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Submerged Culture for Production of Ellagic Acid from Pomegranate Husk by *Aspergillus niger* GH1

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ABSTRACT

Ellagic acid is a bioactive compound derived from the hydrolysis of the ellagitannins. Ellagic acid has important applications in the food, pharmaceutical and cosmetic industry due its beneficial biological properties. Ellagic acid has been obtained from several sources, such as some berries, semi-desert plants, and oak trees. Pomegranate husk has a high content of ellagitannins, which can be bio-converted into ellagic acid by fungal bioprocesses, although there is no industrial biotechnological process available. The aim of this work was to find appropriate conditions for ellagic acid production by *Aspergillus niger* GH1 in submerged culture using pomegranate husk powder. The influence of culture factors was studied by a Box-Bhenken design. The maximum ellagic acid production was 21.19 mg g^{-1} of pomegranate husk powder. Substrate and pH influenced the ellagic acid production. A constant consumption was present in the substrate during the kinetics of hydrolysable tannins (ellagitannins). The maximum protein accumulation was reached at 24 h, which can be associated

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with the activity of some enzymes present in the culture. The best conditions for ellagic acid production in submerged culture were: substrate level of 7.5 g L^{-1}, pH of 5.5, and agitation of 150 rpm. The developed system represents an alternative for large-scale ellagic acid production.

Key words: ***Aspergillus niger*****, Box-Behnken design, ellagitannase, ellagitannins.**

Cultivo sumergido para la producción de ácido elágico a partir de cáscara de granada por *Aspergillus niger* GH1

RESUMEN

El ácido elágico es un compuesto bioactivo derivado de la hidrólisis de los elagitaninos. El ácido elágico tiene importantes aplicaciones en la industria de alimentos, farmacéutica y cosmética, debido a que presenta propiedades biológicas benéficas. El ácido elágico se obtiene de diferentes fuentes, tales como bayas de frutas, plantas del semi-desierto, y corteza de encinos. La cáscara de granada contiene una gran proporción de elagitaninos, los cuales pueden ser biotransformados en ácido elágico mediante bioprocesos fúngicos, aunque no existe producción biotecnológica industrial. El objetivo de este trabajo fue encontrar las condiciones apropiadas para la producción de ácido elágico por *Aspergillus niger* GH1 en cultivo sumergido, a partir del polvo de cáscara de granada. Se utilizó un diseño Box-Bhenken para estudiar la influencia de los factores de cultivo sobre la producción de ácido elágico. La mayor producción de ácido elágico fue de 21.19 mg g^{-1} de cáscara de granada en polvo. El sustrato y el pH influyeron directamente sobre la producción de ácido elágico. Se observó un consumo constante durante la cinética de taninos hidrolizables (elagitaninos) presentes en el sustrato. La máxima acumulación de proteínas se alcanzó a las 24 h, lo cual se puede asociar con la actividad de algunas enzimas presentes en el cultivo. Las mejores condiciones de producción de ácido elágico en cultivo sumergido fueron: una concentración de sustrato de 7.5 g L^{-1}, pH 5.5, y una agitación de 150 rpm. El sistema desarrollado representa una alternativa para la producción de ácido elágico a gran escala.

Palabras clave: ***Aspergillus niger*****, diseño Box-Bhenken, elagitanasa, elagitaninos.**

INTRODUCTION

Ellagic acid (EA) is a secondary metabolite that has generated great interest in recent years due to its biomedical and health benefits[7,9,15,22,25]. EA is present in fruits, such as strawberries, blueberries, blackberries, walnuts, and pomegranate[16]. EA is obtained from the ellagitannins when hexahydroxydiphenic acid

(HHDP) group is released by chemical or enzymatic hydrolysis. The ester bond between glucose and HHDP is cleaved, and thereafter the HHDP undergoes a molecular rearrangement to form EA[1] (**Fig. 1**). EA generated by chemical hydrolysis involves highly polluting methods due to acid substances used[19]. Furthermore, production costs of EA are high and the product yield is small. EA or 4, 4′, 5, 5′, 6, 6′- hexahydroxydiphenic acid 2, 6, 2′, 6′-dilactone has a molecular weight of 302.19 g mol^{-1}. Its chemical structure confers great stability to the molecule. Our Department has been studying bioprocess designs for EA production. The best fungal strains of *Aspergillus niger* (GH1 and PSH) were selected to convert pomegranate ellagitannins into EA, using solid state culture[18]. Furthermore, the ability of *A. niger* to convert ellagitannins from creosote bush (*Larrea tridentata*) into EA has been shown using the same system[2]. Pomegranate husk was used as a sole carbon source for EA accumulation in solid state culture by *A. niger* GH1[20]. Most studies of EA production have been performed on solid state culture. The aim of this study was to evaluate submerged culture as an alternative for EA production by *A. niger* GH1 using pomegranate husk powder.

