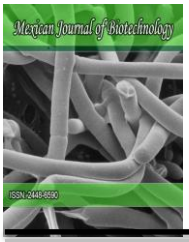

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ORIGINAL RESEARCH

Tween 80-induced esterase production by *Trichoderma harzianum* in submerged fermentation: An esterase activity assay using α-naphthyl acetate as substrate

Producción de esterasa inducida por Tween 80 por *Trichoderma harzianum* en fermentación sumergida: Un ensayo de actividad de esterasa utilizando α-naftil acetato como sustrato

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ABSTRACT

Tween 80 is a surfactant that has been used for several purposes in microbial cultures. In bioremediation studies, the addition of surfactants in the medium is a common practice, since it makes hydrophobic substrates available to the microorganism. In this work, the influence of Tween 80 (100 or 400 µL/L) on the biomass production, protein content, esterase activities and, growth and enzymatic yield parameters of *Trichoderma harzianum* grown in submerged fermentation was evaluated. Assessments of esterase activities through biochemical tests and zymographic assays were carried out using α-naphthyl acetate as substrate. A single band with esterase activity was observed in the different concentrations of Tween 80 tested. However, 400 µL of Tween 80 induced higher esterase production (148.2 U/L) in *T. harzianum* as compared to that esterase activity (79.5 U/L) shown in the medium added with 100 µL of Tween 80 per liter. These results showed that high concentrations (*e.g.* 400 µL/L) of Tween 80 enhanced the esterase activity in *T. harzianum*. The single band with esterase activity revealed on zymograms suggests that this enzyme (of 38 kDa approx.) may be present in both forms as a constitutive as well as an inducible esterase, since it was observed in both glucose- and Tween 80-supplemented media.

Keywords: α-naphthyl acetate, esterase production, fungal growth, liquid fermentation, *Trichoderma harzianum*, Tween 80.

RESUMEN

Tween 80 es un tensoactivo que se ha utilizado para diversos fines en cultivos microbianos. En estudios de biorremediación, la adición de tensoactivos en el medio es una práctica común, ya que hace que los sustratos hidrófobos estén disponibles para el microorganismo. En este trabajo se evaluó la influencia de Tween 80 (100 o 400 µL/L) en la producción de biomasa, contenido proteico, actividades de esterasa, y parámetros de crecimiento y rendimiento enzimático de *Trichoderma harzianum* crecido en fermentación sumergida. Se realizaron evaluaciones de las actividades de esterasa mediante pruebas bioquímicas y ensayos zimográficos utilizando como sustrato α-naftil acetato. Se observó una única banda con actividad de esterasa en las diferentes concentraciones de Tween 80 estudiadas. Sin embargo, 400 µL de Tween 80 indujeron una mayor producción de esterasa (148,2 U/L) en *T. harzianum* en comparación con la actividad de esterasa (79,5 U/L) mostrada en el medio añadido con 100 µL de Tween 80 por litro. Estos resultados mostraron que altas concentraciones (por ejemplo, 400 µL/L) de Tween 80 incrementaron la actividad de esterasa en *T. harzianum*. La única banda con actividad de esterasa revelada en los zimogramas sugiere que esta enzima (de 38 kDa aprox.) puede estar presente en ambas formas como esterasa constitutiva e inducible, ya que se observó en ambos medios, en el medio adicionado con glucosa y en el medio adicionado con Tween 80.

Palabras clave: α-naftil acetato, crecimiento de hongos, fermentación líquida, producción de esterasa, *Trichoderma harzianum*, Tween 80.

1. INTRODUCTION

Fungi are an important group of organisms, which have developed several strategies to adapt and occupy diverse habitats. These organisms live in their mostly macromolecular substrates and have an efficient extracellular enzymatic system for the hydrolysis of a wide range of polymers (Sánchez 2020; Sánchez, 2021). Therefore, fungi have a great biotechnological potential for industrial exploitation (Hyde *et al*., 2019; Sánchez *et al*., 2020). In this context, *Thichoderma* species possess the glycoside hydrolases, which comprise one the most abundant groups of its genome. In addition, this genus also produces carbohydrate esterases, polysaccharide binding proteins, polysaccharide lyases, and auxiliary oxidative enzymes (Kubicek *et al*., 2019).

