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Evaluation of onion waste as a substrate in submerged fermentation for the production of bioactive compounds

Evaluación de residuos de cebolla como sustrato en fermentación sumergida para la producción de compuestos bioactivos

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Resumen

El desarrollo de nuevas tecnologías para el aprovechamiento de residuos agroindustriales, implica su uso como soportes o sustratos para la producción de compuestos bioactivos mediante fermentación sumergida (SmF), utilizando microorganismos que tienen la capacidad de degradar las paredes celulares de los vegetales. El objetivo del estudio fue utilizar residuos de cebolla como sustrato de *Aspergillus niger* para la producción de un extracto rico en compuestos bioactivos utilizando SmF. Las evaluaciones del extracto fermentado se realizaron por triplicado cada 24 h durante 240 h, para evaluar la cinética del proceso de producción de compuestos bioactivos; los datos se analizaron estadísticamente mediante análisis de varianza y las pruebas de comparación de medias se evaluaron con la prueba de Tukey ($p \leq 0.05$). Los resultados obtenidos mostraron la

capacidad de *Aspergillus niger* para utilizar el residuo de cebolla como fuente de carbono, alcanzando un crecimiento máximo de 60.9 mg de glucosamina (GcA)/g de muestra seca a las 144 horas. El crecimiento del microorganismo permitió la producción de metabolitos secundarios, como los fenoles hidrolizables (THP), con una concentración máxima de 14.4 mg de Equivalentes de Ácido Gálico (GAE)/g de muestra seca a las 120 h, y Flavonoides, con una concentración máxima de 4.1 mg de equivalentes de quercetina (QE)/g de muestra seca en 240 h, con una capacidad antioxidante de 51.4 mM de equivalente de Trolox (TE)/g de muestra seca en un tiempo de 240 horas. Se demostró que el uso de residuos de cebolla como sustrato por parte de SmF permite obtener una mejor concentración de compuestos bioactivos de alto interés.

Palabras clave: Residuo agroindustrial, *Aspergillus niger*, compuestos bioactivos, fermentación sumergida.

Abstract

The development of new technologies for the use of agroindustrial waste, implies their use as supports or substrates for the production of bioactive compounds through submerged fermentation (SmF), using microorganisms that have the ability to degrade the cell walls of vegetables. The objective of the study was to use onion waste as a substrate by *Aspergillus niger* for the production of an extract rich in bioactive compounds using SmF. Evaluations of the fermented extract were performed in triplicate every 24 h during 240 h, to evaluate the process kinetics of bioactive compounds production; data were statistically analyzed by analysis of variance, and the tests for comparison of means were evaluated with the Tukey test ($p \leq 0.05$). The obtained results showed the capacity of *Aspergillus niger* to use the onion waste as carbon source, reaching a maximum growth of 60.9 mg glucosamine (GcA)/g dry sample at 144 hours. The growth of the microorganism allowed the production of secondary metabolites, such as Hydrolysable phenols (THP), with a maximum concentration of 14.4 mg Gallic Acid Equivalents (GAE)/g dry sample at 120 h, and Flavonoids, with a maximum concentration of 4.1 mg Quercetin equivalents (QE)/ g dry sample in 240 h, having an Antioxidant Capacity of 51.4 mM Trolox Equivalent (TE)/g dry sample at time of 240 hours. It was demonstrated that the use of onion residues as substrate by SmF allows obtaining a better concentration of bioactive compounds of high interest.

Key Words: Agroindustrial waste, *Aspergillus niger*, bioactive compounds, submerged fermentation.

1. Introduction

The large production of agro-industrial waste generated by the food industry makes necessary to search for alternatives to its valorization. Such valorization involves the sustainable conversion of waste into value-added products. Agri-food wastes are a good source of bioactive compounds (Ben-Othman et al., 2020), being conformed mainly by molecules with health beneficial properties such as antioxidants, antibacterial, anti-tumor, anti-obesity, etc. (Hussain et al., 2020).

