



## Production of cutinolytic esterase by *Fusarium culmorum* grown at different apple cutin concentrations in submerged fermentation

## Producción de esterasa cutinolítica por *Fusarium culmorum* crecido en diferentes concentraciones de cutina de manzana en fermentación sumergida

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Article history:

Received: 4 October 2019 / Received in revised form: 10 November 2019 / Accepted: 18 November 2019 / Published online: 19 November 2019.

<https://doi.org/10.29267/mxjb.2019.4.4.50>

### ABSTRACT

Cutinolytic esterase (i.e., cutinase) is an enzyme that catalyzes the cleavage of ester bonds in cutin and also in diverse soluble and insoluble esters. It has application in several biotechnological areas, acting as biocatalysts in the food industry, in detergents, in biodegradation of polymers and other toxic substances, being important in biorremediation. In this research, specific growth rate, protein content, cutinolytic activity by biochemical tests and polyacrylamide gel electrophoresis, and growth and enzymatic kinetic parameters were determined for *F. culmorum* grown at different apple cutin concentrations (0.2, 2 and 20 g/L) in submerged fermentation. It was observed that biomass, protein content and enzymatic activity enhanced as

cutin concentration increased in the media. A cutinase activity band of around 65 KDa was observed in zymograms of different cutin concentration. An additional cutinase activity band of around 90 KDa was also observed in zymograms of *F. culmorum* grown in 20 g of apple cutin/L. These studies showed that *F. culmorum* used apple cutin as the sole carbon source, which acted as a cutinase inducer. The highest-yielding parameters of cutinase were observed in 2 g of apple cutin/L. This research showed promising results in the cutinase induction for *F. culmorum* using a low concentration of apple cutin.

**Keywords:** Apple cutin, cutinases, *Fusarium culmorum*, submerged fermentation.

## RESUMEN

La esterasa cutinolítica (i.e. cutinasa) es una enzima que cataliza la ruptura de los enlaces éster en la cutina y también en una variedad de ésteres sintéticos solubles e insolubles. Esta enzima tiene aplicación en varias áreas biotecnológicas, actuando como biocatalizadores en la industria alimentaria, en detergentes, en biodegradación de polímeros y otras sustancias tóxicas, siendo importante en la biorremediación. La tasa de crecimiento específica, el contenido de proteínas, la actividad de esterasa cutinolítica mediante pruebas bioquímicas y de electroforesis en gel de poliacrilamida, y los parámetros cinéticos de crecimiento y enzimáticos se determinaron para *F. culmorum* cultivado en diferentes concentraciones de cutina de manzana (0.2, 2 y 20 g/L) en fermentación sumergida. Se observó que la biomasa, el contenido de proteínas y la actividad enzimática aumentaron conforme aumentó la concentración de cutina en los medios. Se observó una banda de actividad de cutinasa de aproximadamente 65 KDa en los zimogramas de las diferentes concentraciones de cutina probadas. Una banda adicional de actividad de cutinasa de aproximadamente 90 KDa fue observada en zimogramas de *F. culmorum* crecido en 20 g de cutina de manzana /L. Estos estudios mostraron que *F. culmorum* empleó la cutina de manzana como la única fuente de carbono, misma que actuó como un inductor de cutinasa. Los parámetros de rendimiento más altos de ésta enzima fueron observados en 2 g de cutina de manzana/L. Esta investigación mostró resultados prometedores en la inducción de cutinasa en *F. culmorum* usando bajas concentraciones de cutina de manzana.

**Palabras clave:** Cutinasas, cutina de manzana, fermentación sumergida. *Fusarium culmorum*.

## 1. INTRODUCTION

Cutinolytic esterase (i.e., cutinase) is known as cutin hydrolase (EC 3.1.1.74) and it is an enzyme that catalyzes not only the cleavage of ester bonds in cutin but also in diverse soluble and insoluble esters, insoluble triglycerides, phthalates, plastics and