On the other hand, surfactants are amphiphilic compounds that possess a hydrophilic polar part and a lipophilic nonpolar part. These molecules decrease the surface or interfacial free energy, which causes reduction in interfacial or surface tension between two phases (Mondal *et al*., 2015). In bioremediation studies, surfactants have been used to enhance biodegradation substrates (Khoramfar *et al*., 2020; Cheng *et al*., 2021; Sánchez-Muñoz *et al*., 2021; Ren *et al*., 2021). In

particular, *Trichoderma harzianum* has been described to be able to degrade the plasticizer di(2-ethyl hexyl) phthalate, which make plastics elastic, flexible and extensible (Aguilar-Alvarado *et al*., 2015). Some ascomycete fungi have been reported to be highly efficient phthalate-degrading organisms due to their secretion of enzymes such as esterase (Chhaya & Gupte, 2013; Aguilar-Alvarado *et al*., 2015; Bouchiat *et al*., 2015; Ahuactzin-Pérez, *et al*., 2016; Ferrer-Parra *et al*., 2018; Sánchez-Sánchez & Sánchez, 2018; González-Márquez *et al*., 2019a,b; Rios-González *et al*., 2019; González-Márquez *et al*., 2020; González-Márquez *et al*., 2021).

The addition of surfactants in the medium is a common practice since it makes hydrophobic substrates available to the microorganism. In particular, Tween 80 namely polyoxyethylene glycol sorbitan monooleate ($C_{64}H_{124}O_{26}$) is a nonionic surfactant extensively used in hydrophobic pollutant biodegradation studies (Bustamante *et al*., 2012; Cheng *et al*., 2017). Furthermore, Tween 80 has also been used to increase the production of xanthan, pullulan and curdlan (Sheng *et al*., 2013; Liang *et al*., 2018; Ghashghaei *et al*., 2018) and exopolysaccharide production of the mushroom *Ganoderma lucidum* in submerged fermentation (Yang *et al*., 2020). The mechanism underlying the increased production of these molecules could be related to enzyme biosynthesis (Zhang & Cheung, 2011; Sheng *et al*., 2013). In this context, it is important to understand how Tween 80 affects microbial growth and enzyme production. Some investigations have described that microorganisms are able to use Tween 80 as a sole source of carbon (Chakrabarty *et al*., 1970; Nielsen *et al*., 2016; Nguyen *et al*., 2018). It has been found that *Fusarium culmorum* was able to use Tween 80 as the sole carbon and energy source and that esterase production was induced by this surfactant (Medina-Flores *et al*., 2020).

In the present work, the influence of Tween 80 (100 or 400 µL/L) on the biomass production, protein content, esterase activities and, growth and enzymatic yield parameters of *T. harzianum* grown in submerged fermentation was evaluated. Assessments of esterase activities through biochemical tests and zymographic assays were carried out using α-naphthyl acetate as substrate.

2. MATERIALS AND METHODS

2. 1. Strain

T. harzianum from the microbial collection from the Research Centre for Biological Sciences (CICB) at the Autonomous University of Tlaxcala (Tlaxcala, Mexico) was used in this research. The strain grew in malt extract agar at 25°C and was preserved in refrigeration at 4°C.

2.2. Growth media and culture conditions

Three liquid culture media were prepared containing the following components per liter: 1) Mineral medium (MM) + 10 g glucose, 2) MM + 100 µL of Tween 80 (v/v), 3) MM + 400 µL of Tween 80 (v/v). MM contained the following constituents, per

liter of distilled water: 1.2 g, $Ca(NO_3)_2$; 0.6 g, K_2HPO_4; 0.23 g, $MgSO_4.7H_2O$; 0.2 g, KCl, and 0.01 g, $FeSO_4.7H_2O$. The pH was adjusted to 6.5 using either 0.1 M HCl or 0.1 M NaOH.
Erlenmeyer flasks of 125 mL containing 50 mL sterilized culture medium were inoculated with a suspension of *T. harzianum* spores to a final concentration of 10^7 spores/mL as reported in our previous study (González-Márquez *et al*., 2019a). Cultures were incubated at 25 °C on an orbital shaker with a speed of 120 rpm. The sampling was carried out at 12-h intervals up to 72 h.