MATERIALS AND METHODS

Raw material. Pomegranate fruits (wonderful variety) were purchased in a supermarket from San Juan de Sabinas, Coahuila, Mexico. Pomegranate husks were obtained by manual separation, and dried at 60 C for 48 h. Dried pomegranate husk was powdered using a mill (Pulvex mini 100), and then passed through a sieve (no. 30, 600 μm).

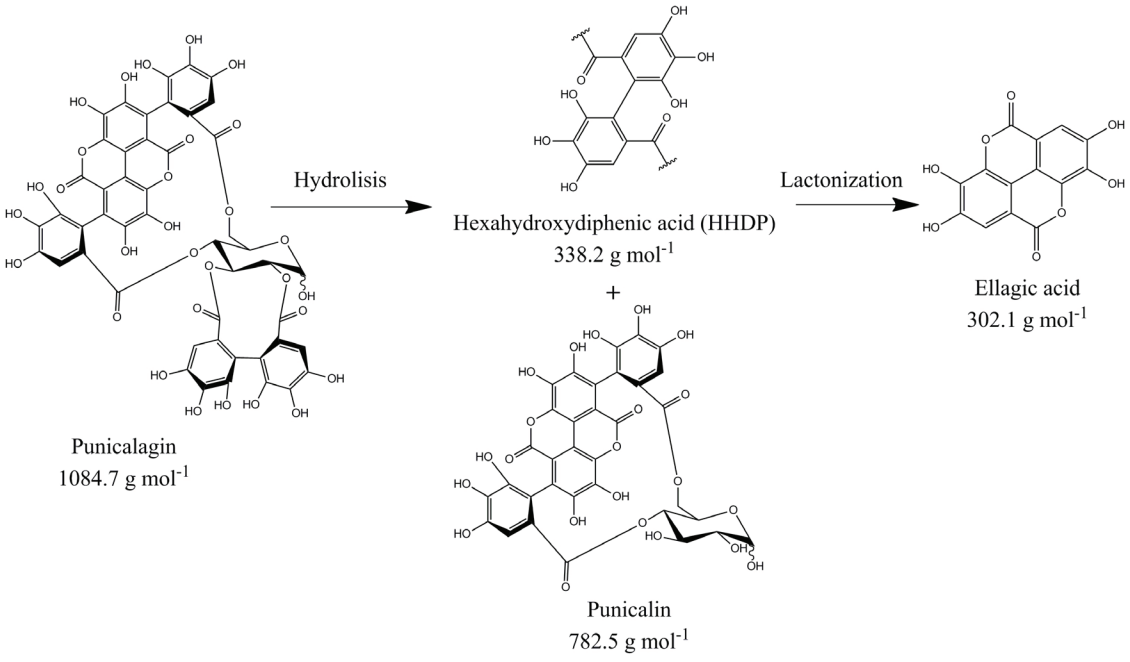

Fig. 1. Hydrolysis of punicalagin to produce ellagic acid.

Fungal strain and cell culture. The strain GH1 of *Aspergillus niger* Tiegh. was used, which is deposited at the FRD-UAC culture collection. Cryopreserved spores were activated on PDA medium (30 mL) at 30 C for five days. Fungal spores were harvested using a sterile solution of 0.01% Tween-80, and counted in a Neubauer chamber.

Submerged culture conditions. Erlenmeyer flasks (125 mL) containing 50 mL of Czapek Dox medium[20] were used. Moreover, the Czapek-Dox medium was supplemented with pomegranate husk powder as a carbon source, and the pH was adjusted using NaOH 1M (**Table 1**). The culture medium was inoculated with 1 x 10^6 spores/g of pomegranate husk powder. The submerged culture was carried out at 30 C for 48 h under constant agitation (Shel Lab S12).

Significant factors for EA production. The selection of factors for EA production included tests of different concentrations of substrate, pH and agitation. Significant factors were identified by a Box-Behnken design[4] (BBD). The experimental matrix of the BBD showing treatments, factors, and responses can be seen in **Table 1**. All treatments were performed in triplicate.

Kinetic evaluation of the best production condition of EA by submerged culture. Treatment five was selected (substrate 7.5 g L^{-1}, pH 5.5, agitation 150 rpm) for further study according to the previous experiment. The EA production by submerged culture was evaluated each 8 h during 72 h at 30 C.