others (Kim *et al.*, 2003; Chen *et al.*, 2013; Nyysölä, 2015; Ferrario *et al.*, 2016; Ferrer-Parra *et al.*, 2018). Cutinase is an extracellular enzyme produced by several phytopathogenic fungi that grow in cutin as sole carbon source. Cutin is a high molecular weight and insoluble biopolymer composed of epoxy and interesterified hydroxy fatty acids that forms the structural component of higher plants cuticle, and that function as a structural support (Pio & Macedo, 2009; Fich *et al.*, 2016). Cutinase shares properties of lipase and esterase in its catalytic action, since it is a serine esterase with the classical Ser-His-Asp triad similar to several lipases and serine proteases (Nyysölä, 2015). This enzyme presents a unique feature of being active regardless the presence of an oil-water interface, making it attractive as a biocatalyst for several promising industrial applications like inter- and intra-transesterification reactions (Pio & Macedo, 2009). These properties of cutinase are being used for several applications in agriculture, in the textile industry, in household detergents, in pharmaceuticals, in processing food and dairy, in biocatalysis, in detoxification of environmental pollutants etc. (Nyysölä, 2015). Therefore, cutinase is an important industrial catalyst that replaces older technologies with environmentally friendly processes (Chen *et al.*, 2013). It is known the importance of the carbon source for the growth of the microorganisms and its influence on the enzyme parameters such as productivity, yield, and specific rate of enzyme production. It has been reported that cutin from the natural and agro industrial wastes offer advantages over pure or synthetic analogues of cutin with respect to cutinase production (Fett *et al.*, 1999; Pio & Macedo, 2009). Plant cutin acts as an important inducer of cutinase enzyme for different fungal species. Several studies have reported that *Fusarium* species are able to produce cutinase (Kim *et al.*, 2007; Fraga *et al.*, 2012; Canavati-Alatorre *et al.*, 2017; De Oliveira *et al.*, 2019). In this research, different concentrations of apple peels cutin were evaluated for growth and cutinase production by *Fusarium culmorum* in submerged fermentation. Fungal specific growth rate, enzymatic activity (biochemical and zymogram analysis), protein content, growth and enzymatic kinetic parameters were determined.

## **2. MATERIALS AND METHODS**

### **2.1. Strain**

*F. culmorum* from the culture collection of the Research Centre for Biological Sciences (CICB) at Universidad Autónoma de Tlaxcala, Mexico was used. This strain was isolated from a recycled paper industry (Aguilar-Alvarado *et al.*, 2015). The strain was grown on malt extract agar (Difco) at 20 °C and stored at 4 °C.

### **2.2. Culture media and culture conditions**

Three liquid culture media were prepared: 1) Mineral medium (MM) + 0.2 g of apple peels cutin/L 2) MM + 2 g of apple peels cutin/L, 3) MM + 20 g of apple peels cutin/L. MM contained (in g/L), 0.6  $\text{KH}_2\text{PO}_4$ , 0.001  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.4  $\text{K}_2\text{HPO}_4$ , 0.05  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.5  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25  $\text{CuSO}_4$ . Each medium was supplemented with 5 g of yeast extract as nitrogen source. The pH was adjusted to 6.5 using 0.1 M HCl or 0.1 M NaOH as required. Flasks of 125 mL 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 malt extract agar (DIFCO). Cultures were incubated at 25 °C for 7 days (168 h) on a rotary shaker at 130 rpm. Analyses were carried out on samples taken at 12-h intervals and performed in triplicate.

### 2.3. Cutin extraction

Cutin was extracted from the cuticle of apples (Golden Delicious, purchased on the local market), using the method described by Macedo & Pio (2005). Apples were peeled and peels were placed in a beaker, and then a buffer solution of sodium oxalate at pH of 3.5 was added. Peels were boiled for 30 min in order to separate the pulp remaining from the peels. Peels were filtered and freeze-dried at -40 °C (for 12 h) and then lyophilized. A solution of methanol and dichloromethane (1:1 v/v) was added to the lyophilized material and then left overnight. Cutin powder was obtained from that solution by rotary evaporation at 45 °C.

### 2.4. Growth and estimation of kinetic parameters

Biomass ( $X$ ) was obtained by filtration of the samples and the specific growth rate was determined from changes in dry weight, using the logistic equation as reported previously (Ahuactzin-Pérez *et al.*, 2016). The specific growth rate ( $\mu$ ) was estimated as reported by Ahuactzin-Pérez *et al.* (2016).  $X_{max}$  corresponds to the maximal biomass produced by the fungus during the exponential growth.

### 2.5. Protein content and pH measurements

Culture supernatants were used to determine the protein production using Bradford (1976) protein assay. 960 mL of Bradford reagent (Bio-Rad) added to a test tube with 40 mL of each supernatant. Protein production was evaluated by using a UNICO spectrophotometer (S-2150 series, DAYTON, NJ, USA) at 595 nm. The pH was measured every 12 h in the supernatant of the cultures using a digital potentiometer (Hanna Instruments, México).

