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Research Article

Enzymatic Biotransformation of Pomegranate Ellagitannins: Initial Approach to Reaction Conditions

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Background: Ellagitannase (Ellagitannin acyl hydrolase) is an inducible enzyme with great potential use in food industry since allows the ellagic acid release from ellagitannins.
Objective: In this work, ellagitannase was produced by the fungus *Aspergillus niger* GH1 in solid state fermentation using polyurethane foam as solid support and pomegranate husk ellagitannins as sole carbon source and ellagitannase inducer and an initial approach to the enzymatic reaction conditions was reached.
Materials and Methods: Ellagitannase was produced by *Aspergillus niger* GH1 in solid state fermentation and the ideal reaction conditions for ellagitannase activity based on ellagic acid quantification as ellagitannins biotransformation product by high performance liquid chromatographic are reported.
Results: The enzyme ideal reaction conditions were: substrate concentration of 1 mg.mL^{-1}, 60 °C and pH 5.0, during 10 min of reaction. The kinetic enzyme constants (V_{max} = 30.34 mM.mL^{-1}.min^{-1} and K_m = 1.48 x 10^3 mM) using punicalagin as substrate were determined.
Conclusion: The assay was completed in a short time and may find application in future studies of ellagic acid production.
Keywords: *Aspergillus niger* GH1, Ellagitannin acyl hydrolase, Ellagitannase assay, HPLC, Pomegranate ellagitannins.

1. Background

Ellagitannase or ellagitannin acyl hydrolase (EAH) is a novel enzyme reported as responsible of ellagitannins (ET's) biodegradation. EAH has been recently associated with ellagic acid accumulation by hydrolysis of ester bonds between glucose and the hexahydroxydiphenic acid (HHDP) group of ETs; after release the HHDP group spontaneously forms ellagic acid by lactonization (1).
Ellagic acid (4,4′,5,5′,6,6′-Hexahydroxydiphenic acid 2,6,2′,6′-dilactone) is a molecule with a molecular weight of 302.19 g.moL^{-1}, its chemical structure gives high stability to a wide range of temperature (up to 300 °C) and pH conditions (1.8-7.3) (2, 3). Due to stability of the molecule and the hydroxyl groups the ellagic acid present biological properties and benefits on human health, such as antioxidant (4), anti-inflammatory (5), antimicrobial (6), antiviral (7, 8), inhibit adipogenesis (9), oxidative stress (10).
To perform this specific enzymatic reaction, ellagitannins, an enzyme extract and a substrate with a suitable level of purity are needed to establish appropriate reaction conditions (11). Previous reports have demonstrated the activity of EAH using pomegranate ellagitannins as a substrate. Different enzyme activities were performed (polyphenoloxidase, cellulase, tannase, β-glucosidase) in order to establish the relationship between enzyme and ellagitannins biotransformation, and EAH was the enzyme directly related to the biotransformation mentioned (12). On the other hand, the EAH activity was analyzed in detail using punicalagin as substrate and the main intermediary compounds were identified, determining a biotransformation pathway formed by punicalagin, punicalin, gallagic acid finally ellagic acid (13).

2. Objective

This paper describes the production by solid state fermentation of EAH, and the definition of ideal reaction conditions for enzymatic activity.

3. Materials and Methods

3.1. Extraction and Analysis of Ppomegranate Husk Ellagitannins

Pomegranate husk ellagitannins was obtained according to Ascacio-Valdés et al., 2010 (14). The extract was submitted to column chromatography using an Amberlite XAD-16. Water was used as the eluent to discard undesirable compounds, and then, ethanol was used as the eluent to obtain a pomegranate total polyphenols (PTP) fraction. Ethanol was evaporated and PTP was obtained as a fine powder. 50 mg of PTP was resuspended in distillated water, filtered through Millex® membrane units of 0.45 µm and collecting in vials for mass analysis. The analysis was performed by electrospray ionization mass spectrometry (ESI-MS) using a Varian 500/MS (California, USA) equipment.All mass experiments were carried out negative mode $(M-H)^{-1}$. Nitrogen was used as nebulizing gas and helium as damping gas. The ion source parameters were: spray voltage 5.0 kV and, capillary voltage and temperature were 90.0 V and 350 °C, respectively. Data were collected and processed using MS Workstation software (V 6.9). Samples were firstly analyzed in full scan modeacquired in the m/z range 50–2000. MS/MS analyses were performed on a series of selected precursor ions (15).