2.3. Calculation of specific growth rate and yield parameters

Biomass concentration (X, dry cell weight) was measured by filtering the mycelial culture through a filter paper. The specific growth rate (μ) was calculated fitting the logistic equation and the exponential model to the biomass data. The biomass yield ($Y_{X/S}$) was estimated as the coefficient of the linear regression of biomass concentration *versus* substrate concentration in grams of biomass/grams of substrate consumed (Ahuactzin-Pérez *et al*., 2016).

2.4. Protein content analysis and pH measurements

Samples were analyzed for protein content using a Bradford assay (Bradford, 1976). 200 μL of Bradford reagent (BIORAD) was added to 100 μL of supernatant and 700 μL of sterile distilled water, mixed, and incubated at room temperature for 10 min. A protein standard curve was made by measuring the absorbance of known concentrations of bovine serum albumin solutions at 595 nm. The absorbance of the solutions was measured at 595 using a spectrophotometer UNICO (S-2150 series Dayton, NJ, USA).
The pH was measured every 12 h in the samples using a digital potentiometer (Conductronic, Mexico).

2.5. Esterase calibration curve

A standard curve for α-naphthol was made by measuring the absorbance of known concentrations of α-naphthol solutions. Each solution contained increasing concentrations (in μg/mL) of α-naphthol (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9), 0.1 mL of a 1% fast red solution and the final volume was made up to 1.0 mL using distilled water. Test tubes containing the different solutions were incubated for 5 min at 37 °C in a water bath and absorbance was measured at 600 nm using a UNICO spectrophotometer (S-2150 series DAYTON, NJ, USA).
The plots of α-naphthol concentration versus absorbance values proved to be linear and a linear regression analysis of the standard curve was carried out to determine the concentration of α-naphthol produced during the esterase assay in each sample.

2.6. Esterase assay and enzymatic yield parameters

Esterase activity was quantified spectrophotometrically following the hydrolysis of α-naphthyl acetate at 600 nm using a UNICO spectrophotometer (S-2150 series DAYTON, NJ, USA). Esterase activity was assayed by adding 100 µL supernatant to 700 µL 0.003 M α-naphthyl acetate (dissolved in a 50 mM of phosphates buffer at pH 7.5 containing 5% acetone), 100 µL of 1% fast red TR solution and 100 µL of deionized water. This mixture was incubated for 5 min at 37 °C in a water bath and then the samples were incubated at 4 °C to stop the reaction. One unit of esterase activity (U) was defined as the amount of the esterase required for the release of 1 µmol of α-naphthol from α--naphthyl acetate per minute at 37 °C and pH 7.5. Esterase specific activities were expressed in U/mg of protein. The esterase yield per unit of biomass ($Y_{E/X}$), esterase productivity (P), and specific rate of enzyme production (q_p) were estimated as previously described (Ahuactzin-Pérez *et al*., 2016). In Table 2, maximal enzymatic activity (E_{max}) is reported as volumetric activity in U/L,

2.7. Zymographic analysis

The polypeptide profiles of the samples with esterase activity were carried out using 0.1% polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970). The analysis was performed using a procedure previously reported with slight modifications (Ferrer-Parra *et al*., 2018). Briefly, 14% 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 200 volts for 1.30 h, and then gels were incubated in a solution containing 0.003 M α-naphthyl acetate (dissolved in a 50 mM of phosphates buffer at pH 7.5 containing with 5% acetone) and 0.005 M fast red TR, which were then incubated at 25 °C for 12 h. Finally, esterase activity was detected by the appearance of red-colored bands in the gels. The gels were imaged using a Gel Doc EZ Imager (BIORAD), and bands in the lanes were assessed for their densities using Image Lab Version 6.0.0 (BIORAD).

2.8. Statistical analysis

All assays were carried out in triplicate. Statistical analyses were conducted using one-way ANOVA followed by Tukey's *post hoc* test using SigmaPlot version 12.0 (Systat Software Inc, San Jose, CA, USA).

3. RESULTS

3.1. Biomass production and pH of the cultures

Figure 1 shows biomass production by *T. harzianum* in media containing different concentrations of Tween 80 (100 and 400 µL/L) and glucose (control medium) as

carbon sources. The highest X_{max} and $Y_{X/S}$ were observed in the medium added with glucose, followed by the media added with 400 and 100 µL of Tween 80/L (Fig. 1, Table 1).