### 2.6. Cutinase activity and estimation of enzymatic parameters

Cutinase activity was evaluated by spectrophotometry at 405 nm by adding 900  $\mu\text{L}$  of substrate and 100  $\mu\text{L}$  of supernatant sample. The reaction mixture contained 1.76% of *p*-nitrophenyl butyrate (*p*NPB), 1.1% of acetonitrile, 11.1% of distilled water, 0.04% of Triton X-100 and was diluted with 0.01 M phosphate buffer (pH 7.5)

(Canavati-Alatorre *et al.*, 2016). One unit of cutinolytic activity was defined as the amount of cutinase required to release one micromole of *p*-nitrophenol in one minute (Macedo & Pio, 2005). Yield of enzyme per unit of biomass produced by the fungus ( $Y_{EX}$ ) was estimated as the relation between maximal enzymatic activity obtained during the exponential growth ( $E_{max}$ ) and  $X_{max}$ . Enzymatic productivity was evaluated at that time when the enzymatic activity was maximal ( $P=E_{max}/\text{time}$ ). The specific rate of enzyme production was calculated from the equation:  $q_p = (\mu) (Y_{EX})$  (Ahuactzin-Pérez *et al.*, 2016).

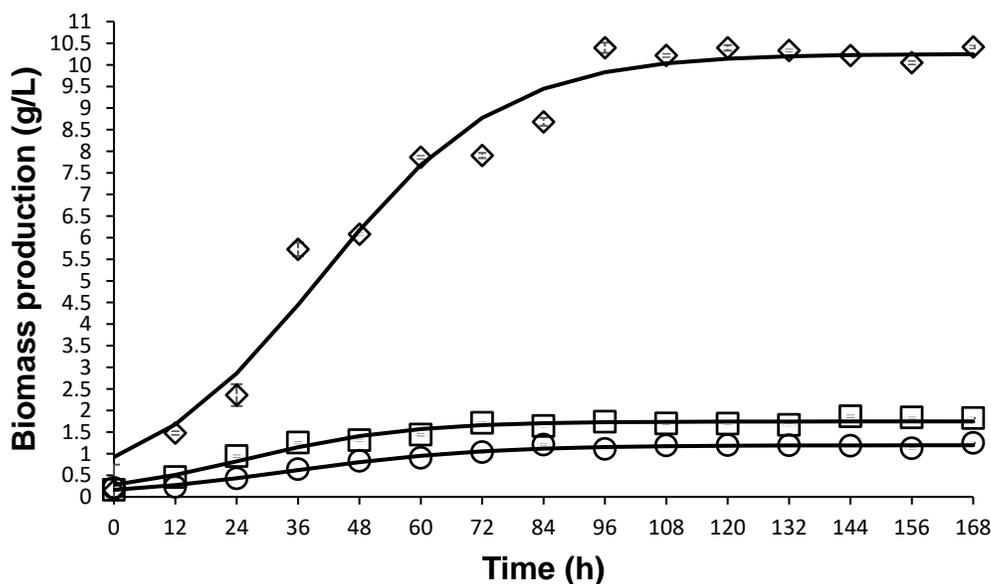
## 2.7. Zymography

The samples with cutinase activity were analyzed by electrophoresis in 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. Precision Plus Protein™ Dual Xtra Standards (Bio-Rad) was used as molecular weight 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 and the procedure was carried out as previously reported (Karpushova *et al.*, 2005; Canavati-Alatorre *et al.*, 2016). Cutinase activity was observed as red-colored bands in the gels.

## 3. RESULTS

### 3.1. Biomass production and specific growth rate

Growth curves of *F. culmorum* on media added with different concentrations of apple cutin are shown in Fig. 1. The highest biomass production was obtained in medium containing 20 g of cutin/L followed by those media added with 2 and 0.2 g of cutin/L (Fig.1; Table 1). However, there were no significant differences in the  $\mu$  values showed in the different media tested (Table 1).



**Fig.1.** Biomass production by *F. culmorum* grown on media supplemented with 0.2 (o), 2 (□) and 20 (◇) g of apple cutin/L in submerged fermentation.