3.2.Microorganism

The *Aspergillus niger* GH1 strain (Food Research Department Collection, Universidad Autonoma de Coahuila, Mexico) was used in this work. The strain was maintained at -50 °C in glycerol-skimmed milk. For inoculum preparation, spores of *A. niger* GH1 were inoculated in 250 mL Erlenmeyer flasks containing 30 mL of potato dextrose agar (PDA-Bioxon) medium and incubated at 30 °C for five days. The culture spores were harvested with sterile solution of 0.01 % Tween-80 and counted in a Neubauer chamber.

3.3.Solid State Fermentation Conditions

Ellagitannin acyl hydrolase (EAH) production was carried out using plastic tray bioreactors (29.0 x 24.5 x 5.5 cm) containing the following fermentable mass: 3 g of support (polyurethane foam sterile) and 7 mL of Czapek Dox salts with the following composition ($g.L^{-1}$): $NaNO_3$ (7.65), KH_2PO_4 (3.04), KCl (1.52) y $MgSO_4.7H_2O$ (1.52). The carbon source and inductor were ellagitannins from pomegranate husk. The medium pH was adjusted to 6.5, and then, the medium was autoclaved (121 °C for 15 min). Fermentable mass was aseptically inoculated with $2x10^7$ $spores.g^{-1}$of support and incubated at 30 °C for 44 h.

3.4.Recuperation of Enzyme Extract

The enzymatic extract (EE) was obtained adding 7 mL of 50 mM citrate buffer pH 5 (mixing 2.101 g of citric acid in 100 mL distilled water and 2.941g of sodium citrate in 100 mL distilled water), compressing the fermentable mass with a sterile syringe and then filtered (Whatman 41). The filtered liquid was dialyzed in buffer using cellulose membranes (12 kDa) under refrigeration, which every 12 h was changed until a clear extract was obtained. The dialyzed extract was ultrafiltered using a column of nominal molecular weight cutoff of 30 kDa.

3.5.Ellagic Acid Quantification by HPLC. Calibration Curve for Ellagic Acid Estimation

Ellagic acid quantification was carried out using HPLC (High Performance Liquid Chromatography) equipment (Varian ProStar System, California, USA) with a Diode Array Detector (PDA ProStar) to 254 nm, according to Ascacio-Valdés *et al*.,2010 (14), under the following operation conditions: 5 µm Optisil ODS column (250 x 4.6 mm), flow rate of 1 $ml.min^{-1}$, sample volume of 10 µL, 30 °C in column for 40 min. Mobile phase A was methanol (wash phase), phase B was acetonitrile, and phase C was 3% acetic acid in gradients (0–20 min 100% C; 20-25 min 80% C and 20% B; 25-26 min 70% C and 30% B; 26-31 min 40% C and 60% B; 31–40 min 70% C and 30%). A calibration curve was made using ellagic acid (Sigma-Aldrich™) from 0-500 $\mu g.mL^{-1}$.

3.6.Ellagitannin Acyl Hydrolase Assay

In order to establish the ideal reaction conditions, the EAH activity was evaluated. The factors substrate concentration (0.5-4.0 $mg.mL^{-1}$), pH (3-7), time (0-120 min) and temperature (20-60 °C) were evaluated. In this study, the effect of pH and temperature on EAH activity were determined by kinetic evaluations during 10 min. Kinetic parameters K_m and V_{max} were established. The values were estimated using the Lineweaver-Burk and Michaelis-Menten models and the solver function of Excel software (Microsoft®). Three replications were performed during all experiments. In the final protocol for the enzyme reaction, the substrate solution was prepared using punicalagin (85 % of purity; proportioned by Food Research Department, Universidad Autónoma de Coahuila) at a concentration of 1 $mg.mL^{-1}$ in 50 mM citrate buffer pH 5.0. The enzymatic extract and buffer (50 mM citrate buffer, pH 5.0) were pre-incubated at 30 °C for 5 min before the enzyme reaction was started. The reaction mixture in the blank and test tube was as follow: 1000 µL of substrate solution and 50 µL of 50 mM citrate buffer pH 5.0 and 1000 µL of substrate

solution and 50 µL of enzyme sample, respectively. The control was prepared with 1000 µL 50 mM citrate buffer pH 5 and 50 µL of enzyme sample. All reaction mixtures were allowed to react for 10 min at 60 °C in a thermal bath (Sheldon Manufacturing, model 1225). The reaction was stopped by adding 1050 µL of absolute ethanol. Samples were sonicated (Ultrasonic cleaner BRANSON, Danbury, USA) for 25 min and filtered through Millex® membrane units of 0.45 µm and collecting in vials. An ellagitannase enzymatic unit was defined as the enzyme amount able to release 1 µmol of ellagic acid per min under the above conditions at pH 5.0 and 60 °C. Ellagic acid was quantified by HPLC using the conditions above mentioned.Protein content was analyzed using bovine serum albumin solution at 100 ppm (10 mg in 100 mL of 50 mM citrate buffer pH 5.0). For assay 100 µL of sample was added with 1000 µL of Bradford reagent. The samples were shaken and allowed to rest five minutes. The absorbance was recorded at 595 nm (16).

