



ORIGINAL ARTICLE

## Effect of different polyphenol sources on the efficiency of ellagic acid release by *Aspergillus niger*



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### KEYWORDS

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**Abstract** Fungal hydrolysis of ellagitannins produces hexahydroxydiphenic acid, which is considered an intermediate molecule in ellagic acid release. Ellagic acid has important and desirable beneficial health properties. The aim of this work was to identify the effect of different sources of ellagitannins on the efficiency of ellagic acid release by *Aspergillus niger*. Three strains of *A. niger* (GH1, PSH and HT4) were assessed for ellagic acid release from different polyphenol sources: cranberry, creosote bush, and pomegranate used as substrate. Polyurethane foam was used as support for solid-state culture in column reactors. Ellagitannase activity was measured for each of the treatments. Ellagic acid was quantified by high performance liquid chromatography. When pomegranate polyphenols were used, a maximum value of ellagic acid (350.21 mg/g) was reached with *A. niger* HT4 in solid-state culture. The highest amount of ellagitannase (5176.81 U/l) was obtained at 8 h of culture when cranberry polyphenols and strain *A. niger* PSH were used. Results demonstrated the effect of different polyphenol sources and *A. niger* strains on ellagic acid release. It was observed that the best source for releasing ellagic acid was pomegranate polyphenols and *A. niger* HT4 strain, which has the ability to degrade these compounds for obtaining a potent bioactive molecule such as ellagic acid.

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**PALABRAS CLAVE**

Polifenoles;  
*Aspergillus niger*;  
 Cultivo en estado  
 sólido;  
 Elagitanasa

## Efecto de las diferentes fuentes de polifenoles sobre la eficiencia de liberación de ácido elálgico por *Aspergillus niger*

**Resumen** La hidrólisis fúngica de los elagitaninos produce ácido hexahidroxidifénico, considerado como una molécula intermedia en la liberación de ácido elálgico. El ácido elálgico tiene importantes y deseables propiedades benéficas para la salud humana. El objetivo de este trabajo fue identificar el efecto de la fuente de elagitaninos sobre la eficiente liberación de ácido elálgico por *Aspergillus niger*. La liberación de ácido elálgico se realizó con tres cepas de *A. niger* (GH1, PSH y HT4) en presencia de diferentes fuentes de polifenoles (arándano, gobernadora y granada), usadas como sustrato. Se empleó espuma de poliuretano como soporte para el cultivo en estado sólido en reactores en columna. Se midió la actividad elagitanasa a cada uno de los tratamientos. El ácido elálgico liberado se cuantificó por cromatografía líquida de alta resolución. Cuando se utilizaron los polifenoles de granada, se alcanzó un valor máximo de 350,21 mg/g de ácido elálgico con *A. niger* HT4 en cultivo en estado sólido. La mayor actividad elagitanasa (5176.81 U/l) se obtuvo a 8 h de cultivo cuando se usaron los polifenoles de arándano como sustrato y *A. niger* PSH. Los resultados demostraron el efecto que tiene la fuente de polifenoles y la cepa de *A. niger* en la liberación de ácido elálgico. Se observó que la mejor fuente para la liberación de ácido elálgico fueron los polifenoles de granada y que la cepa *A. niger* HT4 posee la habilidad de degradar estos compuestos para la obtención de potentes moléculas bioactivas, como el ácido elálgico.