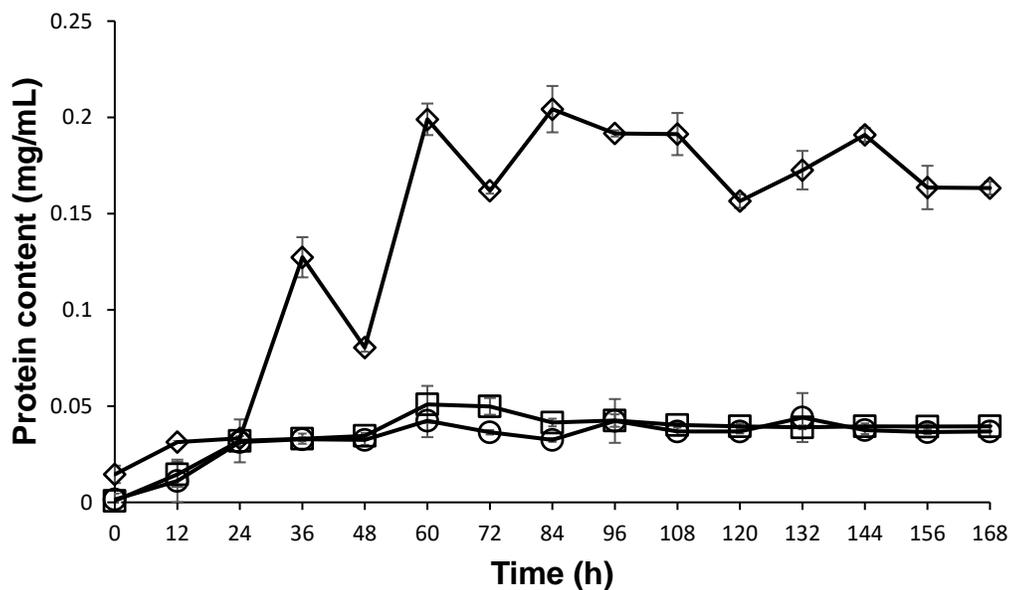
**Table 1.** Growth parameters and yield parameters of cutinase of *F. culmorum* grown in media supplemented with different concentrations of apple cutin under submerged fermentation.

Parameters	Culture media supplemented with apple cutin (g/L)		
	0.2	2 g	20 g
Growth parameters			
$\mu$ (h <sup>-1</sup> )	0.05 ± 0.001 <sup>a</sup>	0.06 ± 0.001 <sup>a</sup>	0.05 ± 0.001 <sup>a</sup>
X <sub>max</sub> (g/L)	1.19 ± 0.01 <sup>c</sup>	1.74 ± 0.01 <sup>b</sup>	10.25 ± 0.01 <sup>a</sup>
Yield parameters of cutinase			
E <sub>max</sub> (U/L)	251.6 ± 0.1 <sup>c</sup>	360.9 ± 0.3 <sup>b</sup>	434.1 ± 0.2 <sup>a</sup>
Y <sub>E/X</sub> (U/g/X)	211.4 ± 5 <sup>a</sup>	207.4 ± 6 <sup>a</sup>	42.4 ± 3 <sup>b</sup>
P <sub>RO</sub> (U/L*h)	1.49 ± 0.01 <sup>b</sup>	3.00 ± 0.01 <sup>a</sup>	3.01 ± 0.01 <sup>a</sup>
q <sub>p</sub> ((U/gX*h))	10.57 ± 0.01 <sup>b</sup>	12.44 ± 0.02 <sup>a</sup>	2.12 ± 0.01 <sup>c</sup>