4. Results

4.1.MS Analysis of Ellagitannins from Pomegranate Husk

As **Figure 1** shows, the MS profiles obtained from pomegranate husk enabled the detection of two compounds at m/z 1083 and m/z 781. The ion at *m/z* 1083 has been assigned to punicalagin, while the ion at m/z 781 corresponding to punicalin.

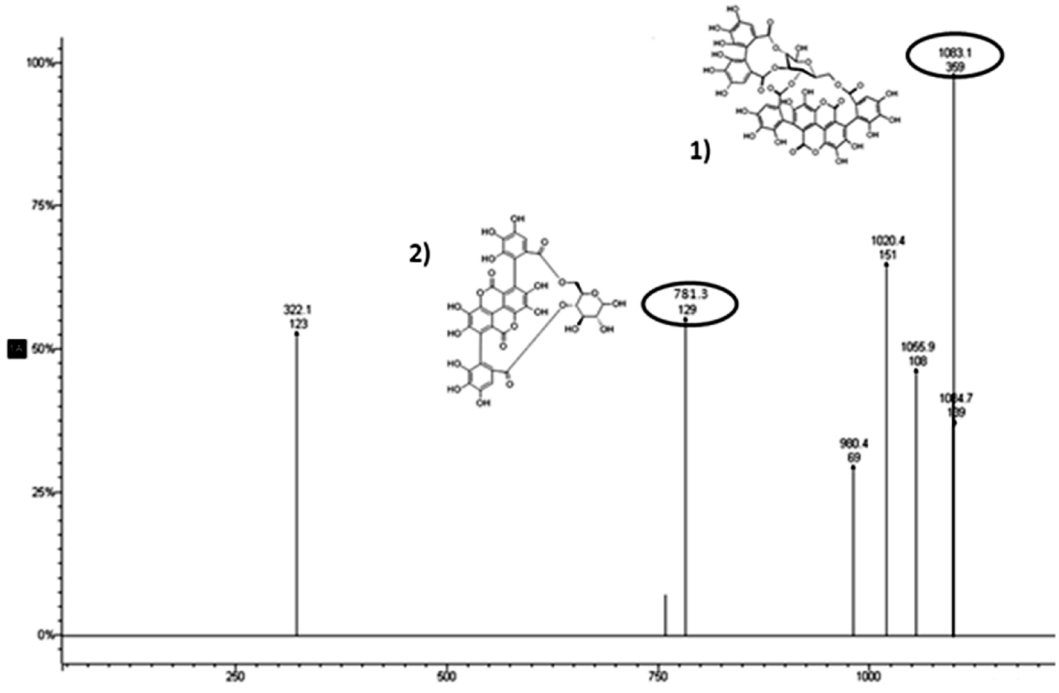

Figure 1. MS profiles obtained from pomegranate husk

4.2.Production of Enzymatic Extract

In this step, an enzymatic extract (1950 mL) was obtained by compression and subsequently dialyzed. The dialyzed extract (100 mL) was submitted to ultrafiltration, yielding 43.5 mL, 65.03±8.56 $U.mg^{-1}$ of ellagitannase activity was detected in 100 mL dialyzed extract, and this activity was increased at 104.62±18.76 $U.mg^{-1}$ in the 43.5 mL extract after ultrafiltration. The ultrafiltration allowed sample concentration by the elimination of low molecular proteins and peptides.

