high concentrations of DEHP induced a high number of isoforms of esterases with diferent molecular weight.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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