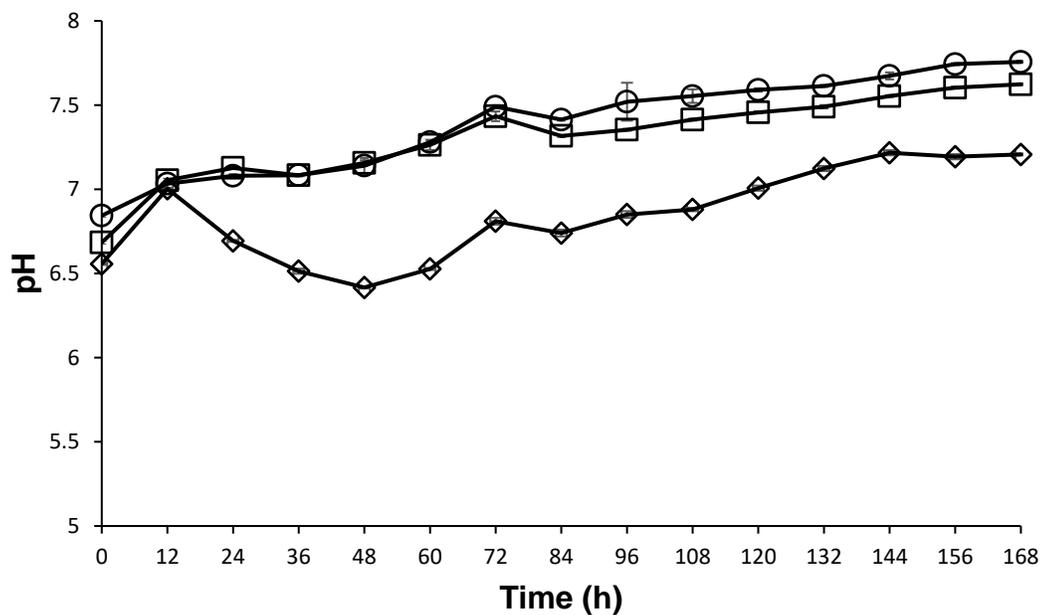
Values are expressed as mean±SEM (n=3); means within the same column not sharing common superscript letters (a, b, c) differ significantly at 5% level. Growth parameters; X<sub>max</sub> and  $\mu$ , were evaluated using a non-linear least squares fitting program (Ahuactzin-Pérez *et al.*, 2016). Yield parameters of esterase were calculated as previously reported (González-Márquez *et al.*, 2019).

### 3.2. Protein content analysis and pH measurement

The results for protein production are shown in Fig. 2. It was observed that *F. culmorum* produced the highest protein content in the culture medium containing 20 g of cutin/L, showing the greatest protein content (0.2 mg/mL) after 84 h of fermentation. The lowest protein content was showed in those media supplemented with 2 and 0.2 g of cutin/L, with a maximum production of approximately 0.05 mg/mL. Fig. 3 shows the pH of the cultures during the fermentation process. The highest values of pH were observed in those media supplemented with 0.2 and 2 g of apple cutin/L, in which the pH value increased to 7.7 approx. after 168 h. The lowest pH values were showed in the media containing 20 g of apple cutin/L, reaching a maximum value of 7.2 after 168 h.



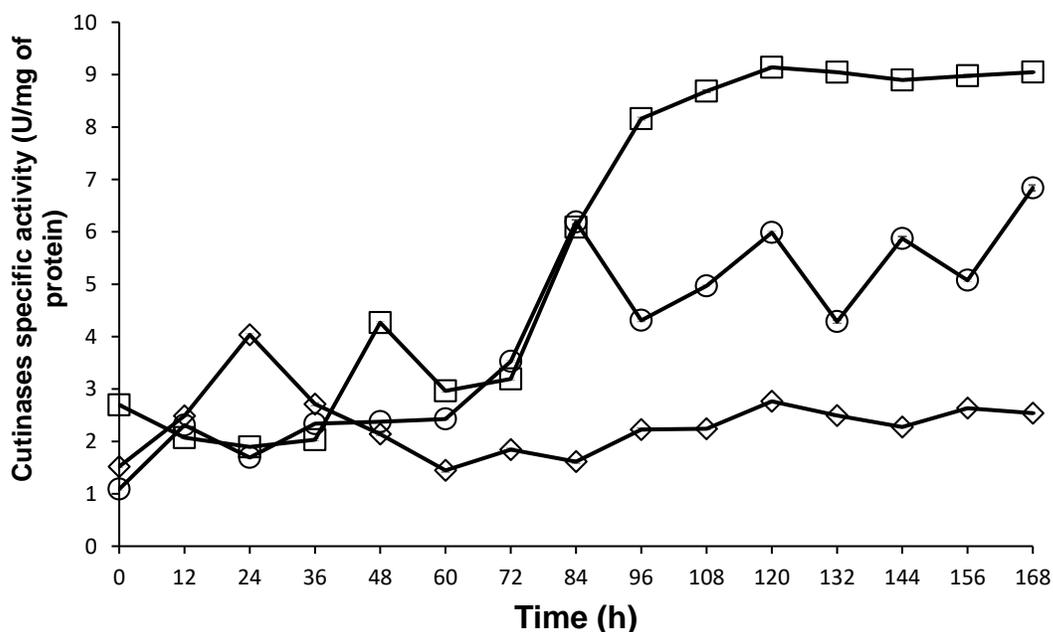
**Fig. 2.** Protein content of *F. culmorum* grown on media supplemented with 0.2 (o), 2 (□) and 20 (◇) g of apple cutin/L in submerged fermentation.