4.3.Definition of Enzymatic Reaction Conditions

EAH was assayed by the method based on ellagic acid quantification, product of ETs hydrolysis. It was observed that enzymatic activity increased with time (**Fig. 2a**) and reached the maximum activity at 10 min of reaction time (300 $U.L^{-1}$), 2.5 times more than that observed at 15 min (120 $U.L^{-1}$). After this the EAH activity decreased until the minimum activity value was reached at the end of assay(120 min). The substrate concentration on ellagitannase activity was evaluated, finding the highest EAH activity at a substrate concentration of 1 $mg.mL^{-1}$ (**Fig. 2b**). Changes in pH values for enzyme activity are shown in **Figure 2c**. Enzymatic activity increased slowly until reaching a maximum value above 120 $U.L^{-1}$ at pH 5. Subsequently, the enzymatic activity decreased until reaching around 20 UL^{-1} at pH 7. **Figure 2d** shows the

relationship between the reaction temperature and the enzymatic activity. The temperature values gradually increased until reaching a maximum enzymatic activity around 500 UL^{-1} at 60 ° C.In **Table 1**, the ideal assay conditions for EAH activity of *Aspergillus niger* GH1 during solid state fermentation are shown.

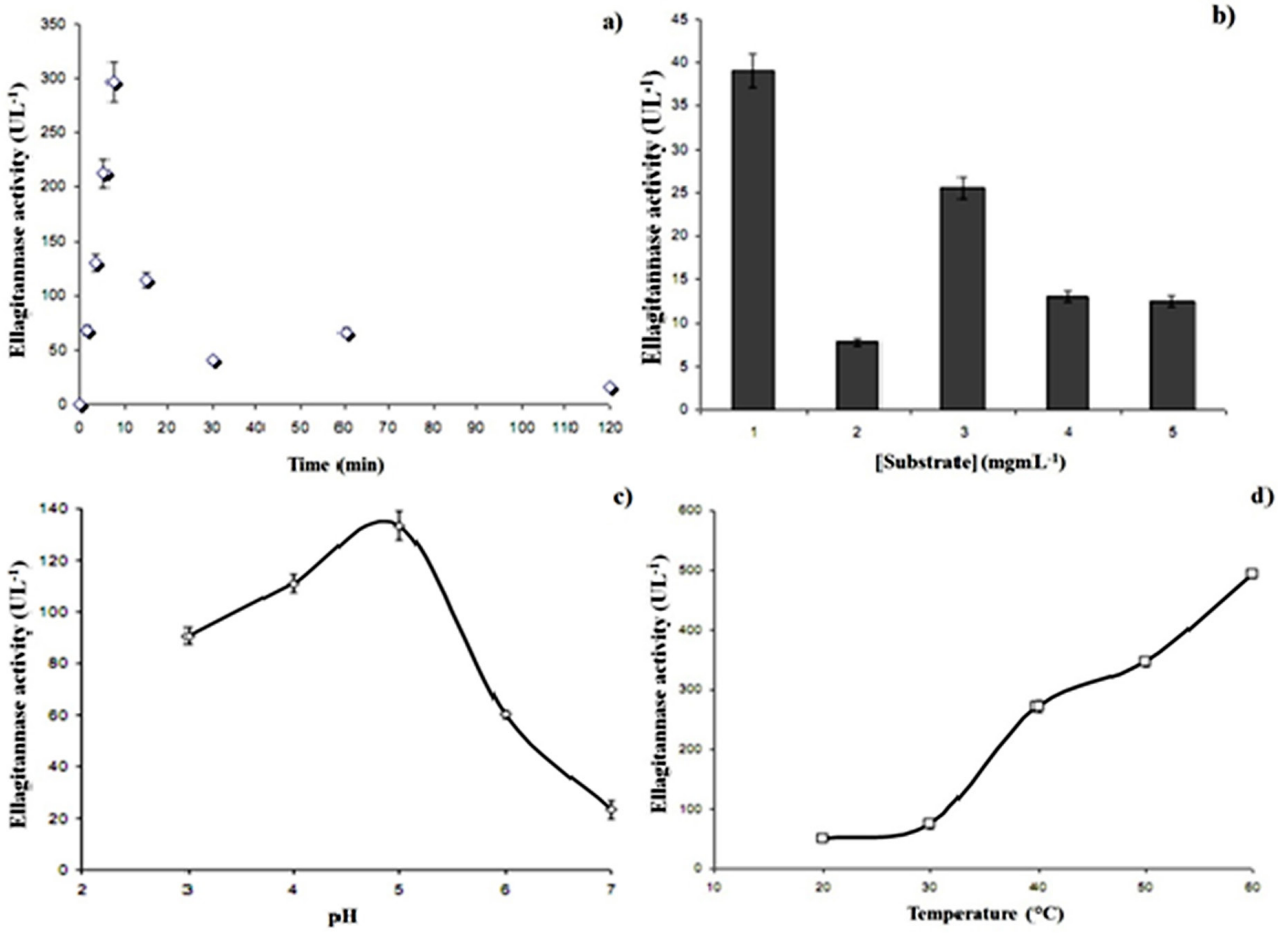

Figure 2. Effect of: **A)** time reaction, **B)** substrate concentration, **C)** pH and **D)** temperature on ellagitannase activity.