Onion (*Allium cepa L.*) is one of the main agricultural products in the world, second only after tomato (Sidhu et al., 2019), and its

production continues increasing, so the waste generated by the industry has caused serious problems. Onion waste is a renewable raw material and their valorization products can be used as a source of functional components (Sharma et al., 2016). Studies have shown that these wastes have been used as raw material for the fermentative production of biogas, microbial lipids, biohydrogen, volatile organic acids, chemicals and biofertilizer, after appropriate processing (Mashad et al., 2019; Blue et al., 2019, 2021; Esercizio et al., 2021). Research refers to onion waste as an excellent source of compounds such dietary fibers, fructooligosaccharides, polyphenols, antioxidants, and minerals (Sagar et al., 2018, 2020).

The vegetable wastes are mainly of lignocellulosic nature, consisting of carbohydrates: cellulose protected by hemicellulose and lignin, and a smaller proportion of protein, pectin, and ashes, used as a source of carbon and energy (Azelee et al., 2020). The main structural component in plant cell walls is cellulose, a polysaccharide composed of a linear chain of β1,4-glycosidic linked D-glucose units (Namnuch et al., 2021). Hemicellulose is an heterogeneous polymer, composed mainly of pentose sugars (D-xylose, L-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids; its composition is highly variable and depends on the plant source (Zhou et al., 2018). Lignin is an amorphous heteropolymer synthesized from phenylpropanoid precursors of guaiacyl alcohol, p-coumaryl alcohol and syringyl alcohol, when bound to hemicellulose and cellulose, act as a barrier to any solution or enzyme, preventing penetration into its lignocellulosic structure for depolymerization (Weng et al., 2021). Onion wastes are mainly composed by glucose, fructose, sucrose, fructooligosaccharides, polyphenols and antioxidants (Krähmer et al., 2021; Pöhnl et al., 2018; Sagar et al., 2021).

In the selection of a suitable process in the conversion of lignocellulosic materials, the submerged fermentation (SmF) technique is a promising method in fungal bioconversion for the production of nutrient-rich compounds (Chang & Webb, 2016). Also, the SmF process allows a better control of the operating parameters due to the liquid media homogenization, thereby improving heat and mass transfer, and favoring nutrient uptake (Coradi et al., 2013; Lima et al., 2019).

Some microorganisms, especially fungi of the genus *Aspergillus*, can degrade this type of vegetal wastes, being one of the main contributors of secondary metabolites from fungal origin (Chang & Webb, 2016). Microorganisms have the capacity to enzymatically hydrolyze lignocellulosic residues, generating simple sugars as a carbon source for their metabolic functions, thus producing various compounds of great interest in industries (Rodríguez-Luna et al., 2020).

The objective of this study was to assess the use of onion waste as substrate in submerged fermentation system with *A. niger* to produce extract rich in bioactive compounds.

2. Materials and methods

2.1. Onion peel wastes

White onion wastes were obtained from a local food market in Saltillo, Coahuila. The residues were dehydrated at 60°C for 48 h, ground in a conventional blender (Oster Model 4127) and sieved (Brand Montinox) at particle sizes of mesh 10 (2.00 mm) and mesh 30 (0.60 mm). The samples were kept in hermetic bags at room temperature until further use.

2.2. Submerged fermentation (SmF) process

A. niger (code KY825168.1) strain was obtained from the collection of the Department of Food Science and Technology of the Universidad Autónoma Agraria Antonio Narro.

The fungus was cultured in potato dextrose agar (PDA) at 30°C for 7 days. For the fermentation process, a minimal Czapek Dox mineral medium was prepared according to the method described by Omojasola & Benu, (2016), with some modifications. Composed by 1.5 g onion residue (additional substrate), 1.5 g glucose (growth inducer) (Sigma-Aldrich), 0.3 g $NaNO_3$, 0.1 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.05 g KCl, and 0.001 $FeSO_4$ (Sigma-Aldrich) in 100 mL of sterile fermentation medium. It was inoculated with *A. niger* at $1x10^6$ spores/mL, where the spore count was performed in a Neubauer chamber. It was incubated at 30°C with 200 rpm (Orbital Shaker incubator, Innova 44) for 240 h and was monitored every 24 h in triplicate, to obtain maximum production and guarantee the reproducibility of the bioactive compounds. The obtained extract was filtered on porous paper, centrifuged at 4500 rpm for 10 min, followed by a filtration through 0.45 µm nylon membranes. The process was carried out for the separation of the fermented extract with the biomass generated by the fungus *Aspergillus niger* in the fermentation process.

