Cutinolytic Esterases are Induced by Growth of the Fungus *Trichoderma harzianum* on Glyceryl Monostearate in Solid-State Fermentation

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Cutinolytic esterase are enzymes utilized in a wide variety of industrial applications, and they are capable of degrading emerging environmental pollutants. Due to the application and importance of these enzymes, it is crucial to develop an efficient method for cutinase production using a cost-effective inductor and an efficient microbial production system. In this work, the growth and cutinolytic esterase production of *Trichoderma harzianum* were evaluated in glucose-yeast extract media containing different glyceryl monostearate (GMS) concentrations (1, 3, and 5 g/L). It was used as inducer in solid-state fermentation. A medium lacking GMS was used as control. Biomass production and enzyme productivity were higher in inducer-added (1 g/L) medium than in the control medium. *T. harzianum* produced constitutive and inducible cutinolytic esterase, in which production was enhanced by GMS. In GMS-added cultures, two bands with cutinolytic esterase activity (60 and 150 kDa approximately) were observed by zymography, which were not observed in control culture. GMS represents a promising inducer for cutinolytic esterase production by fungi. This research represents the first approach for the study of cutinolytic esterase production using a synthetic molecule as an inducer.

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INTRODUCTION

Enzymes are biomolecules of great importance that are produced by microorganisms and can be used in a wide number of biotechnological applications. In particular, esterases are a group of relevant enzymes that are capable of catalyzing both synthesis and hydrolysis reactions (Hernández-Sánchez *et al*. 2019; Rafeeq *et al*. 2021). There is increasing interest in new enzymes with interesting properties, such as esterase with cutinolytic activity or cutinases that have different substrate affinity or optimal activity in a wide range of temperature and pH (Kumar *et al*. 2023). Cutinases are especially interesting due to their peculiar catalytic properties, since they have characteristics of both esterase and lipases (Ferrario *et al*. 2016). Therefore, cutinases can hydrolyze triacylglycerols and esters, catalyzing esterification and transesterification reactions, which provides them a broad versatility, expanding their application in areas

such as biodiesel production, detergents, food industry, and enzymatic degradation of synthetic polymers and toxic substances (Martínez and Maicas 2021).

These enzymes are especially attractive for their use in bioremediation, since they are capable of degrading emerging environmental pollutants through an ecofriendly, effective, and a low-cost technology (Chen *et al*. 2013; Liang and Zhou 2023). Filamentous fungi are the best cutinases producers due to their natural ability to degrade the plant polyester polymer called cutin, which make some of them plant pathogenic (Moisan *et al*. 2019).

The production of cutinases is regulated by microbial growth conditions, carbon source, and fermentation system (Liang and Zou 2023). The enzymatic activity of cutinases is induced by the presence of cutin and cutin monomers in the culture medium (Degani 2015; González-Márquez *et al*. 2019a; González-Márquez and Sánchez 2022). Cutin is the main component of the plant cell wall. It is formed by 16-carbon fatty acids, among which 10,16-dihydroxyhexadecanoic acid and its isomer 9,16-dihydroxyhexadecanoic acid comprise the main components, while a small fraction is formed by 18-carbon fatty acids (Arya and Cohen 2022).

In this context, cutin from apple derivatives and tomato skin have been widely studied and reported as efficient inducers for cutinase production (Martinez and Maicas 2021). However, the use of natural cutin is not economically viable neither to study cutinase production nor to use it as an inducer in industrial processes, due to low yields and difficulty of extraction (Degani 2015; González-Márquez *et al*. 2019a). Therefore, there is a search for effective and more accessible low-cost cutinase inducers, as well as efficient induction and production methods (Medina-Flores *et al*. 2019; Rueda-Rueda *et al*. 2020).