On the other hand, the pH showed a decrease from the starting 6.5 to 4.3 after 72 h of fermentation in the control medium. However, the pH of the cultures added with Tween 80 remained almost constant during the fermentation. The cultures added with 100 µL of Tween 80/L had a slightly lower pH than that showed in medium containing 400 µL of Tween 80/L (Fig. 2).

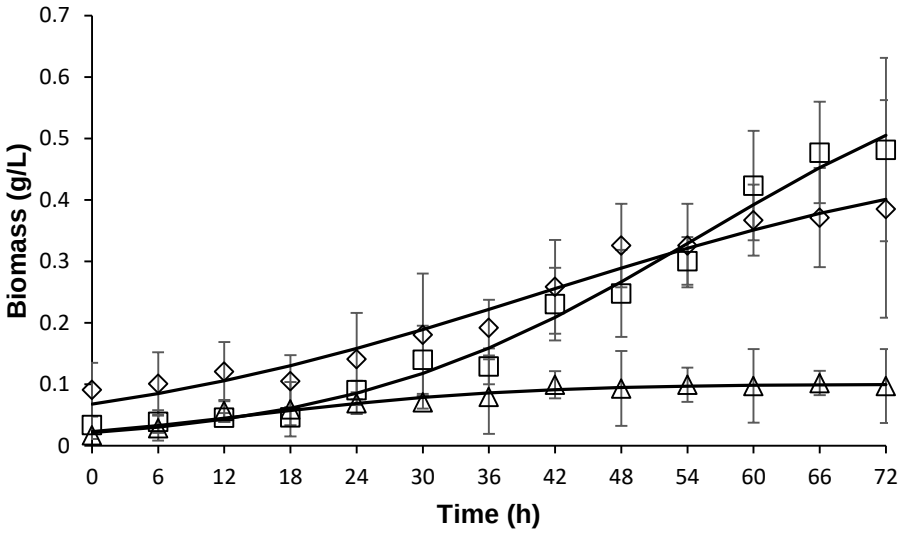

Fig. 1. Biomass production of *T. harzianum* grown in glucose medium (square) and in media added with 100 (triangle) and 400 (diamond) µL of Tween 80/L. Biomass curves were fitted (—) using the logistic equation.

Table 1. Growth parameters of *T. harzianum* grown in glucose-supplemented and in Tween 80-supplemented media in liquid fermentation.

Parameters	**Culture media**		
	Glucose	**Tween (100 µL)**	**Tween (400 µL)**
X_{max} (g/L)	0.7^{a} (0.002)	0.1^{c} (0.001)	0.5^{b} (0.001)
$Y_{X/S}$ (gX/gS)	0.07^{a} (0.001)	0.01^{c} (0.001)	0.05^{b} (0.002)

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.

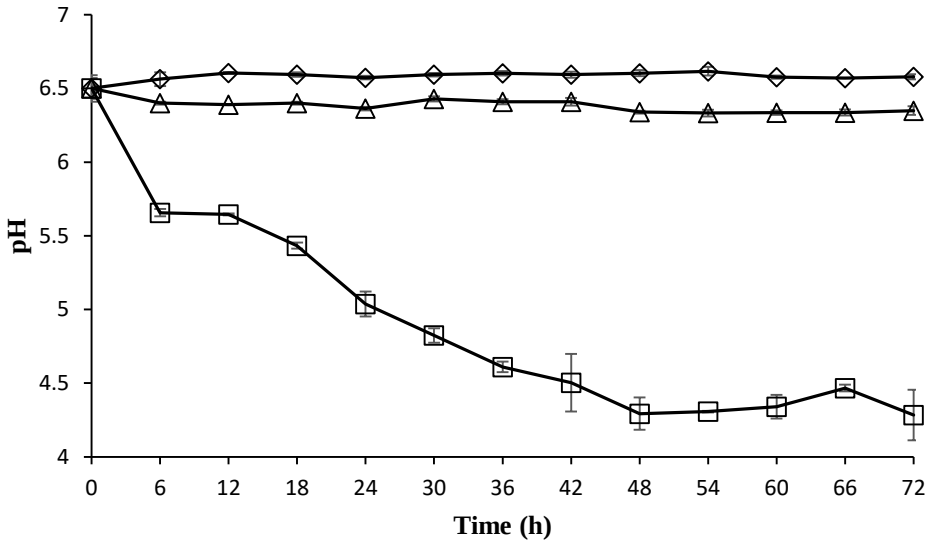

Fig. 2. pH profile of *T. harzianum* grown in glucose medium (square) and in media added with 100 (triangle) and 400 (diamond) µL of Tween 80/L.