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## Introduction

Polyphenols are a wide range of compounds considered to be secondary metabolites of different plant parts: leaves, stems, flowers, fruits, seeds, and others<sup>2</sup>. Polyphenols are divided into three main groups: complex tannins, condensed tannins and hydrolyzable tannins<sup>17</sup>. Ellagitannins are a class of hydrolyzable tannins. These compounds are essentially formed by a hexahydroxydiphenic acid (HHDP) group linked by an ester bond to a glucose<sup>8</sup>. There are over 500 different structures of ellagitannins reported in the literature<sup>13</sup>. Ellagitannins are mainly obtained from the bark and trunks of trees such as oak (*Quercus* spp.)<sup>18</sup> and eucalyptus (*Eucalyptus* spp.)<sup>19</sup>. Ellagitannins are abundant in some red berries<sup>24</sup>, plants of the Mexican desert<sup>6</sup> and pomegranate fruit<sup>22</sup>. Ellagitannins have various beneficial health properties. It has been shown that the ellagitannins present in pomegranate juice can induce apoptosis by activation of the leukemic cell cycle<sup>11</sup>. Punicalagin is an ellagitannin from the pomegranate peel, which has been linked to apoptosis in HT-29 colon cancer cells<sup>23</sup>. Ellagitannins present in leaf extracts, flower extracts, peel, juice and seeds of pomegranate have beneficial effects on the control of obesity, diabetes, cardiovascular disease, hypertension and hypercholesterolemia<sup>5</sup>. Moreover, these polyphenols in pomegranate leaves, obtained by macroporous resin column chromatography have a high antioxidant activity<sup>29</sup>. Ellagitannins can be hydrolyzed by chemical or enzymatic methods to release the HHDP group<sup>15</sup>; this group undergoes a spontaneous molecular arrangement forming a new molecule called ellagic acid (EA)<sup>3</sup>. EA is a secondary metabolite of high industrial interest due to various beneficial effect on human health such as antiviral<sup>16</sup>, anticarcinogenic<sup>20</sup>,

antioxidant<sup>26</sup>, and anti-inflammatory activities<sup>10</sup>. Industrially, EA is obtained with the use of chemicals, involving high production costs and recovery as well as environmental damage. Therefore, it is necessary to develop methods for the biotechnological production of secondary metabolites. There are few studies about obtaining EA by bioprocessing agroindustrial residues such as pomegranate peel and microorganisms that are capable of degrading the ellagitannin compounds present in the waste by solid-state culture (SSC) to generate EA<sup>25</sup>. The aim of this work was to evaluate the effect of different polyphenol sources: cranberry (CP), creosote bush (CBP) and pomegranate (PP) on the hydrolytic efficiency of three different xerophilic *Aspergillus niger* strains, denominated A-GH1, A-PSH and A-HT4, to obtain EA by SSC.

## Materials and methods

### Polyphenol extraction and purification

Cranberries (*Vaccinium macrocarpon* Ait.) were originally from Wisconsin, USA, and were received in vacuum packed bags (350 g) in the month of January of the year 2012. Pomegranates (*Punica granatum* L. var. Wonderful) were originally from California, USA, and were received in cardboard boxes (25 kg), in the same session and year. These fruits were purchased at a supermarket in Saltillo, Coahuila, Mexico. Creosote bush plants (*Larrea tridentate* Sesse & Mocino ex DC. Coville) were manually collected in Federal Highway 57 along 15 km of the Saltillo-Monclova stretch, in Coahuila State, Mexico in the second semester of the year 2011. Pomegranate peel, cranberry and creosote bush

leaves were dehydrated at 60 °C for 48 h, then pulverized in a grinder (PULVEX® Mini 100) to obtain a particle size of 600 µm and taken to constant weight<sup>25</sup>. Polyphenols were extracted according to the methodology proposed by Ascacio-Valdés et al<sup>6</sup>. Substrate was hydrated at 1:5 ratio (100 g substrate/500 ml distilled water) and incubated at 60 °C for 30 min. The substrate mass was filtered through muslin cloth and the extract obtained was centrifuged at 3000 rpm for 15 min. The supernatant was purified by chromatography (Amberlite XAD-resin 16®). The extracts were eluted with distilled water (dH<sub>2</sub>O) to remove soluble compounds. The polyphenolic compound-rich fraction was eluted with 96% ethanol. Finally, the ethanol was evaporated in a heating oven (NAPCO® Model 322) at 60 °C for 24 h. The CP CBB and PP were obtained as a fine powder.

### Support preparation

The use of polyurethane foam (PUF) as inert support for polyphenol biodegradation was assessed. The PUF was cut and ground to a particle size of 0.85 mm, then washed (3×) with hot water (80–90 °C) for 10 min, filtered to eliminate and dried until constant weight<sup>21</sup>.