**Fig. 3.** pH of *F. culmorum* grown on media supplemented with 0.2 (o), 2 (□) and 20 (◇) g of apple cutin/L in submerged fermentation.

### 3.3. Cutinase specific activity

The cutinase specific activity expressed as number of enzyme units per mg of total protein is showed in Fig. 4. The highest cutinase specific activity was showed in that medium added with 2 g of cutin/L, followed by the medium supplemented with 0.2 g of cutin/L after 84 h. The lowest cutinase specific activity was observed in media added with 20 g of cutin/L. However, the greatest  $E_{max}$  was showed in the medium added with 20 g of cutin/L followed by those media added with 0.2 and 2 g of cutin/L (Table 1). Media containing 0.2 and 2 g of apple cutin/L had higher  $Y_{EX}$  than that medium added with 20 g of cutin/L. The greatest  $P_{RO}$  was shown in media supplemented with 2 and 20 g of apple cutin/L, followed by the medium added with 0.2 g of apple cutin/L. The highest-yielding parameters of cutinase ( $Y_{EX}$ ,  $P_{RO}$ , and  $q_p$ ) were observed in medium supplemented with 2 g of apple cutin/L (Table 1).

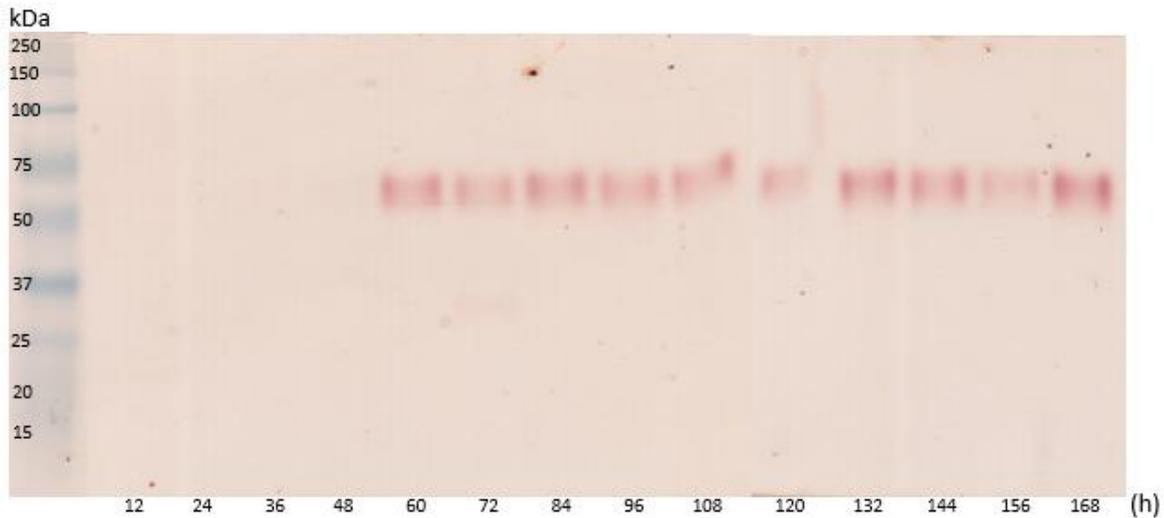


**Fig. 4.** Specific activity of cutinase of *F. culmorum* grown on media supplemented with 0.2 (o), 2 (□) and 20 (◇) g of apple cutin/L in submerged fermentation.