3.2. Esterase calibration curve, protein content, esterase specific activity and enzymatic yield parameters

Figure 3 shows the standard curve for α-naphthol. A linear regression analysis of the standard curve was carried out to calculate the concentration of α-naphthol produced in the esterase analysis.

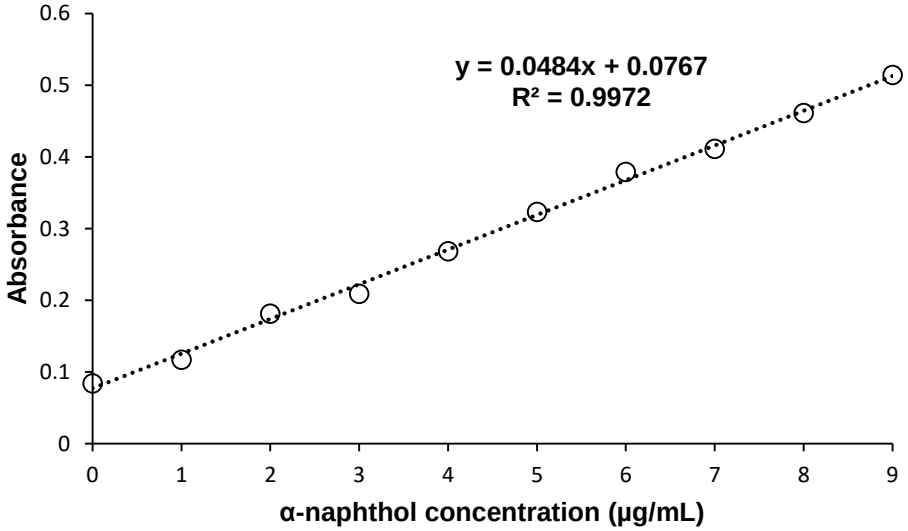

Fig. 3. Standard curve for α-naphthol. A linear regression analysis of the standard curve was carried out to determine the concentration of α-naphthol produced in the esterase assay.

The protein content of the cultures of *T. harzianum* is shown in Figure 4a. It is observed that the medium containing glucose had higher protein content than those media containing Tween 80 as carbon source (Fig. 4a).

Figure 4b shows that the highest esterase specific activity was observed in the medium containing 100 μL of Tween 80/L after 12 h of fermentation. The lowest esterase specific activity was shown after 24 h and until the end of the fermentation in the control medium.

The highest esterase volumetric activity was observed in the control medium followed by those media added with 400 and 100 μL of Tween 80/L (Table 2). The lowest esterase volumetric activity was showed in the medium added with 100 μL of Tween 80/L (Table 2). However, the greatest P_{RO} was observed in the control medium followed by the media containing 400 and 100 μL of Tween 80/L (Table 2).

