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Accumulation of Tannins from Pomegranate Husk Residues (*Punica granatum* L.) by Submerged Fermentation of *Aspergillus* sp.

Acumulación de Taninos a Partir de Residuos de Cáscaras de Granada (*Punica granatum* L.) Mediante Fermentación Sumergida de *Aspergillus* sp.

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Resumen

En la actualidad, el uso y desarrollo de procesos biotecnológicos en diversas industrias cobra especial importancia debido a que estos procesos permiten la obtención de productos a un menor costo y con un menor impacto al medio ambiente. El sistema de fermentación sumergida es ampliamente utilizado en la industria por las ventajas que presenta frente a otros procesos convencionales como la hidrólisis ácida, la maceración y la extracción mediante tecnologías emergentes. A través de un sistema de fermentación sumergida se realizó la cinética de crecimiento de dos cepas del género *Aspergillus* (*Aspergillus niger* GH1 y *Aspergillus niger* HT3) para la posterior evaluación de la acumulación de compuestos fenólicos, azúcares totales y proteínas solubles de la cáscara de granada. Los resultados obtenidos mostraron que la fermentación sumergida es un proceso que permite la acumulación de taninos a partir de la cáscara de granada, además, *A. niger* HT3 fue la cepa que presentó un desarrollo óptimo con una acumulación máxima de 837 mg/L de taninos hidrolizables y 800 mg/L de taninos condensados.

Palabras clave: Taninos hidrolizables, Taninos condensados, azucares, proteínas, bioprocesos.

Abstract

Currently, the use and development of biotechnological processes in various industries are particularly important because these processes allow the obtaining of products at a lower cost and with a lower impact on the environment. The submerged fermentation system is widely used in the industry for the advantages it presents over other conventional processes such as acid hydrolysis, maceration, and extraction using emerging technologies. Through a submerged fermentation system, the growth kinetics of two strains of the genus *Aspergillus* (*Aspergillus niger* GH1 and *Aspergillus niger* HT3) was carried out for the subsequent evaluation of the accumulation of phenolic compounds, total sugars and soluble proteins from pomegranate peel. The results obtained showed that submerged fermentation is a process that allows the accumulation of tannins from pomegranate husk. In addition, *A. niger* HT3 was reported as the optimal microorganism with a maximum accumulation of 837 mg/L of hydrolyzable tannins and 800 mg/L of condensed tannins.

Keywords: Hydrolyzable tannins, Condensed tannins, sugars, proteins, bioprocess.

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INTRODUCTION

In recent years, the development and implementation of biotechnological processes in various industries have gained importance. In this context, submerged fermentation is described as the degradation process of complex molecules into others simpler, this bioprocess is conducted in an aqueous environment using microorganisms (Chisti, 2014).

Submerged fermentation processes are of great importance in the food and pharmaceutical industry. The handling and control of the factors involved in the bioprocess can be easier. In addition, it can maintain greater homogeneity in the system and the recovery of the products is simpler (Zhong, 2011). An important advantage in these bioprocesses is the use of organic waste as a source of carbon and energy for the recovery of metabolites of interest (Vargas- Corredor and Pérez-Pérez, 2018). Some studies that it has been found that agro-industrial residues with potential for their revaluation, among which the pomegranate can be mentioned. Pomegranate fruit has been recognized for its pleasant taste and excellent health benefits (Karimi et al., 2017). These benefits are attributed to the metabolites present in this fruit. Only in Mexico, the production of pomegranate is around 8 thousand tons per year, of which approximately the 40 % by weight corresponds to the peel, these peels are considered as waste, however, several studies show that pomegranate peel contains bioactive compounds of industrial interest. In another study, Buenrostro-Figueroa et al. (2018), evaluated the production of ellagic acid from pomegranate husk polyphenols using *Aspergillus niger* GH1 by solid fermentation on inert supports, the best results of up to 231.22 mg/g of ellagic acid were reported. On the other hand, Salinas-Flores et al., (2019), evaluated two physical extraction methods (maceration and ultrasound) to obtain bioactive compounds present in the pomegranate husk; they evaluated the antioxidant activity of the extracts obtained, and the authors reported a maximum polyphenol was of 71 mg/g approximately with high percentages of antioxidant activity. In addition, the pomegranate husk is currently considered a waste that does not receive any value and because of the way it is disposed of, it can damage the environment, for all the above, the pomegranate husk can be presented as a suitable substrate for producing secondary metabolites of industrial importance.

