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TANNASE PRODUCTION BY THE XEROPHILIC *Aspergillus niger* GH1 STRAIN AND PARTIAL ISOLATION OF THE TANNASE GENE

PRODUCCIÓN DE TANASA POR LACEPA XERÓFILA *Aspergillus niger* GH1 Y AISLAMIENTO PARICAL DEL GEN DE TANASA

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Abstract

Tannase is an enzyme widely used in the food industry, mainly for wine and beer production. This enzyme has been identified in different fungus isolated from Mexican semi-desert plants. Some of these fungal strains such as *Aspergillus niger* GH1 and PSH strain have demonstrated to be excellent enzyme producers. In the present study, tannase was produced under submerged state culture, with its maximum activity at 24 h. At the same time, a fragment of tannase gene from GH1 and PSH strains was isolated and expression of tannase was determinate by RT-PCR (at 16 h,). It was found that tannase sequences of GH1 and PSH strain are 97% similar to the *A. niger* strain SL-5 reported in GeneBank. The translation of GH1 and PSH amino acid sequences demonstrated its identity with tannase.

Keywords: *Aspergillus niger*, submerged culture, tannase gene, tannin acil hydrolyse.

Resumen

La enzima tanasa es muy utilizada en la industria alimentaria, principalmente para la producción de vino y cerveza. Se ha identificado la enzima en hongos asilados de plantas del semi-desierto mexicano. Las cepas de *A. niger* GH1 y PSH han demostrado ser productores de la enzima. En el presente estudio, se obtuvo la enzima por cultivo sumergido con la máxima actividad a las 24 h. Al mismo tiempo, se aisló un fragmento del gen de tanasa de las cepas GH1 y PSH, la expresión del gen se determinó cualitativamente por RT-PCR (a las 16 h). Se encontró que las secuencias de las cepas GH1 y PSH son 97% similares con la secuencia de *A. niger* SL-5 reportada en el GenBank. La secuencia traducida a aminoácidos demostró su identidad con tanasa.

Palabras clave: *Aspergillus niger*, cultivo sumergido, gen de tanasa, tanin acil hidrolasa.

1 Introduction

Tannase or tannin acyl hydrolase (3.1.1.20) catalyzes the hydrolysis reaction of ester bonds present in gallotannins, complex tannins, and gallic acid esters (Mukherjee, 2007). This enzyme is used for quality improvement of beer and wine production. Tannase is used for treatment of juice and solid residues of grape. In addition, this enzyme is employed to clarify some fruit juices and for tannin elimination of contaminants in effluents of the leather industry (Aguilar *et al*., 2001; Ramírez *et al*., 2008). Other important use of this enzyme is for production of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry (Lekha and Lonsane, 1994). Actually, tannase is used as an indicator of colon cancer, manufacture of cosmetics, treatment of agroindustrial wastes for ethanol production, and others (Aguilar-Zarate *et al*., 2014). Tannase has been identified in animals, vegetables and microorganisms. This last one is the most important source because the produced enzyme is more stable; it is produced in great amounts, in constant form, and placed under fermentation techniques, enzyme production and titers of enzymatic activity increase (Lekha and Lonsane, 1997).

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Since Hatamoto *et al*. (1996) described the tannase sequence from *A. oryzae*, which had 1767 bp and 588 amino acids with a signal peptide of 18 amino acids, new source of tannase has been searched. Cruz-Hernández *et al*. (2005) isolated different tannin-degrading strains from Mexican semi-desert plants and soils. Two of the most important isolated strains are *Aspergillus niger* GH1 and PSH, which have been reported as tannase producer strains in both submerged (SmF) and solid state fermentation (SSF) (Belmares *et al*., 2003; Mata-Gómez *et al*., 2009; Flores-Maltos *et al*., 2011). According to the above, the purpose of the present study were identifying, expressing and sequencing the tannase gene from two *Aspergillus niger* GH1 and PSH strains and stablishing genetic relationship of the obtained sequences with other reported sequences of tannase.

2 Materials and methods

2.1 *Strains, medium composition and chemicals*

Aspergillus niger GH1 and PSH strains were obtained from the DIA-UAdeC culture collection. The fungal strains were propagated using the Czapek medium which was prepared with the following composition (g/L): KH_2PO_4 (2.19), $(NH_4)_2SO_4$ (4.38), $MgSO_4{\cdot}7H_2O$ (0.44), $CaCl_2{\cdot}7H_2O$ (0.044), $MnCl_2{\cdot}6H_2O$ (0.009), $NaMoO_4{\cdot}2H_2O$ (0.004) and $FeSO_4{\cdot}7H_2O$ (0.06). These reagents were obtained from Productos Químicos Monterrey (Monterrey, Nuevo Leon, Mex). Tannic acid, methytl gallate and gallic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO).