EA recovery. The fermented broth was filtered through Whatman no. 40 membranes and stored in amber glass vials at 5 C until analysis. EA recovery was as follows: 1.5 mL of sample was placed in Eppendorf tubes and centrifuged at 6000 rpm for 30 min (spectrofuge 24D, Labnet International). The supernatant was discarded, and 1.5 mL of ethanol-formic acid (0.01%) was added to dissolve EA. Samples were homogenized by sonication (Branson 2510) for 15 min. EA was quantified using high pressure liquid chromatography (HPLC).

Quantification of EA by HPLC. Samples were filtered (0.45 µm millipore-millex HN membranes), and analyzed by HPLC system using a modified method of Sepúlveda *et al.*[20]. HPLC system (Varian ProStar model 2301) was used for EA determination under the following operational conditions: column Grace Denali C18 (250 x 4.6 mm), at 30 C, flow rate of 1.0 mL min^{-1}, sample volume of 10 µL, wavelength of 275 nm, and 20 min of time analysis. Mobile phase was: methanol (A) as wash phase, acetonitrile (B) and 3% acetic acid (C). A standard calibration curve of EA (Sigma-Aldrich) from 0-500 µg L^{-1} was performed.

Soluble protein. Protein content was determined according to the method of Bradford[5].

Hydrolysable tannins. Determinations were made according to Makkar *et al.*[17]. Gallic acid (Sigma-Aldrich) was used as a standard (0-500 µg L^{-1}), 800 µL of diluted sample (1:100) was mixed with 800 µL of Folin-Ciocalteu reagent (Sigma-Aldrich); 5 min later, 800 µL of 0.01 M sodium carbonate was added. After 1 min, the samples were measured in a UV-visible spectrophotometer (Varian Cary 50 Bios) at 750 nm.

Statistical analysis. BBD was carried out using 13 treatments (randomized experimental design with factorial arrangement 3^3). Three factors were evaluated (substrate, pH, agitation), and three central points. All treatments were performed in triplicate. The factor

response was the EA production (**Table 1**). Data were analyzed using Statistica 7.0. Pareto chart was used to standardize values, and to evaluate factors in descending order.

RESULTS AND DISCUSION

Significant factors for EA production. The effect of three factors on EA production by *Aspergillus niger* GH1 is shown in **Table 1**. The maximum EA production was achieved in treatments 1, 5, and 7, reaching 20.81, 20.66 and 21.19 mg g^{-1}, while the minimum production was obtained in treatment 12 (2.58 mg g^{-1}). A similar system for EA production by *A. niger* SHL6 using valonia tannins as a carbon source in submerged culture found the effect of culture volume, substrate concentration, pH and other factors. The authors attributed the EA production to the action of an enzyme called valonia tannin hydrolase[10]. Another study evaluated the individual and combined effects of the same parameters for EA production using *A. oryzae* and ellagitannins of oak acorn. Ellagitannins concentration, incubation time and the pH were considered significant parameters for a maximum EA production of 17.7 mg g^{-1} after 84 h of culture[11]. The greatest EA production from cups extract of valonia acorns by pure and mixed culture of *A. oryzae* and *Trichoderma reesei* was 23 mg g^{-1}, obtained at 96 h of culture[12]. Individual and combined effects of five enzymes (ellagitannin acyl hydrolase, β-glucosidase,

Table 1. Matrix from the Box-Behnken design and ellagic acid production by *Aspergillus niger* GH1 in submerged culture.

Treatments	Factors			Response
	Substrate (g L^{-1})	pH	Agitation (rpm)	Ellagic acid (mg g^{-1})
1	7.5	5	200	20.81
2	17.5	5	200	9.09
3	7.5	6	200	16.33
4	17.5	6	200	6.41
5	7.5	5.5	150	20.66
6	17.5	5.5	150	5.94
7	7.5	5.5	250	21.19
8	17.5	5.5	250	7.04
9	12.5	5	150	10.10
10	12.5	6	150	8.68
11	12.5	5	250	8.74
12	12.5	6	250	2.58
13	12.5	5.5	200	8.42

polyphenol oxidase, xylanase, cellulase), hydrolysis time, and particle size were assessed for EA production by *Aspergillus* and *Endomyces* from oak acorns[13]. Maximum EA production (38.17 mg g^{-1}) was obtained after 1.48 h of culture. In the present study, EA production was lower than those previously reported, showing the ability of *A. niger* to degrade ellagitannins present in the pomegranate husk. In order to identify the influence of each factor on EA production, a Pareto chart is shown in **Fig. 2**. Only those factors that passed central dotted line had influence on EA production. Substrate (L) and pH (Q) showed significant influence on EA production at the 95% confidence level. Software analysis indicated that the best production of EA is reached at low values of substrate and pH. Agitation did not have influence on EA production.