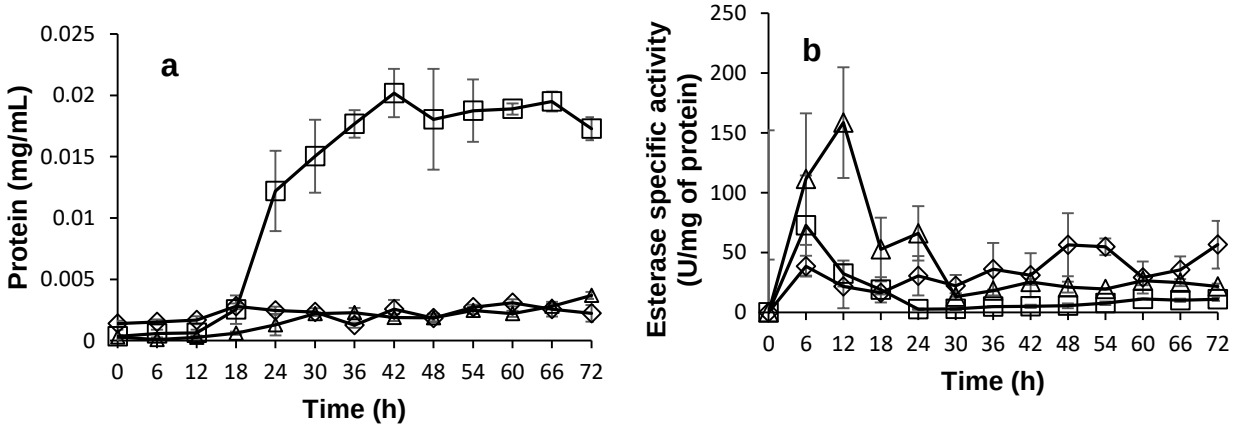

Fig. 4. Protein concentration (a) and esterase specific activity (b) of *T. harzianum* grown in glucose medium (square) and in media added with 100 (triangle) and 400 (diamond) μL of Tween/L.

Table 2. Enzymatic yield parameters of esterase of *T. harzianum* grown in glucose-supplemented and in Tween 80-supplemented media in liquid fermentation.

Parameters	Culture media		
	Glucose	Tween (100 µL/L)	Tween (400 µL/L)
E_{max} (U/L)	212.5[a] (45)	79.5[c] (24)	148.2[b] (19)
$Y_{E/X}$ (U/gX)	303.6[b] (0.5)	795[a] (0.24)	296.4[b] (0.8)
P_{RO} (U/L/h)	3.5[a] (0.003)	1.1[c] (0.002)	2.7[b] (0.001)
q_p (U/h/gX)	18.2[b] (0.001)	63.6[a] (0.002)	11.9[b] (0.001)

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.

3.3. Detection of esterase by zymography

Figures 5, 6 and 7 show the esterase zymograms of *T. harzianum* grown in glucose-containing medium, and in media supplemented with 100 and 400 µL of Tween 80/L, respectively. In all the media, one band with esterase activity was detected after 6 h and until the end of the fermentation with a molecular weight of 38 kDa approximately.

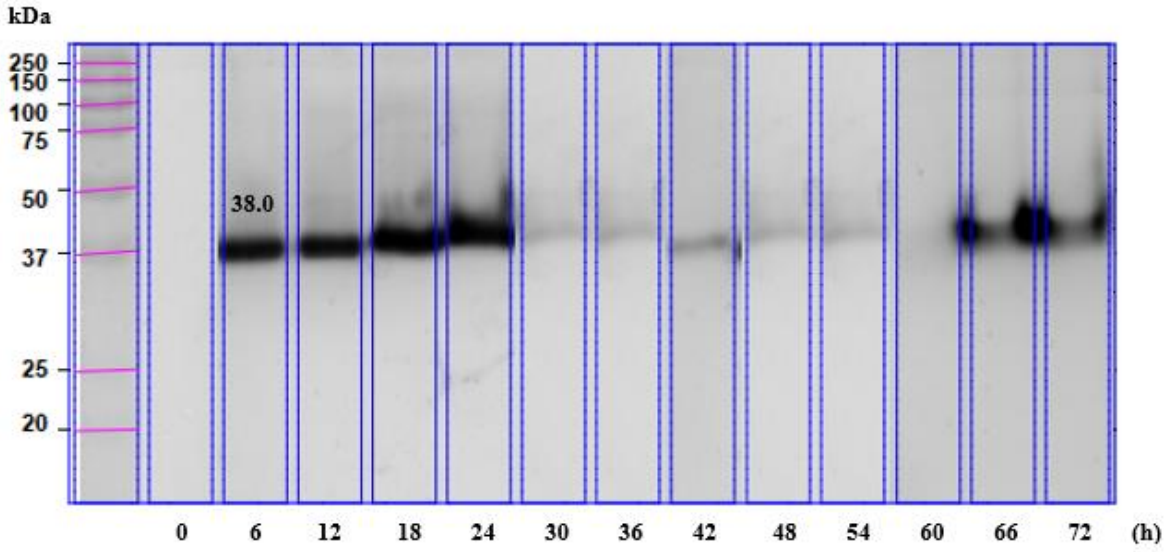

Fig. 5. Esterase zymogram of *T. harzianum* grown in glucose-supplemented medium for 72 h in submerged fermentation.