Glyceryl monostearate (GMS) is an organic molecule synthesized for use as a large-scale oleophilic emulsifier. This emulsifier is made from stearic acid (octadecanoic) and palmitic acid (hexadecanoic) (BASF 2023), having similar components to natural cutin. The effect of GMS on the production of cutinolytic esterase and growth of filamentous fungi is unknown. In this work, the filamentous fungus *T. harzianum* as a hydrolases-producer model was grown on glucose-yeast extract media containing different concentrations (0, 1, 3, and 5 g/L) of GMS as a cutinolytic esterase inducer in solid-state fermentation (SSF). Fungal growth, protein content, glucose consumption, enzyme activity, and enzyme yield parameters were evaluated. This research represents the first approach for the study of cutinolytic esterase production using a synthetic molecule as an inducer.

EXPERIMENTAL

Microorganism and Culture Media

T. harzianum from the culture collection of the Research Centre for Biological Sciences at Universidad Autónoma de Tlaxcala (CICB, Tlaxcala, Mexico) was used. The strain was grown on potato extract agar at 22 °C for 5 d and stored at 4 °C. Cultures were transferred periodically to fresh agar medium for preservation.

The composition of the medium glucose-yeast extract (GYE) was as follows (in g/L): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4$, 0.25; $FeSO_4.7H_2O$, 0.5; $ZnPO_4.7H_2O$, 0.001; $MnSO_4.H_2O$, 0.5. Three media containing different concentrations of cutin® GMS (GMS) were prepared; a) GYE + 1 g of GMS/L,

b) GYE + 3 g of GMS/L, and c) GYE + 5 g of GMS/L. The final pH was adjusted to 7.5 using either 1 M HCl or 1 M NaOH. A GYE medium lacking GMS was used as control.

Inoculation and Culture Growth Conditions

Fragments of mycelium were taken from the periphery of colonies grown on potato-extract agar at 20 °C for 5 d and used as inocula.

Fifteen mL of a sterile culture medium were placed into 250-mL sterile Erlenmeyer flasks containing 0.5 g of treated polyurethane foam (PUF), which was used as an inert support for SSF experiments. PUF was cut into cubes (0.5 cm^3) washed with distilled water, and treated with 0.1 N HCl and 0.1 N NaOH solutions for 24 hours each. Then, it was dried in an oven at 40 °C for 24 h and used. Flasks were inoculated with three mycelial fragments (4 mm in diameter) and incubated at 22 °C for 5 d (total time of fermentation). Analyses were carried out on samples taken at 8 h intervals. Experiments were carried out in triplicate.

Biomass Production and Specific Growth Rate Calculation

Mycelial biomass was filtered from cultures using filter paper (Whatman No. 4). The filter paper was oven dried at 60 °C for 48 h and the specific growth rate (μ) was calculated using the logistic equation as previously specified (Ahuactzin-Pérez *et al*. 2016). Supernatants were collected and used in all the tests.

Protein Content, Glucose Consumption, and pH Measurement

Protein content was measured using the Bradford method (Bradford 1976). 100 µL of supernatant was mixed with 860 µL of sterile distilled water and 40 µL of Bradford reagent (BIORAD). A protein standard curve was undertaken by measuring the absorbance of solutions containing different bovine serum albumin concentrations at 595 nm. Samples were incubated for 10 min at room temperature, and absorbance readings were taken at 595 nm using a spectrophotometer (Jenway 6405UV/Vis, NJ, USA).

Glucose consumption was quantified using the dinitrosalicylic acid reagent (DNS, SIGMA) (Miller 1959). 80 µL of supernatant was mixed with 2 mL of DNS and 920 µL of distilled water. The reaction mixture was boiled in a water bath for 5 min and reaction was stopped by placing the samples on ice. Finally, the absorbance of each sample was read at 575 nm using spectrophotometer (Jenway 6405UV/Vis, NJ, USA). A glucose standard curve was made using known glucose concentrations, which absorbance was measured at 575 nm. pH measurements were taken using a potentiometer (Conductronic® PC45, Mexico).