Tannins are polyphenolic compounds and are considered molecules more abundant in nature and presented in various tissues of plant species (Arbenz and Avérous, 2015). The according to their chemical structure can be classified as condensed and hydrolyzable tannins (Das et al., 2020). Condensed tannins are phenolic plant secondary compounds formed from flavan-3-ol units, including (−)-epicatechin, (+)-catechin, (−)-epigallocatechin, and (−)-epicatechin-3-O-gallate, linked by carbon-carbon bonds (Li and Duan, 2019). On the contrary, the hydrolyzable tannins are composed of a glucose nucleus attached to compounds of ellagic acid and gallic these tannins are divided into two families: the gallotannins, which produce gallic acid and its derivatives from hydrolysis; and the ellagitannins, which produce ellagic acid (Sharma, 2019). The importance of these compounds lies in the biological activities attributed to them, which provide health benefits. Among these have been mentioned the antioxidant, antimicrobial, antifungal, and antiviral activity among others (Diaz-Herrera et al., 2019).

The objective of this study was evaluated the growth capacity of strains of *Aspergillus* (*A. niger* GH1 and *A. niger* HT3) for the accumulation of tannins and his relationship with decrease of total sugars and soluble proteins obtention from pomegranate husk using submerged fermentation.

MATERIALS AND METHODS

Raw material

The pomegranate fruits were collected in Cuatrociénegas, Coahuila, Mexico. The husk will be removed by hand and dehydrated at 60 °C for 48 h. The samples will be pulverized until obtaining a particle size smaller than 1 mm (Sepúlveda et al., 2012).

Microorganism

A. niger GH1 and HT3 strains for DIA/UADEC (Departamento de Investigación en Alimentos/Universidad Autónoma de Coahuila). The strains were reactivated according to the methodology described by Sepúlveda et al., (2018).

Submerged fermentation system

For fermentation kinetics, Erlenmeyer flasks (250 mL) containing 50 mL of Czapek-Dox medium were utilized, with the following composition (gL^{-1}): $NaNO_3$ (15.6); KH_2PO_4 (6.08); $MgSO_4 \cdot 7H_2O$ (3.04); KCl (3.04). The medium culture was inoculated with $1x10^6$ spores and pomegranate husk was incorporated as a source of carbon and energy. Fermentation was conducted at 200 rpm and 30 °C. The fermentation extract was recovered through simple filtration with filter paper. The content of hydrolyzable tannins, condensed tannins, total sugars, and soluble protein and the biomass produced were evaluated directly in the fermentation extract according to the next methodologies. The samples were recovered for 144 h every 24 h.

Biomass determination

Biomass was determined by the difference in dry weight. The content of each flask was suction filtered with filter paper previously weighed. Then the filter paper was dried at 60 ºC to obtain a constant weight (Rodríguez-Pérez et al. 2017).

Total sugars determination

The determination of the total sugar content was made according to the methodology described by Boshagh, (2021); 250 µL of the fermentation extract was taken and placed in a test tube. Then 250 µL of 5% phenol was added, leaving a cold-water bath for 5 min. Subsequently, 1 mL of H_2SO_4 was added and left to boil for 5 min. Finally, the sample was allowed to

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cool to room temperature for 5 min and the sample was read at an absorbance of 480 nm.

Soluble protein determination

The soluble protein content was performed using the method described by (Mæhre et al., 2018). A pattern curve was made using bovine serum albumin as standard at 1000 ppm, 0.1 ml of the sample was placed in test tubes, then 5 ml of the Bradford reagent was added, stirred, and left to stand. Finally, the absorbance.

Hydrolyzable tannins determination

The hydrolyzable tannins were determined using the Folin-Ciocalteu spectrophotometric method using gallic acid as a reference curve according to the methodology reported by De León-Medina et al., (2020) with some modifications; 800 µL of the sample were placed in a test tube, then 800 µL of the Folin-Ciocalteu reagent were added and mixed, leaving them to react for 5 min, after this time, 800 µL of sodium carbonate (0.01 M) were added and mixed, with a new resting period of 5 minutes. Finally, the solution was diluted with 5 ml of distilled water and its absorbance was read on the spectrophotometer at 790 nm.

Condensed tannins determination

Condensed tannins were determined by the spectrophotometric method of HCl-Butanol using catechin as a reference standard according to the methodology reported by Sepúlveda et al., (2020) with some modifications. A standard solution of catechin at 1000 ppm was prepared.

RESULTS

Biomass production in submerged fermentation

In Figure 1 shows the biomass kinetics production of *A. niger* GH1 and *A. niger* HT3. In the case of both microorganisms, there is no latency phase which indicates that the fungi of this genus adapt quickly to the environment. Later they presented an exponential growth phase within the first 24 h, in the case of *A. niger* GH1 a stationary phase can be observed until 96 h hand in the case of *A. niger* HT3 an increase was observed again at 120 h. Both microorganisms it can see the cell death phase where nutrients have been depleted and mycelial growth begins to decrease. *A. niger* GH1 presents a higher biomass production reached approximately 0.45 mg/L at 72 h. Biomass production is associated with the production performance, in Figures 5 and 6 can be appreciated that the maximum concentration of tannins is reached at 72 h, when is present maximum biomass concentration.