### Microorganisms and culture media

*A. niger* GH1, *A. niger* PSH and *A. niger* HT4 strains were supplied by DIA-UAdC (Departamento de Investigación en Alimentos, Universidad Autónoma de Coahuila). These strains have the ability to degrade substrates rich in polyphenols<sup>3,12,22</sup>. The strains were activated in potato dextrose agar (PDA-Bioxon), and incubated at 30 °C for 5 days. Spores were harvested with 0.01% Tween-80 and counted in a Neubauer® chamber. Pontecorvo culture medium having the following composition (g/l): NaNO<sub>3</sub> 6.0, KH<sub>2</sub>PO<sub>4</sub> 1.52, KCl 0.52 MgSO<sub>4</sub>·7H<sub>2</sub>O 0.52, ZnSO<sub>4</sub> 0.001, FeCl 0.85 and 1 ml trace metals was used. The trace metal solution contained (mg/l) Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O 10, MnCl<sub>2</sub>·4H<sub>2</sub>O 50, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 50 and CuSO<sub>4</sub>·5H<sub>2</sub>O 250. The carbon source (30 g/l) was supplemented with either CP, CBP or PP<sup>7</sup> and the initial culture medium pH was adjusted to 6.5 with 1 M NaOH. An inoculum size of 2 × 10<sup>7</sup> spores per g of culture material was used.

### SSC conditions

The SSC was prepared as follows: 0.5 g of PUF was homogenized with 1.16 ml of culture medium (70% humidity) and packed in columns (0.5 cm × 5 cm) at a packing density of 0.08 g/cm<sup>3</sup>. The solid-state culture was performed for 32 h at 30 °C, with sampling every 8 h (two columns by treatment)<sup>9</sup>. The enzymatic extract of the culture was obtained by adding 1.16 ml of 50 mM citrate buffer (pH 5) within each column, the mass culture was mixed and the extract was obtained by manual pressing. To recover the EA, an ethanol–formic acid (1.16 ml of 0.01%) solution was added<sup>25</sup> and the extracts obtained were filtered (0.45 µm) and frozen until analysis.

### Ellagitannase activity assay

Ellagitannase activity was carried out according to the methodology suggested by De la Cruz et al<sup>12</sup>. Ellagitannins 1 mg/ml in 50 mM citrate buffers, pH 5, were used as enzyme substrate. A substrate control (1 ml ellagitannins and 50 µl of 50 mM citrate buffers, pH 5), enzyme control (1 ml of 50 mM citrate buffers, pH 5 and 50 µl of enzymatic extract) and the reaction mixture (1 ml of ellagitannins and 50 µl of enzymatic extract) were prepared. The reaction was done in a water bath for 10 min at 60 °C. The reaction was stopped by adding 1050 µl of absolute ethanol. Then, samples were sonicated for 25 min, filtered through 0.45 µm nylon membrane and collected in vials. One ellagitannase enzymatic unit was defined as the enzyme amount needed to release 1 µmol of EA per min, under the conditions described.

### HPLC analysis

Quantification of EA was performed on a Varian ProStar model 2301 HPLC. A column Pursuit XRs 5µm C18 (150 mm × 46 mm) and, (A) methanol (washing phase), (B) acetonitrile and (C) acetic acid 3% (mobile phase). The flow rate was 1 ml/min, at a column temperature of 30 °C. Standard curve of EA (≥95%, Sigma–Aldrich®) from 0 to 500 µg/ml was prepared<sup>7</sup>.

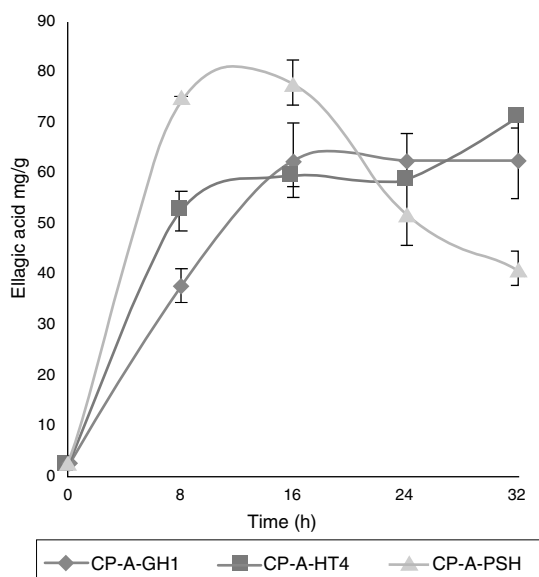
### Data analysis

The effect of the different polyphenol sources and strains on EA accumulation was assessed under a 3 × 3 factorial design with two replicates. Data were analyzed by ANOVA using SAS 9.0 and means were compared by Tukey's multiple range procedure ( $p \leq 0.05$ ) when needed.