Enzyme Assays and Enzyme Yield Parameters Calculation

Esterase activity was measured using ρ-nitrophenyl butyrate (ρNPB) as substrate (Ferrer-Parra *et al*. 2018). The reaction mixture contained 100 µL of supernatant and 900 µL of a solution containing the following components: 10 µL of ρNPB (1.76% in acetonitrile) (v/v), 790 µL of 50 mM phosphate buffer at pH 7.5, and 0.04% (v/v) Triton X-100. The reaction mixture was incubated at 37 °C for 5 min and absorbance reading were taken at 405 nm using a spectrophotometer (Jenway 6405UV/Vis, NJ, USA). Cutinolytic esterase activity was expressed in IU (International Units)/L. One international unit (IU) of cutinolytic esterase activity converts one micromole of ρNPB into one micromole of ρ-nitrophenol per minute at 37 °C and pH 7.5 (Speranza and Macedo 2013).

The enzyme specific activity (E_{esp}) is expressed as IU of enzyme per mg of protein (IU/mg of protein).

The theoretical yield of the enzyme in relation to biomass ($Y_{E/X}$) was estimated as the ratio between the maximum enzyme activity obtained during the exponential growth (E_{max}) in IU/L and the maximum biomass production (X_{max}) in g/L (Ahuactzin-Pérez *et al*. 2016). The productivity at the maximum enzyme activity (P_{RO}= E_{max}/time of fermentation) and the specific rate of enzyme production ($q_p=\mu Y_{E/X}$) were calculated as previously reported (Hernández-Dominguez *et al*. 2017; González-Márquez *et al*. 2019b).

Zimographic Analysis

The polypeptide profiles of the samples with esterase activity were analyzed using 0.1% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Leammli 1970). The separating and packing gel contained 12% and 4% acrylamide, respectively. A broad-spectrum molecular weight marker (2-250 kDa, BIO-RAD) was used. Sample buffer contained: 0.5 M Tris-HCl pH 6.8, 35% glycerol, 0.01% bromophenol blue, and 10% SDS. Samples were separated into 0.75 mm thick gels on a Mini Protean Tetra Cell electrophoresis system (Bio-Rad) at 100 volts for 90 min. After running, the gels were washed in 100 Mm Tris-HCl buffer at pH 6.5 with 0.05% Triton X-100 for two 5-minute intervals at 4 °C.

Subsequently, the gels were incubated for 3 h at room temperature in a solution with 3 mM α-naphthyl acetate, 1 mM Fast Red TR (Sigma) and 100 mM phosphate buffer at pH 7.5 (Karpushova *et al*. 2005; Canavati-Alatorre *et al*. 2016; Ríos-González *et al*. 2019). Finally, the gels were placed on a vinyl acetate sheet, and the activity of esterase was detected by the appearance of red bands, which image was then digitalized.

Statistical Analysis

Statistical analysis was carried out using a factorial Analysis of Variance (ANOVA) and post Hoc Tukey HSD test for multiple comparisons (significance level of $p < 0.05$) using the SigmaPlot® version 12.0 software. Parameters were measured in triplicate in three separate experiments.

RESULTS AND DISCUSSION

Biomass Production and Glucose Consumption

Figure 1 shows biomass production (X) of *T. harzianum* grown in GMS-added media and control medium. The fungus reached the stationary phase after approximately 40 h and 56 h in GMS-added media, and in control medium, respectively.

Maximum biomass (X_{max}) production was shown in medium added with 5 g of GMS/L (10.6 g/L), followed by 3 of GMS/L (9.8 g/L) and 1 g of GMS/L (8.8 g/L). The lowest biomass production was shown in the control medium (7.6 g/L) (Table 1). GMS-added media had the highest μ values. The lowest μ value was obtained in control medium (Table 1).