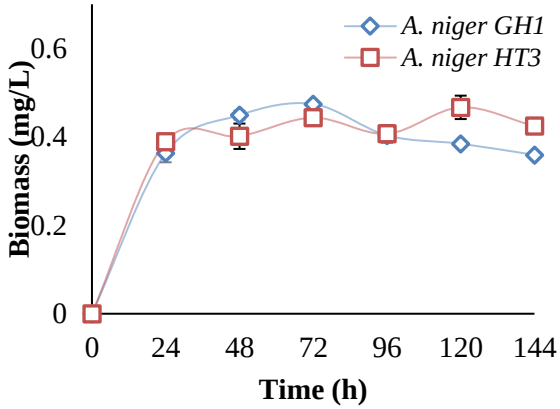

Figure 1. Biomass production in submerged fermentation.

Total sugar accumulation in submerged fermentation

The kinetics of consumption of total sugars shown in Figure 2, where a maximum decrease is observed in the first 24 h of the submerged fermentation using each of the microorganisms, this indicates that during this time the microorganism began to decrease the sugars present in the substrate. In Figure 1 the greatest increase in the mycelial mass occurs at 24 h, same in which the highest consumption of sugars is presented, after this, there is a trend of small decreases up to 144 h, which is explained that the microorganism begins to take carbon from other sources to stay active until it reaches the stage when it begins to inactivate and decreased his biomass cell.

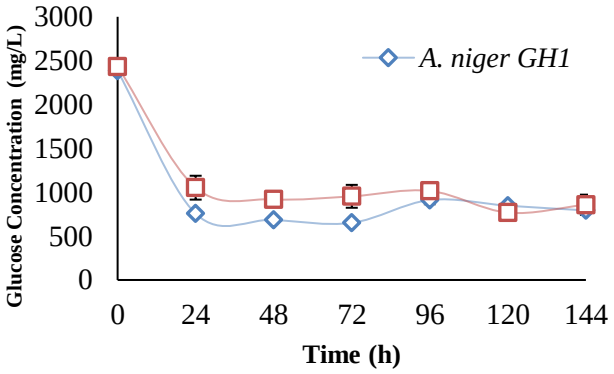

Figure 2. Decrease of total sugars in submerged fermentation.

Soluble Proteins in submerged fermentation

The soluble protein content is shown in Figure 3. The maximum quantification of protein was presented at 24 h in submerged fermentation using *A. niger* HT3, while for *A. niger* GH1 the maximum concentration occurs at 48 h, after this time, for both microorganisms, the proteins have a small decrease over time. The protein content is associated with the growth of the microorganism, so we can see that after 120 h there is a drop in the production of proteins for both microorganisms, this indicates that the microorganism decreased your growth cell. Sepúlveda et al., (2014) in a similar study in submerged fermentation, reported the enzymes ellagitannase, tannase,

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xylanase, and β-glucosidase as the enzymes responsible for the degradation of ellagitannins from pomegranate husk. In another study Kassara et al., (2022) evaluated the total concentration of protein of red wine produced from *V. vinifera* and interspecific (*Vitis* spp.) hybrids, the results obtained showed concentrations in a wide range from 23 to 380 mg/L, in addition, it was found that a higher concentration of tannins in the wine was also correlated with greater heat stability of the wine protein.

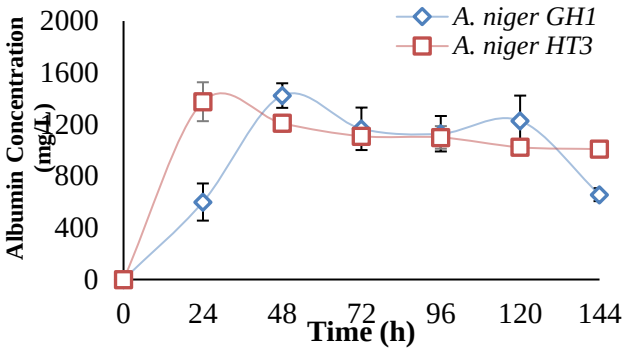


Figure 3. Determination of soluble proteins in submerged fermentation.