The results showed that the pH and agitation had some effect on EA production. The EA yield increases at low pH and medium level of agitation. This is due to the presence of enzymes, such as ellagitannase, which degrade ellagitannins at pH 5[3]. Agitation is an essential parameter in submerged culture for suitable mixing of the medium and fungal growth. The EA yield increases at low level of substrate and medium level of agitation. The EA yield depends on the substrate (ellagitannins) as carbon and energy source for fungal

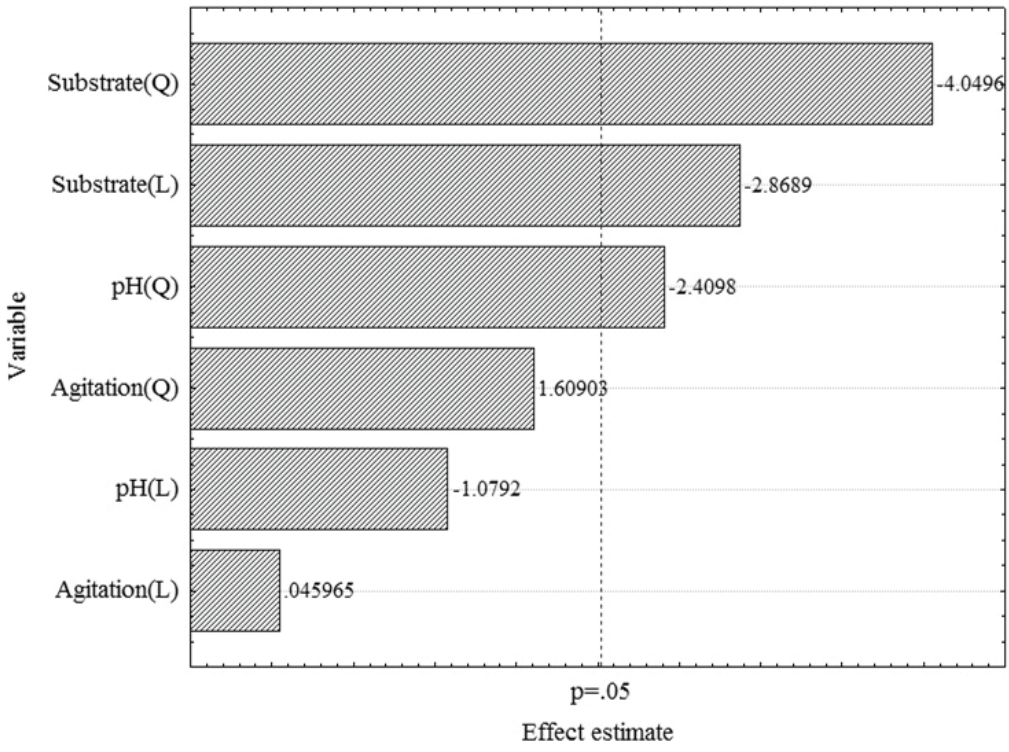

Fig. 2. Pareto chart describing the influence of factors evaluated on ellagic acid production by *Aspergillus niger* GH1. L= Lineal (influence at two certain points of the process). Q= Quadratic (influence at three certain points of the process).

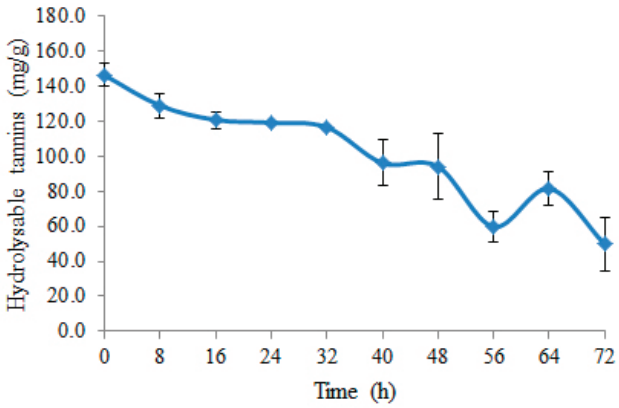

Fig. 3. Total hydrolysable tannins consumption of pomegranate husk by *Aspergillus niger* GH1 in submerged culture.

growth. High values of EA production are obtained at low level of substrate and pH.