Table 1. Ellagitannase assay conditions

Condition	Initial	Adequate
Substrate concentration	(1 $mg.mL^{-1}$)	(1 $mg.mL^{-1}$)
Reaction time	120 min	10 min
pH	5.0	5.0
Temperature	30 °C	60 °C

4.4.Kinetic Properties of Ellagitannase Enzyme

V_{max} is modified by pH and temperature, while K_m is also positively dependent on temperature (17). The maximum velocity (9 $mM.mL^{-1}.min^{-1}$) was obtained at pH 5.0, which was two times more than that obtained at pH 4 and 6 (**Fig.3a**). The pH 5.0 corresponds to the value adequate to enzymatic extract, reaching the highest ellagitannase activity at 60 °C and 10 min of reaction time (**Fig. 3b**). This effect is because high temperature enhances substrate solubility and interaction with the enzymatic extract.

4.5 Estimation of Kinetic Parameters (K_m and V_{max})

Michaelis-Menten constants were determined from Lineweaver & Burk(18) plots (**Fig. 4a and 4b**). K_m (mM) indicates enzyme affinity by the substrate, when the value is small, the affinity is major. V_{max} ($mM.mL^{-1}.min^{-1}$) is the point at which all active sites are saturated and therefore activity is constant, that is to say, the maximum velocity with which this enzymatic reaction is carried out.

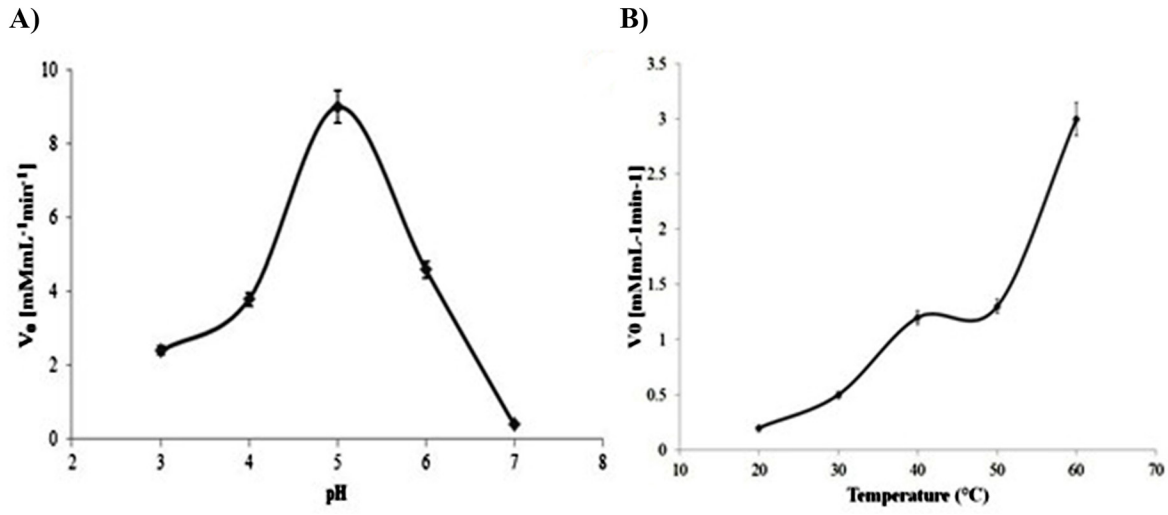

Figure 3. Effect of **A)** pH and **B)** temperature on maximum ellagitannase activity.