## Results

### Kinetics of EA accumulation

Culture kinetics of A-GH1, A-PSH and A-HT4 strains and three culture media enriched with CP, CBP and PP were studied. The kinetic profile when using CP as polyphenolic substrate (Fig. 1) showed the highest EA accumulation (74.56 mg/g) with the A-PSH strain at 8 h of culture followed by the A-HT4 strain (69.55 mg/g) at 32 h of culture and A-GH1 (64.35 mg/g) at 32 h of culture. The A-HT4 strain produced 13.69% more EA than the A-GH1 strain; however the former strain also required a 4-fold increase in the culture time required by the A-GH1 strain. Therefore, in terms of process time, A-GH1 resulted in higher EA accumulation than A-HT4. Fig. 2 shows the EA accumulation profile when using CBP. Total EA accumulation by the different strains was 182.92, 158.54 and 125.97 mg/g using A-GH1, A-HT4 and A-PSH strains respectively; however, A-HT4 reached 158.54 mg/g in an 8 h culture while A-GH1 reached in 32 h culture. Once again, a lower EA accumulation value (158.54 mg/g) could be observed in one of the strains tested (A-HT4) but a culture time 4 times lower than in strain A-GH1 that showed the highest EA accumulation (182.92 mg/g). In contrast, when using PP as substrate, EA

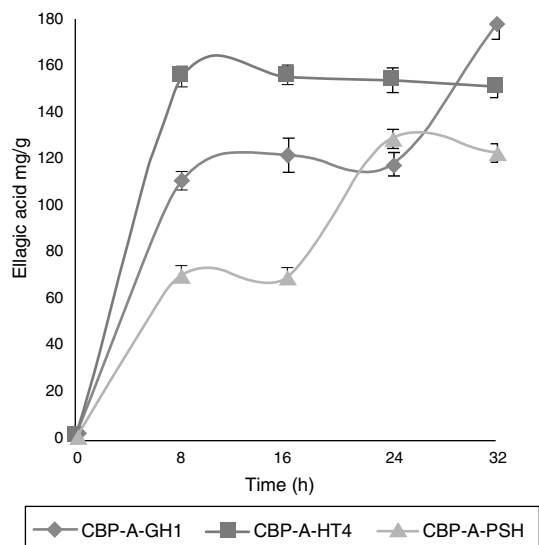


**Figure 1** EA accumulation kinetics using: (◆) CP-A-GH1; (■) CP-A-HT4; (▲) CP-A-PSH in SSC.

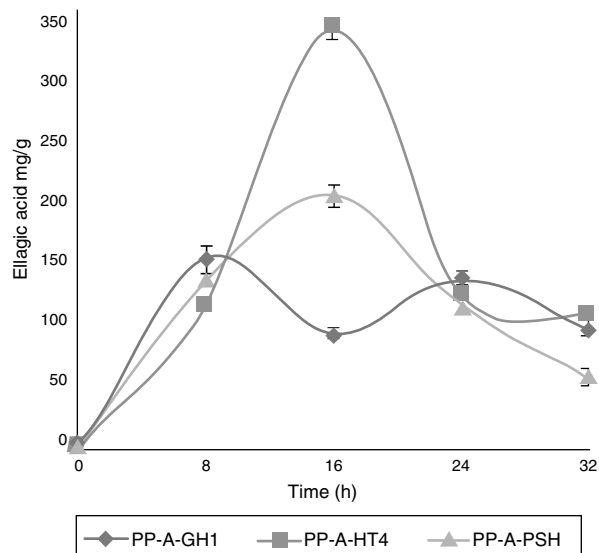
accumulation was 163.03, 350.21 and 200.33 mg/g for A-GH1 (8 h culture), A-HT4 (16 h of culture) and A-PSH (16 h culture) respectively; the highest EA accumulation occurred at 16 h of culture when using A-HT4 and no EA increase was observed at any other time (Fig. 3).