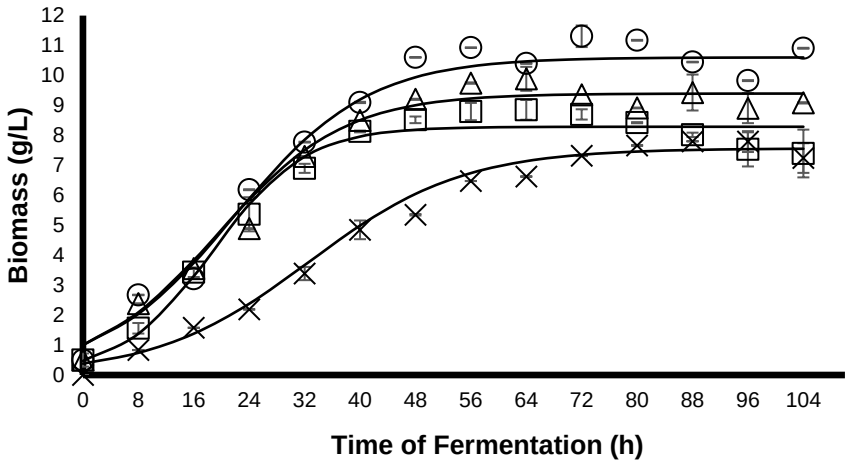

Fig. 1. Biomass production of *T. harzianum* grown in GYE (x), GYE+1 g GMS/L (□), GYE+3 g GMS/L (Δ) and GYE+5 g GMS/L (○) in SSF.

Table 1. Growth Kinetic and Enzyme Yields Parameters of *T. harzianum* Grown in Different GMS Concentrations in SSF

PARAMETER	CULTURE MEDIA			
	GYE	GYE + 1 g GMS/L	GYE + 3 g GMS/L	GYE + 5 g GMS/L
μ (h^{-1})	0.06^{b} (0.01)	0.11^{a} (0.04)	0.10^{a} (0.01)	0.10^{a} (0.01)
X_{max} (g/L)	7.6^{d} (0.02)	8.8^{c} (0.03)	9.8^{b} (0.01)	10.6^{a} (0.02)
E_{max} (UI/L)	420.23^{c} (0.09)	428.9^{b} (0.07)	420.11^{c} (0.03)	433.01^{a} (0.02)
E_{esp} (UI/mg)	49.59^{b} (0.08)	56.47^{a} (0.04)	33.81^{c} (0.03)	46.80^{b} (0.03)
$Y_{E/X}$ (UI/gX)	55.29^{a} (0.01)	48.76^{b} (0.03)	42.86^{c} (0.03)	40.85^{c} (0.01)
P_{RO} (UI/L/h)	4.78^{c} (0.02)	6.70^{a} (0.03)	5.83^{b} (0.02)	6.01^{b} (0.01)
q_p (UI/gX/h)	3.32^{c} (0.01)	5.36^{a} (0.04)	4.28^{b} (0.05)	4.08^{b} (0.02)

Note: Means with the same letter in the same row do not differ significantly. Numbers in parentheses correspond to the standard deviation of three independent experiments.

Glucose consumption by *T. harzianum* is shown in Fig. 2. Glucose decreased by approximately 50% after 40 h of fermentation, when fungus reached the stationary phase. Glucose was completely consumed from the culture after 72 h in control medium, whereas a small amount of glucose remained after 104 h in GMS-added media.

These results showed that *T. harzianum* was capable of using GMS as carbon source, due to the production of enzymes that degrade the polyester polymers into monomer assimilable by this fungus. Martins *et al*. (2014) demonstrated that suberin, a plant polyester with molecular structure similar to cutin, was used as carbon source by the fungus *Aspergillus nidulans*. It was reported that suberin undergoes ester hydrolysis and fatty acid oxidation, releasing fatty acids, which are broken down through β-oxidation, and then transformed in acetyl-CoA, which enter into the Krebs cycle. Furthermore, González-

Márquez *et al*. (2019a) reported that the fungus *Fusarium culmorum* was capable to use apple cutin at different concentrations (0.2, 2 and 20 g/L) as the sole carbon source. It was found that cutin acted as a cutinase inducer, and the maximum biomass production was observed when using 20 g of apple cutin/L.

Ahmed *et al*. (2017) evaluated the growth of *T. harzianum* using different concentration of rice polishing as substrate under optimal conditions and found that the μ value ranged from 0.09 to 0.23 h^{-1}. In the present research, μ values were higher in GMS-added media (0.1 h^{-1}) as compared to control media (0.06 h^{-1}), showing that GMS enhanced the fungal growth. The μ values obtained using a synthetic polymer were within the range of those previously reported for the growth of this fungus in natural substrates.