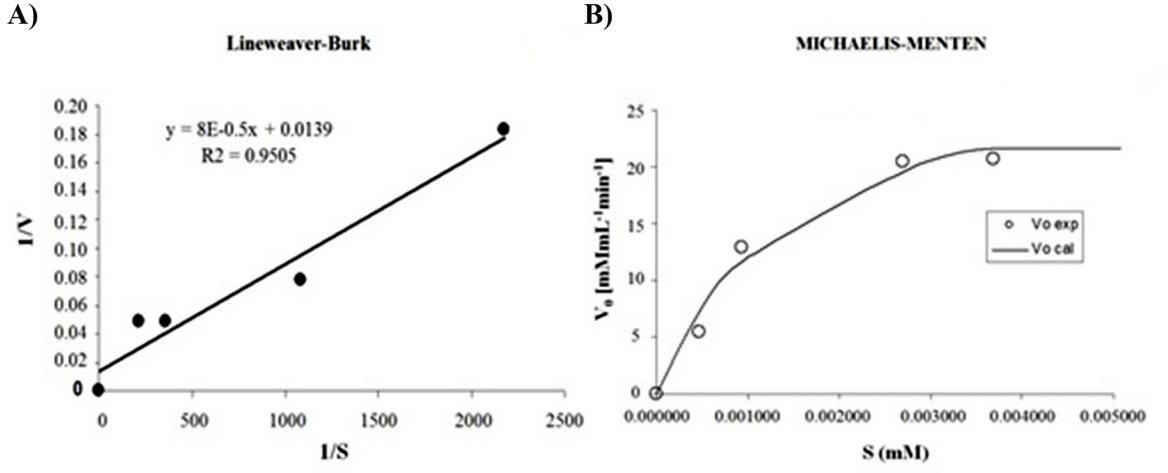

Figure 4. A) Lineweaver-Burk plot for determination of kinetic constants of ellagitannase and **B)** graph model of Michaelis-Menten.

V_{max} and K_m for *A. niger* GH1 ellagitannase are shown in **Table 2**. Kinetic values are considered more accurate, due to a base linearization of Lineweaver-Burk applied to the model of Michaelis-Menten. It is important to mention that the results were obtained using a concentrated enzyme, not purified, in order to establish the ideal reaction conditions. This is the first report about the influence of substrate concentration, time, temperature and kinetic values for EAH, a novel enzyme responsible for ellagitannins biodegradation.

Table 2. Kinetic parameters of ellagitannase enzyme

Parameters	Lineweaver-Burk	Michaelis-Menten
K_m	5.75 x 10^3	1.48 x 10^3
V_{max}	71.94	30.34
R^2	0.9505	0.9911

5. Discussion

Punicalagin and punicalin are two ellagitannins molecules that release ellagic acid after hydrolysis (19, 20). The pH effect on enzymatic activity is determined by several factors, like the active site structure and nature of its amino acids. The enzymes are very sensitive to changes in pH, and they have the best performance at pH specific value (21). The results have shown that the

best enzymatic activity was at 4-5 pH range, but pH 5is the ideal value for enzymatic activity. The enzyme is active at acidic pH and the activity decreased as the pH approached the alkaline range. At pH 6 and 7, no enzyme activity was detected. Any pH change has an effect on protein structure and the enzymatic activity, a decrease in enzymatic could be due to enzyme inactivation or its instability at pH values higher than 5 (22). The highest ellagic acid production level using *A.s niger* SHL6 was reported at pH values of 4 to 5 (23). Tannase produced by *A. niger* GH1 under similar culture conditions has a stable enzymatic activity in pH range 3.5 - 8with ideal pH5.5 (24).

It was observed that at high temperature (in the evaluated range), the maximum EAH activity was reached. A highest value of EAH activity at 60 °C was found (500 $U.L^{-1}$), which is almost 10 times higher than the EAH activity observed at 20 °C. At 60 °C. It has been reported that temperature has an important role in enzymatic reactions because it can increase the speed of reaction, which increases the number of collisions between enzymes (at the molecular level) and substrate. However, it has also been reported that at values higher than the ideal for enzyme activity the ionization state of the protein is affected, and this can cause a decrease in enzyme activity (25).

So based on the above, it has been demonstrated that an ideal temperature value can increase the solubility of the species, which favors the enzyme-substrate interaction (17).This report deals with the initial approach to the reaction conditions of an enzyme capable of hydrolyzing ellagitannins. The maximum temperature value evaluated was 60 °C, based on the temperature of action of the enzyme tanase produced by *A. niger* which has a hydrolase-like activity on the gallotannins that is similar to that of the enzyme evaluated here (26, 27).

The obtained pattern of enzyme activity with respect to substrate concentration shows that using 1 $mg.mL^{-1}$ the highest enzyme activity is obtained, but at higher concentrations this value decreases. The decrease in enzyme activity may be due to the formation of complexes between the molecules involved and the enzyme under reaction conditions. It has been reported that compounds such as punicalagin are capable of reacting with enzymes by forming covalent bonds and this causes the inactivation of enzymes. On the other hand molecules such as ellagic acid derived from the biotransformation of punicalagin are also able to form covalent interactions with macromolecules such as enzymes and cause their inhibition. The product can generate complexes with proteins at different sites of the catalyst, modifying protein conformation. Ellagic acid has been reported to react with the proline groups of proteins (24, 27, 28).