2.3. Characterization

2.3.1. Fungal biomass

Fungal biomass was evaluated spectrophotometrically by the glucosamine content method (Medina-Morales et al., 2012; Díaz-Herrera et al., 2020). Samples were hydrolyzed to release glucosamine from the cell wall, subsequently combining with acetylacetone to form a pyrrole compound, which upon reaction with p-dimethylaminobenzaldehyde forms a red colored compound at an absorbance of 530 nm. The calibration curve was performed with glucosamine (0.001- 0.2 mg/ml) under the same conditions as the sample. The results are expressed in mg glucosamine/g dry sample (mg GcA/gds).

2.3.2. Total sugars

Total sugars were determined by the phenol-sulfuric method, with modification (Dubois et al., 1956). For the assay, 250 μL of the fermented extract were taken, then 250 μL of 5% phenol were added (Sigma-Aldrich), leaving it in a cold-water bath for 5 min, then 1 mL of H_2SO_4 (Jalmek) was added and taken to hot bath at 30°C for 5 min. It was left to rest at room temperature and reading was taken at an absorbance of 470 nm (UV/VIS Spectrophotometer Brand Thermo Scientific G10S). Glucose (Sigma-Aldrich) 0.1 % was used as standard. Values are expressed as mg/gds.

2.3.3. Reducing sugars

The reducing sugars determination was performed with the dinitro-salicylic acid (DNS) method (Miller, 1959) with modifications. For the test, 1 mL of the fermented extract was added, plus 1 mL of DNS, then it was placed in a water bath for 5 min at 90°C.Then in a cold-water bath for 5 minutes to stop the reaction, and 5 mL of distilled water was added. Finally, sample are left at room temperature and reading was taken at an absorbance of 540 nm (UV/VIS Spectrophotometer Brand Thermo Scientific G10S). Glucose (Sigma-Aldrich) 0.1% was used as a standard. Values are expressed as mg/gds.

2.3.4. Total hydrolysable phenols (THPs)

Its quantification was carried out with a modified methodology (Makkar et al., 1993), 800 μl of fermented extract was added, then 800 μl of the Folin-Ciocalteu reagent (Sigma -Aldrich). The assay was mixed and allowed

to react for 5 minutes, then 800 μl of 0.01 M Na_2CO_3 (Biopack) was added, allowing it to stand at room temperature. Finally, the solution was diluted in 5 mL of distilled water, the reading was taken at 790 nm in a Thermo Fisher G10S Uv-Vis spectrophotometer. Gallic Acid (Fagalab) 1% was used for the standard. The results for total phenols were determined as gallic acid equivalents (GAE). Values are expressed as mg of GAE/g of dry sample (mg GAE/gds).

2.3.5. Total flavonoids

The determination of total flavonoids was carried out with a modified methodology (Nurcahyo et al., 2020). One mL of the fermented extract was added, then 1 mL of 2% aluminum chloride (Fagalab) prepared in methanol was added. It was left for 1 hour in the dark, then a reading was taken in a Thermo Fisher G10S Uv-Vis spectrophotometer at 546 nm. Quercetin was used for standard curve (0-50 mg/mL). The results were determined as quercetin equivalents (QE). Values are expressed as mg QE/g dry sample (mg QE/gds).