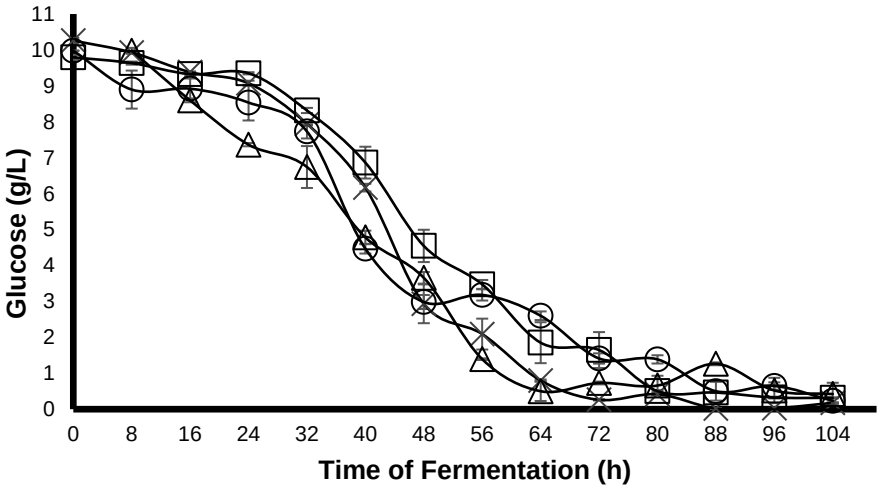

Fig. 2. Glucose consumption of *T. harzianum* grown in GYE (x), GYE+1 g GMS/L (□), GYE+3 g GMS/L (Δ) and GYE+5 g GMS/L (○) in SSF

pH Measurements in the Cultures

Figure 3 shows the pH variation of cultures of *T. harzianum* during fermentation. The pH value of the cultures decreased during the first 24 h of fermentation to a minimum of 3.4 in control medium. The pH of the cultures then increased and reached 8.6 after 104 h of growth in control medium. In general, GMS-added media showed a similar pH value during the fermentation, which after 24 h decreased, and then increased, reaching pH 7.2 by the end of the cultivation.

The pH value in the cultures is an indicator of the substrate degradation process. Acid molecules were released into the media during the first 24 h of fermentation, decreasing the pH value, whereas amino compounds were formed after 32 h, which increased the pH value (González-Márquez *et al*. 2020; González-Márquez and Sánchez 2022).

Gonzalez-Márquez *et al*. (2019a) studied the growth of the fungus *F. culmorum* using different apple cutin concentrations (0.2, 2, and 20 g/L) under submerged fermentation conditions and found that the pH of the cultures increased from the initial pH 6.5 to pH 7.5 after 168 h. The pH variation of the fungal cultures depends on the substrate composition and fermentation system. Moreover, fungi modify the pH of their surroundings to meet their growth needs (Vylkova 2017; Sánchez *et al*. 2020).

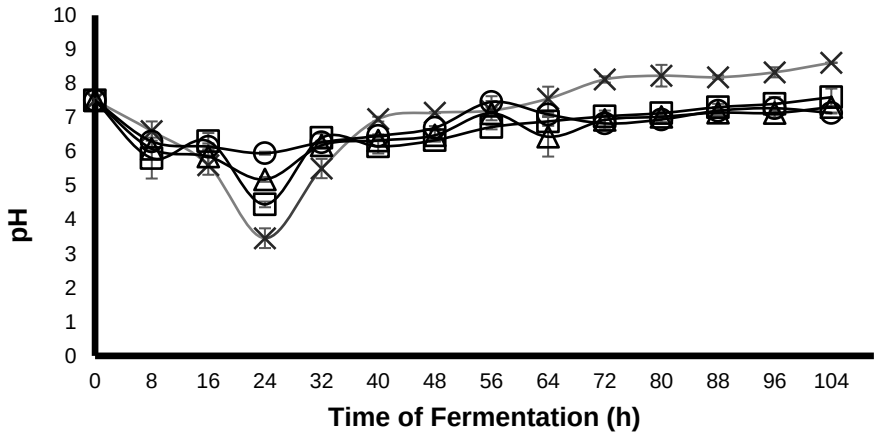

Fig. 3. pH of cultures of *T. harzianum* grown in GYE (x), GYE+1 g GMS/L (□), GYE+3 g GMS/L (Δ) and GYE+5 g GMS/L (○) in SSF