2.2 *Submerged fermentation*

Aspergillus niger GH1 and PSH strains were grown on potato dextrose agar (PDA) at 30° C for 6 days. Fungal spores were harvested using 0.1% Tween 80 and were counted in a Neubauer chamber. 1×10^6 spores / mL were used as inoculums. Erlenmeyer flasks (250 mL) with 30 mL of Czapek medium and tannic acid (25 g/L) were employed as reactors. The pH was adjusted at 5.0, inoculum of 1×10^6 spores / mL was added to the culture medium. Reactors were incubated at 35°C and shaked in a multi-wrist Shaker system at speed 4 and 250 rpm. Kinetic of tannase production was monitored for 72 h and samples were taken every 12 h. The experiment was carried out in duplicate.

2.2.1 Tannase assay

The filtered enzymatic extracts were dialyzed with a buffer of citrate (pH 5.0) for 24 h using a cellulose membrane. The tannase activity was determined employing the technique reported by Sharma (2000). Briefly, in this step, it was used 0.01 M methytl gallate as substrate, which was prepared in 0.05 M citrate buffer (pH= 5.0). In addition, acid gallic (100 ppm) was used as standard. Absorbance was measured at 520 nm using a Thermo Spectronic Biomate spectrophotometer. One unit of tannase was defined as the amount of the enzyme able to release one μmol of gallic acid per minute under the assayed conditions (reaction time: 25 min. and temperature: 30°C).

2.2.2 Protein assay

Protein content was measured using Bradford (1976) technique. Serum bovine album at 1000 ppm was used as standard. Absorbance was measured at 595 nm using a Thermo Spectronic Biomate spectrophotometer.

2.3 *PCR protocol for tannase gene amplification*

Fungal biomass was produced in Czapek medium, using glucose (30 g/L) as carbon source. Inoculum of 200 μL was added to Erlenmeyer flask of 250 mL, which contained the culture medium. The flask was incubated at 200 rpm, at 30°C during 5 days. Subsequently, the biomass was washed three times with distilled sterile water, then biomass was frozen using liquid N_2. Fungal biomass was macerated to obtain a white and fine powder. DNA extraction technique used TES extraction buffer (50 mM pH 7.5 Tris-HCl, 20 mM EDTA and 1% SDS) as reported by Barth and Gallardin (1996). Some modifications were realized; the addition of PEG was eliminated from the technique. On the other hand, DNA washes were performed with phenol/chloroform/isoamyl alcohol (25:24:1), addition of 7.5 M ammonium acetate and cold 100% ethanol (v/v) was included. Polymerase Chain Reaction (PCR) was performed as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min. Then, a final elongation at 72°C for 5 minutes was done. TAN1 (5'-gRTAgAACTggTACCA-3') and TAN 2 (5'-ACCTCTCCTggCAgATYg-3')

oligonucleotides at 10 mM, previously designed (Cerda-Gómez, 2006) were employed for PCR protocol. Paq5000 DNA Polymerase (5U/μL) Agilent Technologies was used in this procedure. DNA of *Aspergillus niger* Aa-20 was used as positive control. This strain was reported previously as tannase producer.

2.3.1. Sequencing and bioinformatics analysis

Sequencing reactions were performed by Sanger method and using TAN1 and TAN2 oligonucleotides. The obtained sequences were analyzed in Bioedit (Hall, 1999) and Blastn 2.2.27 (Zhang *et al*., 2000; Larkin *et al*., 2007). Finally, phylogenetic analysis was conducted using Mega 5.1 (Tamura *et al*., 2011).

2.4 Tannase gene expression

Other submerged culture was performed using the conditions mentioned previously. Samples were monitored every 6 h. Biomass was collected and RNA was extracted using Trizol Reagent. Then, cDNA was obtained by RT-PCR according to Durón-Vázquez (2011). Similarly, PCR was done as mentioned above.