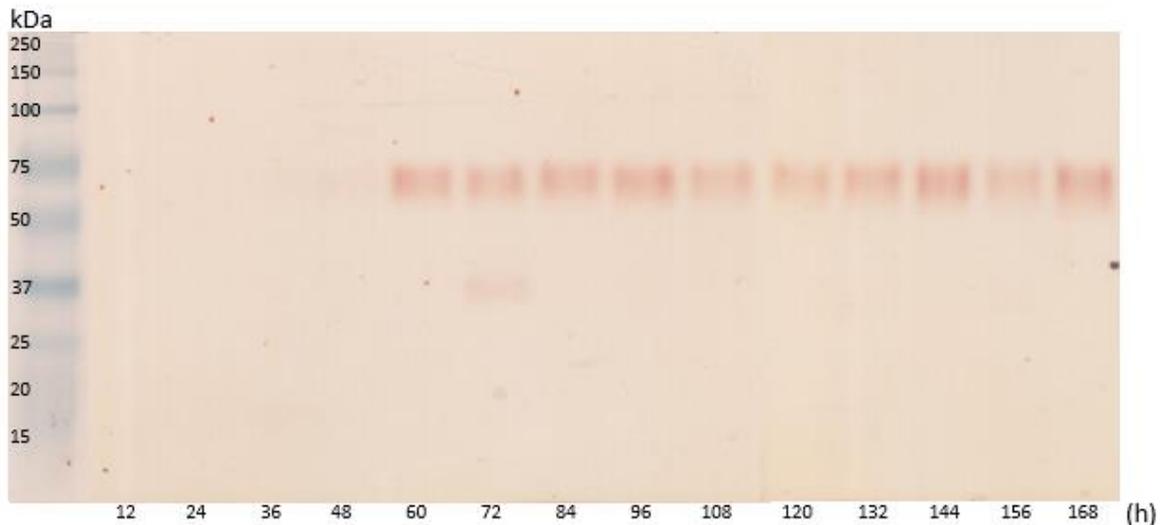
### 3.3. Cutinase analysis by zymography

$E_{max}$  data were confirmed by zymography. Cutinase zymograms of *F. culmorum* grown in 0.2 and 2 g of apple cutin/L are showed in Fig. 4 and Fig. 5, respectively. In these two zymograms, similar cutinase activity bands of around 65 KDa aprox were observed after 60 h of fermentation (Figs. 4 and 5). Zymogram of *F. culmorum* grown on medium supplemented with 20 g of cutin/L is shown in Fig. 6. This zymogram showed higher color intensity in the bands and more cutinase activity

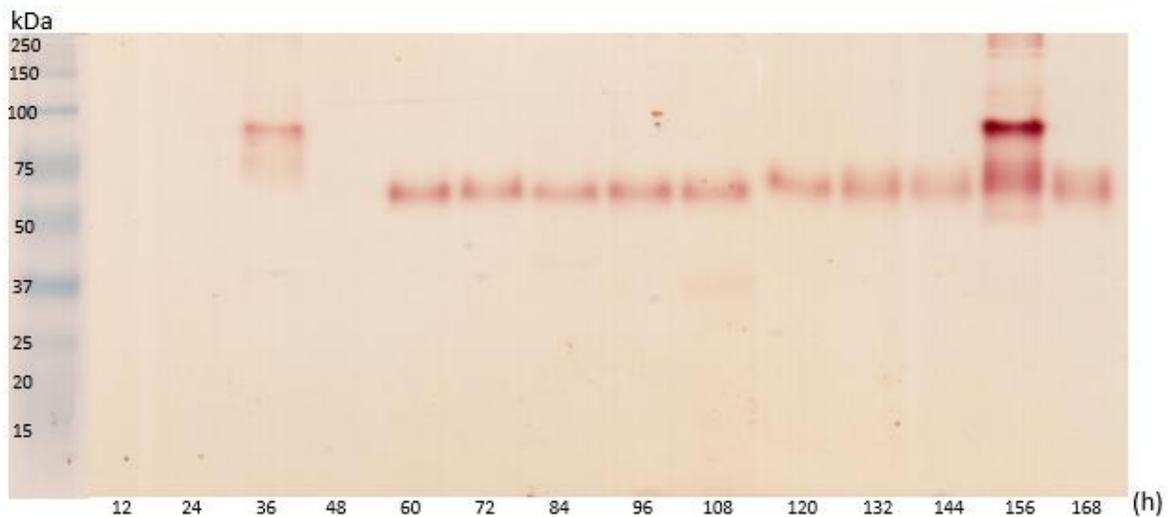
bands than the rest of the zymograms. In this zymogram, a cutinase activity band of around 65 KDa, similar to that observed in zymograms of *F. culmorum* grown in low apple cutin concentration (0.2 and 2 g of apple cutin/L) was observed after 60 h and during all the fermentation (Fig. 6). Additionally, a cutinase activity band appeared after 36 h with a molecular weight of 90 KDa approximately, which was also observed after 156 h (Fig. 6).