It is important to mention that this work was developed using a concentrated enzymatic extract, however, the presence and activity of EAH has been demonstrated in extracts produced by *A. niger* GH1 using punicalagin isolated from pomegranate husk as a substrate; enzymatic activities such as polyphenol oxidase, cellulase, tannase have been evaluated and it has been shown that EAH has direct activity on this substrate (12, 13).

As above mentioned, this is the first report about the influence of several factors on EAH activity. However, it is necessary to carry out the enzyme purification and to develop the optimization of the ideal conditions reported, in order to explore other reaction conditions and improve the enzymatic activity and the release of ellagic acid.

6. Conclusion

The EAH enzyme was produced by *A. niger* GH1 in solid state fermentation, using pomegranate husk extract and polyurethane foam as a solid support. The ideal reaction conditions for EAH activity were established as follows: substrate concentration of 1 $mg.mL^{-1}$, 60 °C of temperature and pH 5.0, during 10 min of reaction.

The results obtained in this work represent an important advance in the topic of the biotransformation of ellagitannins, because it is necessary to develop methodologies that allow the recovery of high-added value compounds such as ellagic acid, which has a great importance in the food, medicine, pharmaceutics area etc. This compound could be used for the formulation of functional foods or as a nutraceutical agent.

Declaration of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

1. Aguilera-Carbo A, Hernández J, Augur C, Prado-Barragan L. Ellagic acid production from biodegradation of creosote bush ellagitannins by *Aspergillus niger* in solid state culture. *Food Bioproc Technol.* 2009;**2**:208-212. doi: 10.1007/s11947-008-0063-0

2. Bala I, Bhardway V, Hariharan S, Kumar M. Analytical methods for assay of ellagic acid and its solubility studies. *J of Pharma and biomedical anal.* 2006;**40**:206-210. doi: 10.1016/j.jpba.2005.07.006

3. Sepúlveda L, Ascacio-Valdés J, Rodríguez-Herrera R, Aguilera-Carbó A. Ellagic acid: biological properties and biotechnological development for production processes. *Afr J Biotechnol.* 2011;**10**:4518-4523. doi: 10.5897/AJB10.2201

4. Nuncio-Jáuregui N, Nowicka P, Munera-Picazo S, Hernández F. Identification and quiantification of major derivatives of ellagic acid and antioxidant properties of thinning and ripe Spanish pomegranates. *J Funct Foods.* 2015;**12**:354-364. doi: 10.1016/j.jff.2014.11.007

5. Mansouri M, Hemmati A, Naghizadeh B, Mard S. A study of the mechanisms underlying the anti-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. *Indian J Pharmacol.* 2015;**47**:292-298. doi: 10.4103/0253-7613.157127

6. Abuelsaad A, Mohamed I, Allam G, Al-Solumani A. Antimicrobial and immunomodulating activities of hespiridin and ellagic acid against diarrehic *Aeromonas hydrophila* in a murine model. *Life Sci.* 2013;**93**:714-722. doi: 10.1016/j.lfs.2013.09.019

7. Park S, Kwon M, Yoo J, Choi H J, Ahn Y J. Antiviral activity and possible mode of action of ellagic acid identified in *Lagerstroemia speciosa* leaves toward human rhinoviruses. *BMC Complement Altern Med.* 2014;**14**:1-8. doi: 10.1186/1472-6882-14-171

8. Jassim SA, Naji MA. Novel antiviral agents: a medicinal plant perspective. *J Appl Microbiol.* 2003;**95**:412-427. doi: 10.1046/j.1365-2672.2003.02026.x

9. Okla M, Kang I, Kim D, Gourineni V. Ellagic acid modulates lipid accumulation in primary human adipocytes and human hepatoma Huh7 cells via discrete mechanisms. *J Nutr Biochem.* 2015;**26**:82-90. doi: 10.1016/j.jnutbio.2014.09.010

10. Türk G, Sönmez M, Çeribaşi A, Yüce A, Ateşşahin A. Attenuation of cyclosporine A-induced testicular and spermatozoal damages associated with oxidative stress by ellagic acid. *Int Immunopharmacol.* 2010;**10**:177-182. doi: 10.1016/j.intimp.2009.10.013

11. De la Cruz R, Ascacio-Valdés J, Buenrostro J, Sepúlveda L. Optimization of ellagitannse production by *Aspergillus niger* GH1 by solid-state fermentation. *Prerp Biochem Biotechnol.* 2015;**45**:617-631. doi: 10.1080/10826068.2014.940965