3 Results and discussion

Tannase production by *A. niger* GH1 was detected and its maximum activity was 344 U/L at 24 h of culture. In the next hours, a decrease was observed (Figure 1). This behavior is due to proteolysis as described by Aguilar *et al*. (2002) whom observed that to high tannic acid concentration (100 g/L), this activity is minor; and at low tannic acid concentration, the protease activity of *A. niger* Aa-20 was high in the culture medium. Other authors have reported the tannase activity from different *Aspergillus niger* isolates, where the enzymatic activity is higher than that obtained in the present study (Table 1). This tendency may be due to inoculums size, tannic acid concentration and fermentation time. However, similarly results were obtained by De la Cerda *et al*. (2011) whom used *A. niger* GH1 for tannase production. Considering these factors, tannase production should be optimized. By the way, specific activity was calculated, in this case 4 U/mg of protein were showed at 24 h of SmC. Minor values of protein has been reported for *A. niger* AUMC 4301 of 0.038 U/ mg protein at 5 days of incubation (El-Fouly *et al*., 2010).

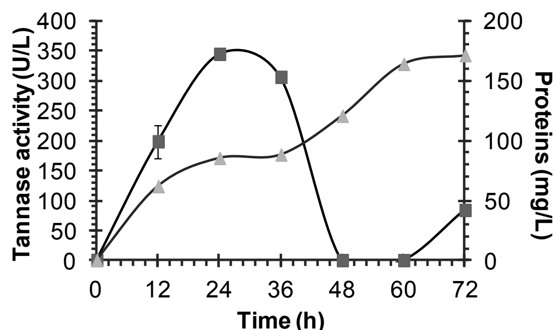

Fig. 1. Tannase (■) and proteins (▲) secretions detected during Submerged Culture from *Aspergillus niger* GH1.

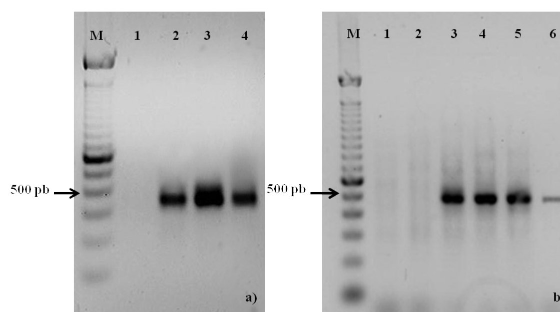

Fig. 2. PCR amplification of a tannase gene fragment (a). M: molecular size marker, 1: water nuclease free as negative control, 2: positive control (Aa-20), 3 and 4 GH1 and PSH DNA genomic, respectively. (b) Tannase expression at different times during SmC. 1) 6 h; 2) 12 h; 3) 18 h; 4) 24 h; 5) 30 h and 6) 36 h. Electrophoresis was done using 1 % (w/v) agarose gel, TAE-1X buffer and the gel was stained using ethidium bromure.

A single band of 412 pb was detected after the PCR and RT-PCR analyses (Fig. 2a and 2b, respectively), which corresponded to a fragment of tannase gene. Alignments realized in Blastn 2.2.28+ of the tannase sequences obtained in the present study demonstrated that PSH (310 pb) and GH1 (298 pb) sequences had 97% similarity with *A. niger* strain SL-5(Acc. No. JN848716.1) begins at 893position. In addition, similarity of 95% with *A. niger* CBS 513.88 (Acc. No. XM_001402449.1) in 1106 position was detected; this is confirmed by genome sequencing of the strain, according to Pel *et al*. (2007). Other values found: 91% with *Aspergillus awamori* strain BTMFW032 (Acc. No.GQ337057.2) (Beena *et al*., 2010) and 87% with tannase from *A. niger* (TanAni) (DQ185610.2) begins in 2928 nucleotide. Meanwhile, the gene expression was detected after 18 h (Figure 2b) in SmC, assuming a decrease at 36 h, this can

Table 1. Different studies about tannase activitiy from *Aspergillus* species reported previously.

***Aspergillus* species**	**Tannic acid (g/L)**	**Inoculum (spores/ mL)**	**Tannase activity [U/L] time (h)**	**Reference**
niger HA37	10	1 x 10^7	1400 (12)	Aissam *y col.* (2005)
niger Aa-20	25	2 x 10^7	2000 (40)	Aguilar *y col.* (2001)
niger GH1	12.5	1 x 10^7	537 (30)	Cruz-Hernández *y col.*, 2006
niger FETL FT3	10	1 x 10^7	1410 (144)	Darah *y col.* (2011)
niger GH1	12.5	5 x 10^6	313(20)	De la Cerda *y col.* 2011.
niger PSH	12.5	5 x 10^6	200 (24)	De la Cerda *y col.* 2011.
niger Aa-20	12.5	5 x 10^6	200 (24)	De la Cerda *y col.* 2011.
niger GH1	25	1 x 10^6	344 (24)	Present study