### Ellagitannase activity and productivity

Ellagitannase activity reached maximum value of 5176.81 U/l when using CP as carbon source and A-PSH at 8 h of SSC (Fig. 4). Other strains reached maximum values in the first 8 h and then these values were kept constant. Table 1 shows effect of substrates, strains and interaction on ellagitannase activity and EA productivity. EA

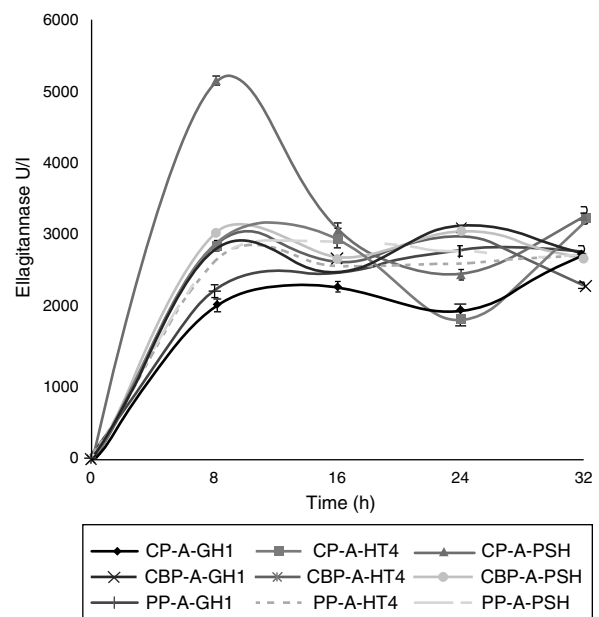


**Figure 2** EA accumulation kinetics using: (◆) CBP-A-GH1; (■) CBP-A-HT4; (▲) CBP-A-PSH in SSC.



**Figure 3** EA accumulation kinetics using: (◆) PP-A-GH1; (■) PP-A-HT4; (▲) PP-A-PSH in SSC.

productivity was defined as the ratio among the maximum accumulation of EA per culture time. In Fig. 5, the values of EA productivity are displayed at a time of 16 h, which corresponds to the time of maximal EA accumulation obtained. In Table 2, the individual effect of each variable, validated by a Tukey test on ellagitannase activity and EA productivity is shown. Then the highest productivity (21.89 mg/g/h), was obtained when using the *Aspergillus* strain coded as A-HT4 and CP as source. It was observed that when A-HT4 and CP were used, a maximum productivity of 21.89 mg/g/h was reached.



**Figure 4** Values of ellagitannase activity, (◆) CP-A-GH1; (■) CP-A-HT4; (▲) CP-A-PSH; (X) CBP-A-GH1; (\*) CBP-A-HT4; (●) CBP-A-PSH; (|) PP-A-GH1; (-) PP-A-HT4; (- -) PP-A-PSH.

**Table 1** Analysis of variance for EA productivity and ellagitannase activity

Responses	Source of variation	df <sup>a</sup>	MS <sup>b</sup>	p-Value	F-test
EA productivity	Replicate	1	0.00	0.98	0.00
	S <sup>c</sup>	2	1252.51	<.0001	858.68
	X <sup>d</sup>	2	487.89	<.0001	334.43
	S*X	4	382.99	<.0001	262.52
$R^2 = 0.997$					
Ellagitannase activity	Replicate	1	17034.93	0.26	1.50
	S <sup>c</sup>	2	43399.04	0.07	3.82
	X <sup>d</sup>	2	230928.25	<.0001	20.32
	S*X	4	112108.11	<.0001	9.86
$R^2 = 0.917$					

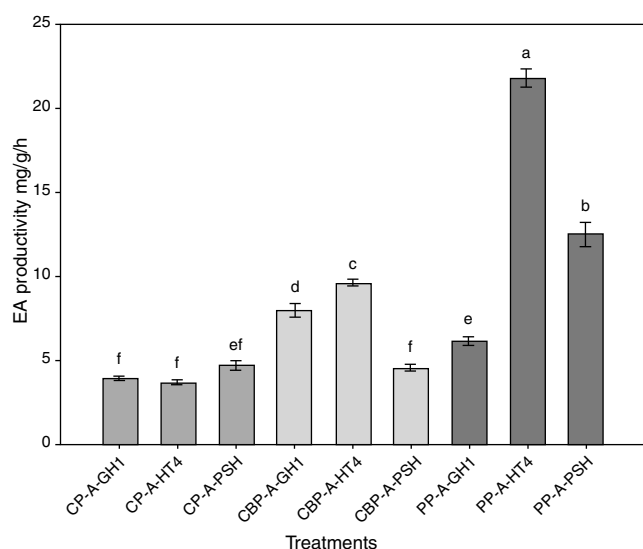
S\*X = combined effect of substrate and strains.