12. Ascacio-Valdés JA, Buenrostro J, De la Cruz R, Sepúlveda L. Fungal biodegradation of pomegranate ellagitannins. *J Basic Microbiol.* 2014;**54**:28-34. doi: 10.1002/jobm.201200278

13. Ascacio-Valdés JA, Aguilera-Carbó A, Buenrostro J, Prado-Barragán A. The complete biodegradation pathway of ellagitannins by *Aspergillus niger* in solid state fermentation. *J Basic Microbiol.* 2016;**56**:329-336. doi: 10.1002/jobm.201500557

14. Ascacio-Valdés J, Aguilera-Carbó A, Martínez-Hernández J, Rodríguez-Herrera R, Aguilar C N. *Euphorbia antisyphilitica* residues as a new source of ellagic acid. *Chem Pap.* 2010;**64**:528-532. doi: 10.2478/s11696-010-0034-6

15. Hernández C, Ascacio-Valdés J, De la Garza H, Wong-Paz J. Polyphenolic content, in vitro antioxidant activity and chemical composition of extract from *Nephelium lappaceum* L. (mexican rambutan) husk. *Asian Pac J Trop Med.* 2017;**10**:1201-1205. doi: 10.1016/j.apjtm.2017.10.030

16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976,**72**:248-254. doi: 10.1016/0003-2697(76)90527-3

17. Wang G, Post WM, Mayes MA, Frerichs JT, Sindhu J. Parameter estimation for models of ligninolytic and cellulolytic enzyme kinetics. *Soil Biol Biochem.* 2012;**48**:28-38. doi: 10.1016/j.soilbio.2012.01.011

18. Lineweaver H, Burk D. The determination of enzyme dissociation constants. *J Amer Chem Soc.* 1934;**56**:658-666. doi: 10.1021/ja01318a036

19. Ascacio-Valdés JA, Buenrostro-Figueroa JJ, Aguilera-Carbo A, Prado-Barragán A. Ellagitannins: Biosynthesis, biodegradation and biological properties. *J Med Plants Res.* 2011;**5**:4696-4703. doi: 10.5897/JMPR

20. Johanningsmeier SD, Harris GK. Pomegranate as a functional food and nutraceutical source. *Annual Rev Food Sci Technol.* 2011;**2**:181-201. doi: 10.1146/annurev-food-030810-153709

21. Sabu A, Kiran GS, Pandey A. Purification and characterizaction of tannin acyl hydrolase from *Aspergillus niger* ATCC 16620. *Food Technol Biotechnol.* 2005;**43**:133-138. doi: 577.152.311:582.288

22. Battestin V, Macedo GA. Tannase production by *Paecilomyces variotii*. *Biores Technol.* 2007;**98**:1832-1837. doi: 10.1016/j.biortech.2006.06.031

23. Huang W, Ni J, Borthwick A. Biosynthesis of valonia tannin hydrolase and hydrolysis of valonia tannin to ellagic acid by *Aspergillus* SHL 6. *Proc Biochem.* 2005;**40**:1245-1249. doi: 10.1016/j.procbio.2004.05.004

24. Aguilar C, Gutierrez-Sanchez GR. Review: Sources, properties, applications and potential uses of tannin acyl hydrolase. *Food Sci Technol Inter.* 2001;**7**:373-382. doi: 10.1106/69M3-B30K-CF7Q-RJ5G

25. Mukherjee G, Banerjee R. Effects of temperature, pH and additives on the activity of tannase produced by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. *World J Microbiol Biotechnol.* 2006;**22**:207-212. doi: 10.1007/s11274-005-9022-3

26. Ramírez-Coronel MA, Viniegra-González G, Darvill A, Augur C. Novel tannase from *Aspergillus niger* with β-glucosidase activity. *Microbiology.* 2003;**149**:2941-294. doi: 10.1099/mic.0.26346-0

27. Ramos E, Mata-Gómez M, Rodríguez-Durán L, Belmares R. Catalytic and thermodynamic properties of a tannase produced by *Aspergillus niger* GH1 grown on polyurethane foam. *Appl Biochem Biotechnol.* 2011;**165**:1141-1151. doi: 10.1007/s12010-011-9331-y

28. Edelmann A, Lendl B. Toward the optical tongue: flow-through sensing of tannin−protein interactions based on FTIR spectroscopy. *J Amer Chem Soc.* 2002;**124**:1471-1474. doi: 10.1021/ja026309v