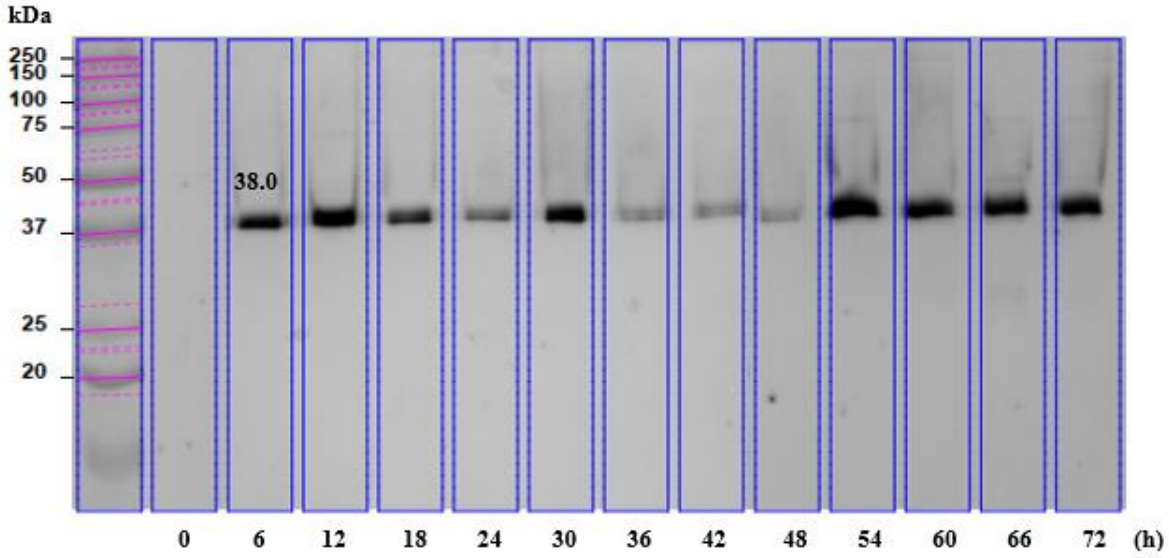

Fig. 6. Esterase zymogram of *T. harzianum* grown in 100 µL of Tween/L-supplemented medium for 72 h in submerged fermentation.

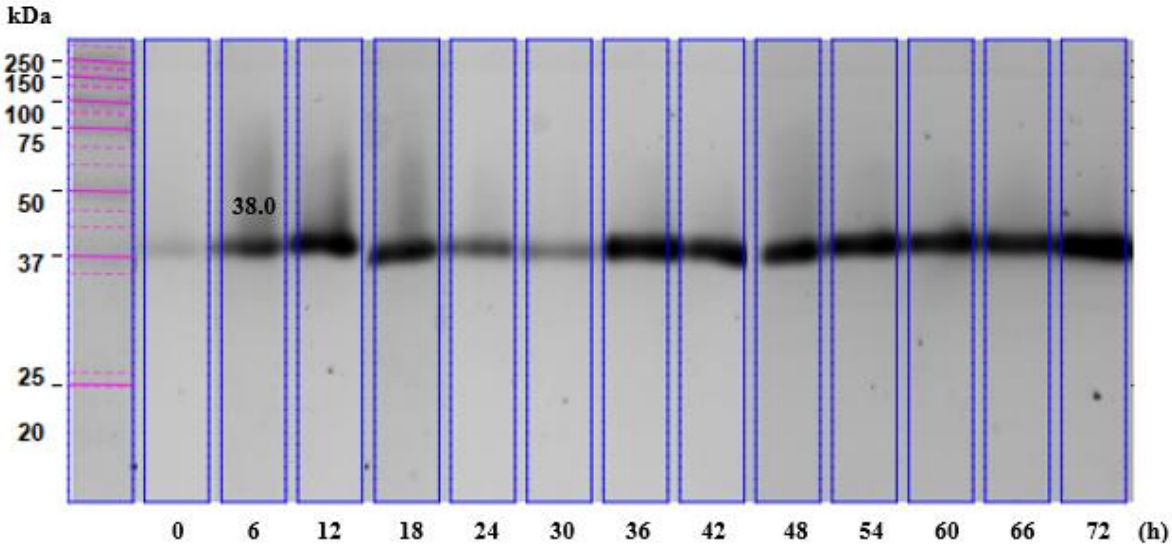

Fig. 7. Esterase zymogram of *T. harzianum* grown in 400 µL of Tween/L-supplemented medium for 72 h in submerged fermentation.