**Fig. 5.** Zymogram of *F. culmorum* grown on medium supplemented with 0.2 g of cutin/L in submerged fermentation.



**Fig. 6.** Zymogram of *F. culmorum* grown on medium supplemented with 2 g of cutin/L in submerged fermentation.



**Fig. 7.** Zymogram of *F. culmorum* grown on medium supplemented with 20 g of cutin/L in submerged fermentation.

#### 4. DISCUSSION

Cutinase is a hydrolytic enzyme that degrades cutin, thus natural cutin has been used as a growth substrate to demonstrate cutinases identity (Chen *et al.*, 2013). It has been reported that cutinases are produced by filamentous fungi (e.g. *Fusarium* species) when grown in media containing cutin or its hydrolysate (Purdy & Kolattukudy, 1973; Lin & Kolattukudy 1978; Degani, 2015). In the present work, apple peels cutin was used to induce cutinase in *F. culmorum*. It has been reported that cutin isolated from apple peels is a good cutinase inducer, since it yielded 9.64 U/mL as compared to 6.77 U/mL of cutinase using watermelon cutin (Chaudhari & Singhal, 2015). It was observed that *F. culmorum* grew in media supplemented with apple peels cutin as sole carbon source, using this substrate as a carbon source, since the biomass production and protein content were enhanced as cutin concentration increased in the media. The pH of the cultures also increased at the end of the fermentation, which could be due to the basic compounds (cutin breakdown products) releasing into the media. Degani (2015) studied production of cutinase by *Fusarium oxysporum* and reported that cutinase activity increases according to the amount of cutin added to the culture medium. It has been reported Induction of cutinolytic esterase activity during growth of fungal pathogens, showing that the enzymatic activity is regulated by the source and concentration of cutin (Hawthorne *et al.*, 2001). Castro-Ochoa *et al.* (2012) reported cutinase production by *Aspergillus nidulans* using olive oil, triacylglycerides and fatty acids as inducers.

In the present research, the  $E_{\max}$  (U/L) of *F. culmorum* raised by increasing cutin concentration, being in accord with that previously reported (Hawthorne *et al.*, 2001; Degani, 2015). However, the  $Y_{E/X}$  (U/g/X) was higher in those media supplemented with low apple cutin concentrations (0.2 and 2 g/L) than in that medium added with 20 g of cutin/L (the highest cutin concentration tested). Macedo & Pio (2005) reported that *F. oxysporum* showed the best conditions for the production of extracellular cutinase in a medium added with 10 g of cutin/L. Canavati-Alatorre *et al.* (2016) studied cutinase production by *F. culmorum* in media supplemented with glucose and apple cutin and found a  $E_{\max}$  of 406 U/L, which is similar to that observed in our results (using 20 g of cutin/L). Several techniques have been developed in order to identify cutinases in SDS-PAGE gels (Karpushova *et al.*, 2005; Castro-Ochoa *et al.*, 2012; Yang *et al.*, 2013). The use of these techniques have revealed fungal cutinases of about 22 kDa and 29 kDa (Castro-Ochoa *et al.*, 2012; Degani, 2015) and cutinolytic esterase produced by bacteria ranged in size from 29 to 47 kDa (Inglis *et al.*, 2011). Soliday & Kolattukudy (1976) reported that *Fusarium roseum culmorum* produced cutinases and non-specific esterases that hydrolyzed cutin, with a molecular weight of approximately 24.3 kDa. Canavati-Alatorre *et al.* (2016) studied cutinase production by *F. culmorum* grown on media supplemented with both glucose and apple cutin and observed cutinase activity bands with a molecular weight of approximately 50 kDa. In the present research, a cutinase activity band of around 65 kDa was observed in zymograms of *F. culmorum* grown in the different apple cutin concentrations and an additional cutinase activity band of around 90 kDa was also observed in zymograms of *F. culmorum* grown in the highest cutin concentration tested (20 g/L). These studies showed that *F. culmorum* used apple cutin as the sole carbon source, which acted as a cutinase inducer. The highest-yielding parameters of cutinase were observed in 2 g of apple cutin/L. This research showed promising results in the cutinase induction for *F. culmorum* using a low concentration of apple cutin.

## ACKNOWLEDGEMENTS

We are thankful to the Mexican Council for Science and Technology (CONACyT) for financial support (project No. 1549; Fronteras de la Ciencia). We also are grateful to CONACyT for providing a doctoral scholarship (No. 555469) to Angel González Márquez.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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