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>Tannase from PSH strain

TDSWTLSIPSTGGEYVTKFVQLLNIDNLENLDNVTYDTLVDWMNIGMIRY
IDSLQTTVIDLTTFKESGGKMIHYHGESDPSIPTASSVHYWQSVRQAMYP
NT

> Tannase from GH1 strain

TDSWTLSIPSTGGEYVTKFVQLLNIDNLENLDNVTYDTLVDWMNIGMIRY
IDSLQTTVIDLTTFKESGGKMIHYHGESDPSIPTASSVHYWQSVRQAM

>Fragment tannase gene from GH1 strain

CCACTGACTCCTGGACTCTGAGCATCCCCTCTACCGGTGGCGAGTACGTT
ACCAAGTTCGTGCAGCTCCTCAACATCGATAACCTGGAGAACCTCGACAA
CGTCACCTACGACACCCTGGTCGACTGGATGAACATCGGTATGATTCGCT
ACATTGACAGTCTCCAGACCACCGTCATCGACCTCACCACCTTCAAGGAG
TCCGGTGGTAAGATGATCCACTACCATGGTGAATCCGACCCCAGTATCCC
CACCGCCTCGTCCGTCCACTACTGGCAGTCTGTCCGTCAGGCCATGTA

>Fragment tannase gene from PSH strain

CCACTGACTCCTGGACTCTGAGCATCCCCTCTACCGGTGGCGAGTACGTT
ACCAAGTTCGTGCAGCTCCTCAACATCGATAACCTGGAGAACCTCGACAA
CGTCACCTACGACACCCTGGTCGACTGGATGAACATCGGTATGATTCGCT
ACATTGACAGTCTCCAGACCACCGTCATCGACCTCACCACCTTCAAGGAG
TCCGGTGGTAAGATGATCCACTACCATGGTGAATCCGACCCCAGTATCCC
CACCGCCTCGTCCGTCCACTACTGGCAGTCTGTCCGTCAGGCCATGTACC
CCAACACCAC
```

Fig. 3. Phylogenetic relationship among tannase sequences from *Aspergillus* species reported in GenBank and the tanasse sequences from *A. niger* GH1 and PSH obtained in the present study. NJ model with 1000 bootstrap re-sampling and Tamura 3-parameter substitution model was employment using MEGA 5.1 program.

be confirmed with the tannase activity decrease at the same time (Figure 1) by reasons discussed before. The phylogenetic relationship for homogeneity assumption was constructed (Figure 3). Sequence of *A. niger* GH1 and PSH were grouped with *A. niger* strain SL-5, this is interpreted as higher similarity and common ancestor for the gene.

Finally, amino acid sequence were aligned, the 4, 42 and 91positions are detected as Trp, which is localized in core proteins and play an important role on its stability and it is a very conserved codon, thus, it is difficult that present a mutation. However, if a mutation is showed, generally, Trp is substituted by other aromatic amino acid such as Phe and Tyr, as in 4 and 92 positions. Tannase sequences were evaluated in Motif Scan (Sigrist *et al.*, 2010). First, sequence 12-99 amino acids were confirmed that our sequence corresponded to tannase and feruloyl sterase, the last enzyme is involved in cosmetic and health industries (Camacho-Ruiz *et al.*, 2014). Position between 63-65 and 93-95 are denominated as protein kinase C phosphorylation site. In addition, positions between 11-14, 38-41, 57-60 and 63-66 are recognized as cistein kinase II phosphorylation sites. Protein kinases are responsible for proteic function regulation; the post translation modification is fast and reversible (Campbell *et al.*, 2007). These data were confirmed using NetPhos 2.0 tool (Blom *et al.*, 1999), where seven phosphorilation sites were detected in GH1 tannase sequence: 2 of Ser (10, and 67 positions), 3 of Thr (38, 56 and 63 positions) and 2 of Tyr (50 and 90 positions). Those amino acids are very reactive because of its hydroxyl group.

Conclusions

Tannase activity by *A. niger* GH1 was observed using Submerged Culture with the maximum activity at 24 h. In addition, a fragment of tannase gene from *A. niger* GH1 and PSH was identified by PCR and RT-PCR. These genes are highly similar to those reported previously. Tannase amino acid sequence was confirmed by bioinformatic tools and was identified as tannase and feruloyl sterase with sites of proteic function regulation. Future studies are necessaries for isolate the full tannase gene and to study its biochemical properties.

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