<sup>a</sup> Degree of freedom.

<sup>b</sup> Mean square.

<sup>c</sup> Substrate (polyphenols).

<sup>d</sup> Strains (*A. niger*).



**Figure 5** EA productivity, (▨) CP-A-GH1; CP-A-HT4; CP-A-PSH; (■) CBP-A-GH1; CBP-A-HT4; CBP-A-PSH; (▩) PP-A-GH1; PP-A-HT4; PP-A-PSH.

**Table 2** Substrate and strain effect on EA productivity and ellagitannase activity

Substrate	EA productivity (mg/g/h)	Ellagitannase activity (U/l)
PP	40.59 a	2721.38 a
PCB	22.06 b	2671.36 a
PC	12.13 c	2837.17 a
<b>Strains</b>		
A-HT4	35.11 a	2733.28 b
A-PSH	21.76 b	2944.31 a
A-GH1	17.92 c	2552.33 c

Means with the same letter, in the same column, are not significantly different according to the Tukey's multiple range test ( $p < 0.05$ ).

## Discussion

The A-PSH strain has been reported to have the ability to produce EA by hydrolysis of the pomegranate peel by SSC<sup>22</sup>. Moreover, this strain also showed the ability to degrade hydrolyzable tannins from extracts of creosote bush (*L. tridentata*) and tarbush (*Flourensia cernua*) releasing EA and gallic acid by SSC<sup>28</sup>. Furthermore, the A-GH1 strain has been reported for the hydrolysis of polyphenolic compounds from pomegranate peel, reaching 17 mg/g of EA at 32 h by SSC<sup>4</sup>. Vattem and Shetty (2003) reported the use of cranberry pomace and *Lentinus edodes* strain for EA production reaching a maximum accumulation of 350 µg/g of EA by dried cranberry pomace and they attributed the EA accumulation to the β-glucosidase enzyme<sup>27</sup>. In this work, when A-HT4 strain and PP are used, a maximum of 350.21 mg/g of EA at 16 h is reached. It has also been reported that the pomegranate peel is an excellent carbon source for EA production, using an *A. niger* strain<sup>1</sup>. Recent studies mention that there is a possible set of enzymes that are responsible for the release of the HHDP group and formation consequence of EA<sup>7</sup>.

The values of ellagitannase activity obtained in this study are 166-fold higher than those reported by Aguilera-Carbó et al.<sup>4</sup>. These authors reported values of 44.5 U/l using pomegranate peel as carbon source and A-GH1 at 48 h of culture time. Similar studies obtained maximum values of ellagitannase activity (2189.94 U/l) at 30 h of SSC using PP as carbon source and several fungal strains (*A. niger* PSH, *A. niger* GH1, *A. niger* HT4 and *A. niger* HC2)<sup>12</sup>. Several enzymes responsible for ellagitannin hydrolysis have been reported; however, the authors found that only ellagitannase, clearly associated with EA accumulation on SSC, reached maximum values (200.04 U/l) at 12 h of SSC using PP and *A. niger* GH1 strain<sup>7</sup>. The authors reported that EA production is higher using partially purified polyphenols, since these molecules have a glucose core, making them more susceptible to attack by microbial enzymes<sup>14</sup>. There are a few studies about the use of polyphenols from plants, barks or fruits SSC for obtaining secondary metabolites.

The productivity obtained with CP and the A-HT4 strain is higher compared with that in previous reports using valonia tannins as a carbon source, reaching values of 0.92 U/l/h<sup>11</sup>. The A-GH1 strain has been associated with the release of EA from creosote bush extracts by SSC, reaching a productivity of 1.22 U/l/h<sup>4</sup>.

In conclusion, it was possible to obtain EA from rich substrates in polyphenolic compounds using *A. niger* strains in SSC. A-HT4 reached a maximum EA value (350.21 mg/g of PP at SSC). When CP and the A-PSH strain was used, high values of ellagitannase activity (5176.81 U/l) were obtained at 8 h of SSC. The EA release mechanism is unknown due to EA complexity; however ellagitannase is one of the enzymes that may be responsible for the hydrolysis of the ester link in the HHDP group to generate EA.

## Conflict of interest

The authors declare that they have no conflicts of interest.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this investigation.

**Confidentiality of data.** The authors declare that no patient data appears in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appears in this article.

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