Protein Content and Enzyme Activity

In general, protein production by *T. harzianum* increased during the fermentation in all media, showing a decrease at 104 h in media containing 1 and 5 g of GMS/L. Medium added with 5 g of GMS/L showed the highest protein content from 56 to 96 h, whereas the lowest protein content was observed at 32 h in control medium (Fig. 4). Cutinolytic esterase activity of *T. harzianum* grown in the different culture media is shown in Fig. 5. Enzyme activity was positively correlated with the concentration of GMS added into the culture medium, cutinolytic esterase activity was enhanced by high GMS concentration.

Media containing 0, 1, and 3 g GMS/L had the lowest enzyme activity values during the first 40 h of fermentation. The highest enzymatic activity was observed during the first 48 h of fermentation in medium containing 5 g of GMS/L. In all media, the enzyme activity increased during the first 48 h of fermentation and then was kept constant (approximately 400 U/L). This result reflects the fungal growth curve; the end of the exponential phase of growth was observed at 48 h, beginning the stationary phase after that time of fermentation. Enzyme yield parameters showed that the E_{max} (433 UI/L) was observed in media added with 5 g of GMS/L, whereas the highest E_{esp}, P_{RO} and q_p (56.4 UI/mg, 6.7 UI/L/h and 5.4 UI/gX/h, respectively) were observed in media containing 1 g of GMS/L (Table 1). These results showed that 1 g of GMS/L was the best inducer concentration for fungal growth and enzyme production.

González-Márquez *et al*. (2019a) studied the growth of *Fusarium culmorum* and the effect of different apple cutin concentrations (0.2, 2 and 20 g/L) as inductor added into liquid media, observing that biomass, protein content and enzymatic activity were positively correlated with the apple cutin concentration added into the medium. However, the highest P_{RO} (3 UI/L/h) and q_p (12 UI/h/gX) were observed in media added with 2 g of apple cutin/L.

This research shows that cultures of *T. harzianum* showed about twice as high P_{RO} in that media containing 1 g of GMS/L in SSF than *F. culmorum* grown in medium added with 2 g of apple cutin/L in liquid fermentation. These results suggest that SSF fermentation is a more efficient fermentation system than liquid fermentation as reported previously (Viniegra-González *et al*. 2003) and that GMS/L is an efficient inducer for cutinolytic esterases production.

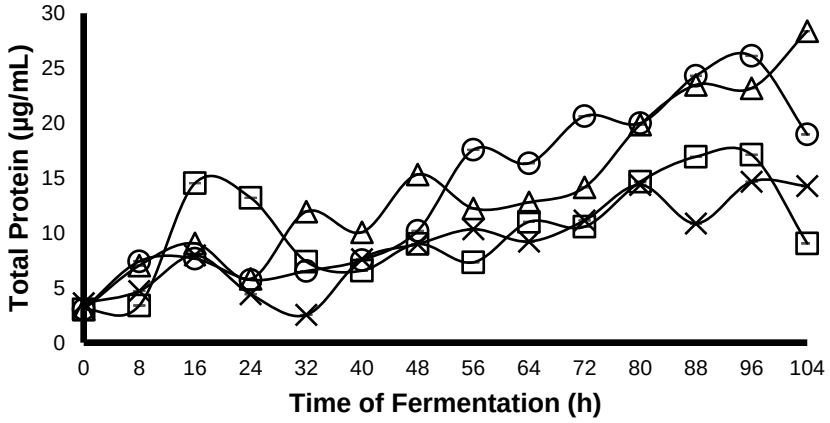

Fig. 4. Protein content of *T. harzianum* grown in GYE (x), GYE+1 g GMS/L (□), GYE+3 g GMS/L (Δ) and GYE+5 g GMS/L (○) in SSF