Hydrolyzable tannins in submerged fermentation

The concentration of hydrolyzable tannins concerning time shown in Figure 4 for both microorganisms the maximum accumulation of hydrolyzable tannins occurs at 72 h, *A. niger* HT3 presented the highest accumulation of 837 mg/L while *A. niger* GH1 reached a maximum accumulation of 532 mg/L. Abdon-Aguilar et al., (2018) reported an accumulation of approximately 1000 mg/L of hydrolyzable tannins using pomegranate husk and *A. niger* GH1 in submerged fermentation, however in this study a modified Czapeck medium and conditions different from those used in the present study. There are few studies about the tannins obtention using submerged fermentation with filamentous fungi taking advantage of agro-industrial waste. In another study where was evaluated phenolic compounds of cereal vinegar and fruit vinegar in China, Ren et al., 2017 reported cereal vinegar exhibited higher hydrolyzable tannins contents than fruit vinegar, reaching until 2200 mg/L. On the other hand, bioactive compounds from of grape wastes (pomace, skin, and seeds) were investigated. Total tannins contents of grape by-products varied between 31.2 mgGAE/g (molasses skin) and 98.97 mgGAE/g (wine seed); 96.93 mgTAE/g (grape juice pomace) and 138.67 mgTAE/g (molasses pomace), respectively. The authors concluded that process methods, such as pressing, and fermentation had affected the extraction efficiency and the source of grape by-product influence on the tannins contents (Gülcü et al., 2019).

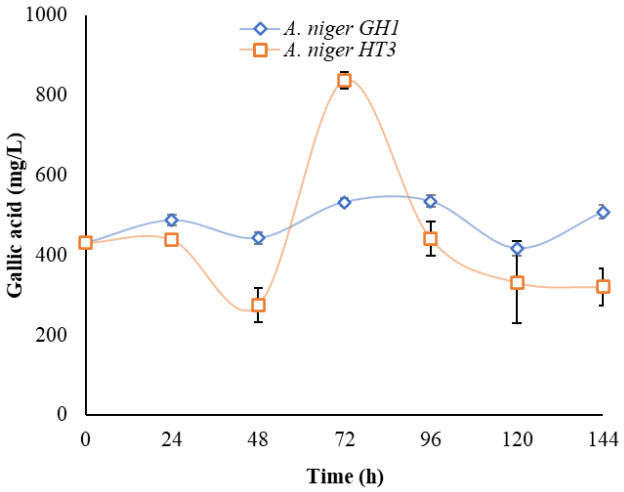
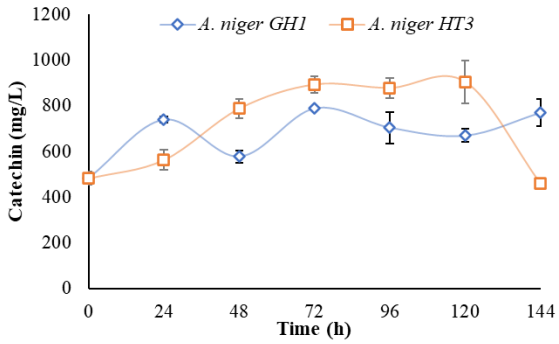


Figure 4. Accumulation of hydrolyzable tannins in submerged fermentation.

Condensed tannins in submerged fermentation

The accumulation of condensed tannins shown in Figure 5, for both microorganisms, the maximum accumulation of condensed tannins occurred at 72 and 120 h, however *A. niger* HT3 presented an amount higher than that obtained by GH1 greater than 800 mg/L. Exist few research about the accumulation of condensed tannins from pomegranate husk in submerged fermentation by filamentous fungi but can mentioned similar works. Adebo et al., (2018) reported accumulation of condensed tannins in fermentation process from sorghum ting, reaching approximately 13 mg/g of catechin. The authors attribute the corresponding increase in catechin, to the release of these bioactive compounds after fermentation with *Lactobacillus* strains. In another study Ju et al., 2021 evaluated condensed tannin concentration of spinal grapes and wines, condensed tannin profiles were evaluated by high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD). The content of condensed tannins depended a lot on the variety of the fruit, the content varying from 0.30 mg/g to 7.80 mg/g (in skins), from 3.12 mg/g to 8.82 mg/g (in seeds), and from 62.60 mg/L to 225.90 mg/g/L (in wines).

Figure 5. The accumulation of condensed tannins in



submerged fermentation

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CONCLUSIONS

The pomegranate husk proved to be an important source of carbon and energy for the growth of fungi of the genus *Aspergillus*. Important yields of condensed and hydrolyzable tannins were obtained through the submerged fermentation process. *A. niger* HT3 proved to be more effective for this bioprocess in the accumulation of tannins from the pomegranate husk compared to *A. niger* GH1 obtaining values of approximately 1.3 times more.

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