4. DISCUSSION

Tween 80 is a surfactant that has been used for several purposes in microbial cultures. This surfactant has been used to enhance the enzymatic hydrolysis of cellulolytic material in the industry (Jin *et al*., 2016), in agricultural applications (Wang *et al*., 2003), to decrease the surface and interfacial tensions for increasing substrate bioavailability in bioremediation studies (Uzoigwe *et al*., 2015; Wijaya *et al*., 2016; Yang *et al*., 2021) among other applications (Lee & Ha, 2003).

Akpinar & Ozturk (2017) have suggested that Tween 80 enhances permeability of the cell membrane, which facilitate enzyme production and it can also protect the enzyme structure and its activity. It has been reported that Tween 80 can be mineralized to CO_2 and H_2O through β-oxidation. In this context, the cleavage of the ether linkages would form primary alcohol, which would be subsequently oxidized to aldehydes and then to carboxylic acids which would enter the Krebs cycle (Nguyen *et al*., 2018).

Several studies have reported that Tween 80 can be used as carbon and energy source by microorganisms and/or as enzyme inducer. *Acinetobacter baumannii* was able of utilizing Tween 80 as its sole carbon and energy source (Nguyen *et al*., 2018). It was also found that Tween 80 was degraded by *Lasiodiplodia theobromae* (Cao *et al*., 2020) and that this surfactant increased the biomass and the intracellular polysaccharide content in *Lentinula edodes* (Li *et al*., 2018). Nielsen *et al*. (2016) showed that Tween 80 (0.1%) enhanced the growth rate of *Staphylococcus aureus* in batch cultures, and it also increased the total biomass when *S. aureus* was grown as biofilms. Moreover, *Mycobacterium smegmatis* was capable to produce tween hydrolyzing esterase (Tomioka, 1983). In addition, It has been reported that laccase showed its maximum value at a Tween 80 concentration of 0.1% (Elsayed *et al*., 2012). Similarly, the addition of Tween 80 to the culture medium enhanced laccase activity during the degradation of bisphenol A by *Pleurotus sajor-caju* (Teodoro *et al*., 2018). In particular, Tween 80 has been shown to be a laccase inducer in *Pleurotus eryngii* grown in solid state fermentation (Akpinar & Ozturk, 2017). Studies on the production of extracellular α-glucosidase and β-glucosidase by the yeast *Aureobasidium pullulans* showed that the addition of Tween 80 to a 24 h old culture led to a three-fold increase in yield of extracellular α-glucosidase (Okagbue *et al*., 2001). Furthermore, Tween 80 enhanced the lipase activity during the degradation of ketoprofen ester by *Candida rugose* (Liu *et al*., 2000). Investigations on *Trichoderma viride* have revealed that Tween 80 decreases enzyme adsorption to its substrate but enhances enzymatic activity (Kim *et al*., 2006).

The present research have shown that *T. harzianum* was able to use Tween 80 as a carbon and energy source, which is in accord to those findings reported previously for *F. culmorum* (Medina-Flores *et al*., 2020) and for other microorganisms (Nguyen *et al*., 2018; Cao *et al*., 2020; Li *et al*., 2018; Nielsen *et al*., 2016). It has been reported that different concentrations of Tween 80 (100, 400 and 600 µL/L) induced esterase production by *F. culmorum* and that Tween 80 concentration was positively correlated with the number of esterase isoforms produced by *F. culmorum* (Medina-Flores *et al*., 2020). It was found that the higher the Tween 80 concentration (400 and 600 µL/L), the more number of esterase

isoforms were induced in *F. culmorum*. However, lower concentration (100 µL/L) of Tween 80/L did not show a significant effect on the induction of the esterase activity in that fungus (Medina-Flores *et al*., 2020).
In the present study, a single band with esterase activity was observed in the different concentrations of Tween 80 tested (100 and 400 µL/L). However, 400 µL of Tween 80 induced in *T. harzianum* higher esterase activity in comparison to that activity shown in the medium added with 100 µL of Tween 80 per liter.
These results showed that high concentrations (*e.g.* 400 µL/L) of Tween 80 enhanced the esterase activity in *T. harzianum*. The single band with esterase activity revealed on zymograms suggests that this enzyme (of 38 kDa approx.) may be present in both forms as a constitutive as well as an inducible esterase, since it was observed in both glucose- and Tween 80-supplemented media.

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