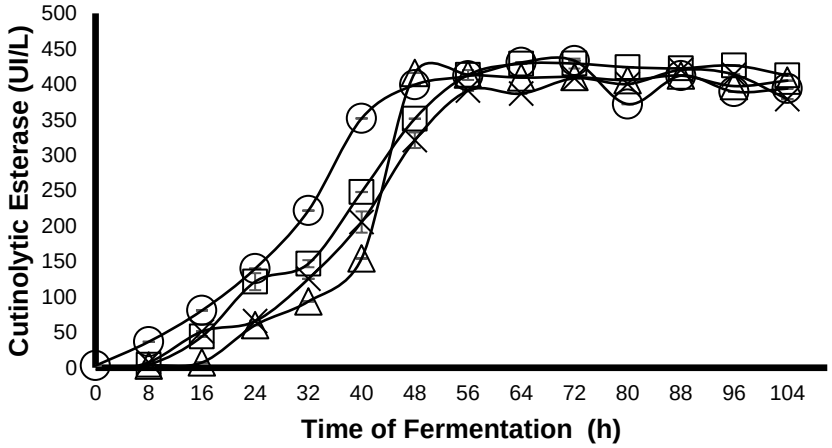

Fig. 5. Cutinolytic esterase activity of *T. harzianum* grown in GYE (x), GYE+1 g GMS/L (□), GYE+3 g GMS/L (Δ) and GYE+5 g GMS/L (○) in SSF

Zimographic Analysis

Zymographic analysis showed a band with cutinolytic esterase activity with a molecular weight of approximately 25 kDa in control medium (Fig. 6a). The influence of 1, 3 and 5 g of GMS/L as inductor of the cutinolytic esterase activity is observed in Figs. 6b, 6c, and 6d, respectively. In zymogram of control culture, a band with cutinolytic esterase activity of approximately 25 kDa was visualized from 32 h to the end of the fermentation (Fig. 6a). In all zymograms of GMS-added cultures, two additional bands to that observed in control cultures, were shown having a molecular weight of approximately 60 and 150 kDa. The staining intensity of the bands with cutinolytic esterase activity showed on the gels was enhanced as the GMS concentration increased. In cultures added with 1 g of GMS, bands with molecular weight of approximately 25, 60, and 150 kDa (lightly stained) appeared from 24, 64, and 80 h, respectively, to the end of the fermentation (Fig. 6b). In general, zymograms of media containing 3 and 5 g of GMS/L showed three bands, with molecular weights of approximately 25, 60 (lightly stained), and 150 kDa (lightly stained), which appeared from 8, 48, and 72 h, respectively, to the end of the fermentation (Figs. 6c and 6d). These results showed that GMS induced the production of

two isoenzymes of approximately 60 and 150 kDa in *T. harzianum*. The band of approximately 25 kDa is a constitutive isoenzyme (as it was revealed in control medium). It appeared during all the fermentation in media containing 3 and 5 g of GMS/L. *T. harzianum* increased the production of that isoenzyme (25 kDa) with increasing GMS concentrations.