Kinetic evaluation of the best production condition of EA by submerged culture. Three treatments showed similar values of EA production. Treatment 5 (substrate 7.5 g L^{-1}, pH 5.5, agitation 150 rpm) was selected, because it had the slowest agitation meaning low production costs. The effect of fermentation time on hydrolysable tannins content is shown in **Fig. 3**. A decrease in hydrolysable tannins concentration was observed, reaching up to 57% consumption at 56 h of culture. This decrease showed the ability of *A. niger* GH1 to degrade hydrolysable tannins present in the pomegranate husk powder. In a solid state culture of creosote bush (*Larrea tridentata*) extracts and *Aspergillus niger* PSH, it was reported a consumption of 16% hydrolysable tannins[24]. The increase of hydrolysable tannins at 64 h culture may be due to the ability of *A. niger* GH1 to cleave other tannins, which can be detected by UV-vis spectrophotometric techniques[8]. A gradual increase in the protein content was observed in the experiments, reaching a maximum at 24 h (**Fig. 4**). Protein content decreases after this time. There are few studies about the relationship among enzymes and ellagitannins hydrolysis. In co-fermentation of *A. niger* and *Endomyces fibuliger*, weak ellagitannin acyl hydrolase, β-glucosidase, and polyphenol oxidase enzymes were observed[13]. When ellagitannins (punicalin) were used as a carbon source in solid state culture of *A. niger* GH1, the action of different enzymes was recorded (cellulase, tannase, xylanase, β-glucosidase, polyphenoloxidase, ellagitannase), and the ellagitannase was associated with EA production[3]. A high correlation between EA yield and enzyme activities (ellagitannin acyl hydrolase, cellulose, xylanase) has also been reported, suggesting that enzymes studied could act synergistically to enhance EA production[14].

The time effect on EA production is shown in **Fig. 5**. The maximum concentration of EA (138.44 mg g^{-1}) was recorded at 8 h, using *A. niger* GH1 in submerged culture. After this time, values decreased to zero. This was due to the

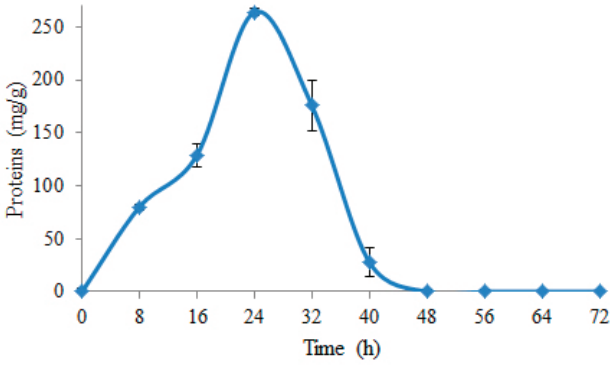

Fig. 4. Protein accumulation of pomegranate husk by *Aspergillus niger* GH1 in submerged culture.

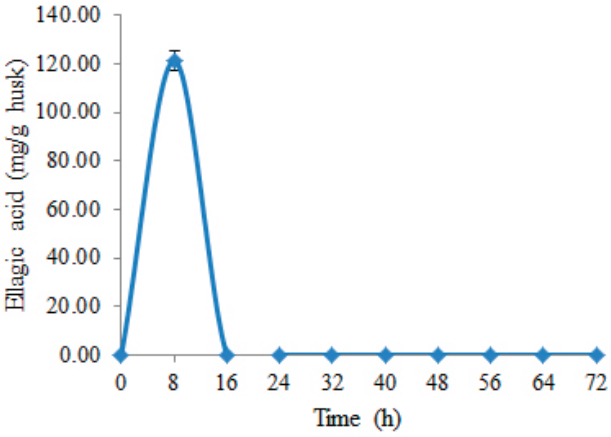

Fig. 5. Effect of time on ellagic acid production of pomegranate husk by *Aspergillus niger* GH1 in submerged culture.

ability of the fungal strain to use EA as a carbon source. However, these phenomena have not been previously described. *A. niger* and *Candida utilis* have been used for EA production, reaching a maximum (21.1 mg g^{-1}) after seven days[21]. Cranberry pomace was used as substrate to produce EA by *Lentinula edodes* in solid state culture, reaching a maximum of 350 mg g^{-1} after five days[23]. Agro-industrial by-products have also been evaluated for EA production[6]. In conclusion, EA production from pomegranate husk powder by *A. niger* GH1 in submerged culture was demonstrated. The bioprocess reached 138.44 mg g^{-1} of EA. The substrate and pH were important factors affecting EA production. Protein production is associated to the activity of enzymes involved in the hydrolysis of ellagitannins, as well as the capacity of *A. niger* GH1 to degrade hydrolysable tannins and to produce EA. The bioprocess developed in this study represents an alternative for large-scale EA production.

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