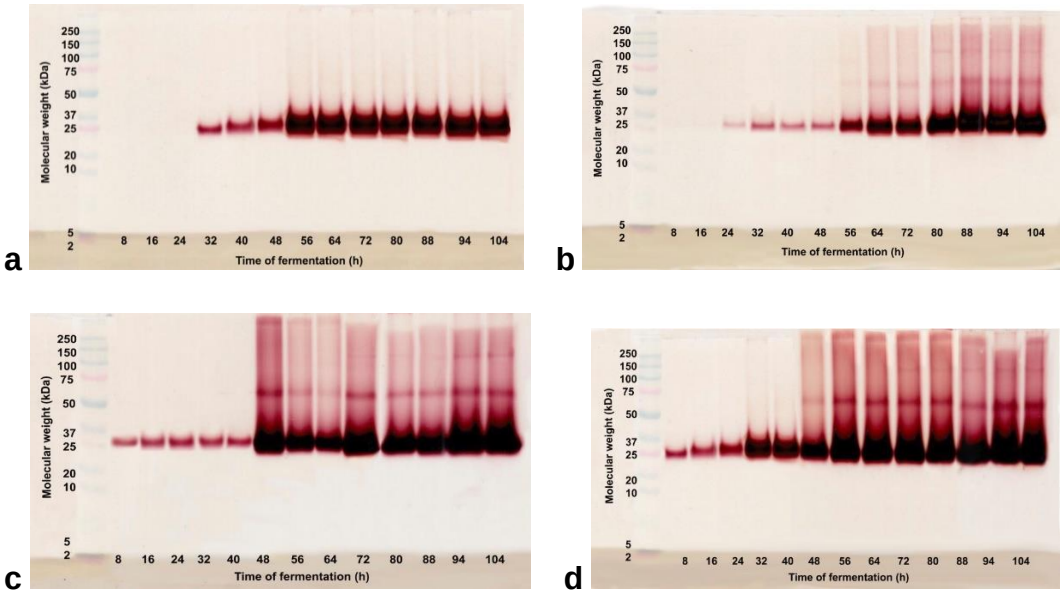

Fig. 6. Zymogram of cutinolytic esterase of *T. harzianum* grown in GYE (a), GYE+1 g GMS/L (b), GYE+3 g GMS/L (c) and GYE+5 g GMS/L (d) in SSF

Hawthorne *et al*. (2001) studied the inducer effect of different concentrations (ranging from 80 mg/L to 2.5 g/L) of apple cutin and other fruits cutin in strains of *Fusarium solani* and observed that cutin was able to induce cutinolytic esterase in liquid medium. Furthermore, Degani (2015) found that *Fusarium oxysporum* was able to produce a cutinolytic esterase with a molecular weight of approximately 20 kDa, using raw apple cutin as an inductor in liquid medium. In addition, Macedo and Pio (2005) studied the use of different fruit cutin as cutinases inducer and reported that *F. oxysporum* grown in a medium added with 1% cutin and incubated for 12 d showed the best performance for cutinase production.

In bacteria and in some filamentous fungi (*i.e*. *F. oxysporum*), natural cutin acted as cutinases inducer; however, cutinases production was repressed by the presence of glucose in the medium (Fett *et al*. 2000; Macedo and Pio 2005). In the current study, enzyme production was not repressed by glucose. In fact, GMS enhanced enzyme production in the different media tested.

Rubio *et al*. (2008) reported that *T. harzianum* produced cutinases, which were induced by olive oil and the cutin monomer 16-hydroxy-hexadecanoic acid. However, cutinase excretion was repressed by glucose. In this case, the theoretical cutinase molecular weight was of 26 kDa. Castro-Ochoa *et al*. (2012) reported that *Aspergillus nidulans* produced a cutinase induced by olive oil, which had a molecular weight of 29 kDa.

In the present research, a band showing a high molecular weight of 150 kDa was observed, which could be a protein having a polymeric structure formed by six subunits of proteins of 25 kDa (which was observed throughout the fermentation). In this context, it has been reported that most enzymes might exist as polymeric structures (oligomers), and

some of them can reversibly dissociate and reassociate in response to an effector ligand (Traut 1994). However, further analysis needs to be performed on the type, catalysis and properties of the enzymes produced by the fungus during its grown on GMS-added media.

This research highlights the importance of studying the use of alternative cutinase inducers to natural cutin, identifying of efficient microorganisms in the production of cutinolytic esterases, and evaluating the use of different production methods. Further studies are required to determine efficient methods and promising candidate organisms for cutinases production, as well as investigate the catalytic performance of new isolated cutinases.

CONCLUSIONS

1. *Trichoderma harzianum* produced constitutive and inducible cutinolytic esterase, which production was enhanced by GMS.
2. *T. harzianum* produced cutinolytic esterases with molecular weight of approximately 25 kDa and isoenzymes of higher molecular weight by adding GMS.
3. GMS is an efficient inducer for cutinolytic esterases production.

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