2.3.6. Antioxidant capacity

Antioxidant compound determination was performed by DPPH (2,2- Diphenyl-1-picrylhydrazyl; Sigma-Aldrich, U.S.A.) (Yeoh & Ali, 2016), with some modifications, at a concentration of 60 % w/v, in a methanolic solution. The fermented extract and the DPPH radical were placed in a 50:50 ratio. It was left to stand at room temperature for 30 minutes in darkness. A reading was taken in a Micro-plate reader (BIOBASE EL-10A ELISA) at an absorbance of 530 nm. Trolox solution (standard) was prepared at a concentration of 0-1000 mM with methanol. Antioxidant capacity results are determined as Trolox equivalents (TE). Values are expressed as mM TE/gds.

2.4. Statistical analysis

The Minitab® (version 17.1.0) statistical package was used to process the data. An analysis of variance (ANOVA) and Tukey mean comparison test ($p \leq 0.05$) were performed. The results were expressed as means of the values obtained from the kinetic process.

3. Results and discussion

3.1. *Aspergillus niger* growth kinetics

Fungal invasion is associated with the easily assimilable carbon sources available due to the biodegradation of lignocellulosic materials and different phenolic compounds present in onion peels, as well as synthesis of various enzymes and production of bioactive compounds. Rhim et al., (2014) mention that onion skin is mainly composed of cellulose, followed by lignin, hemicellulose, and a minor number of extractives, such as phenolics and fructooligosaccharides. Therefore, the valorization of these polysaccharides leads to a potential source of carbon and energy for fermentation and biotransformation processes (Choi et al., 2015a).

The glucosamine content was used as an indirect measure to do an estimate of the growth of the fungus *A. niger*. Figure 1 shows the fermentation progress, where the maximum production of 60.9 mg GcA/gds was reached at the 144 hours. The early exponential growth phase of *A. niger* since the first 24 h, could be associated with the assimilation of glucose as carbon sources (Kriaa & Kammoun, 2015).

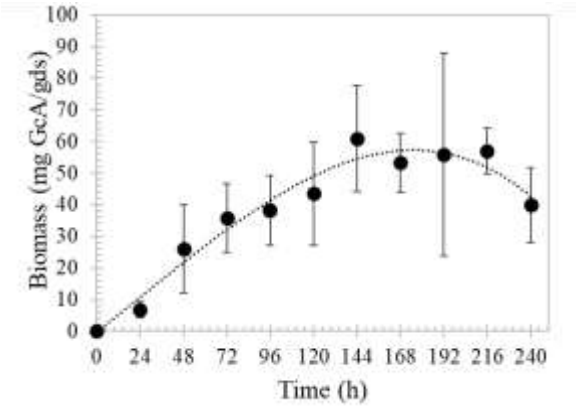

Figure 1. Growth of *A. niger* by SmF using glucose and onion as substrates.

However, once the glucose is consumed, the stationary phase is achieved after 168 hours, where some microbial cells may undergo autolysis with the release of several compounds, thus increasing their concentration in the extract (Lima et al., 2019). Also, Oshoma et al., (2017) mention that the decrease in biomass yield may be the result of nutrient depletion, necessary for fungal growth and biomass formation.

The kinetic behavior of the biomass is attributed to the fermentation time and the age of the microorganism. Likewise, it shows a good behavior with respect to the production of fungal biomass in submerged fermentation, where *A. niger* assimilate the carbon sources provided with glucose and by the onion wastes, and favor the production of cells.

3.2. Kinetics of total and reducing sugars

The onion waste is made up of a high content of soluble sugars (monomers and disaccharides) (Mashad et al., 2019) and a low lignin content (Kim et al., 2017). Cho et al., (2021), mentioned that the first sources of substrate available in onion peel are dissolved sugars (glucose, fructose and sucrose), monosaccharides important in the fermentation process.

The results of substrate consumption as a function of submerged fermentation time are shown in Figure 2. As the hours of fermentation elapse, it is observed that the microorganism is able to consume the sugars present in the culture medium, starting with 655.1 mg/gds of total sugars and 945.7 mg/gds of reducing sugars, until they reach their minimum value at 240 hours. The significant decrease in sugars is due to the microorganism metabolism taking advantage of the easily assimilated carbon sources available in the media.

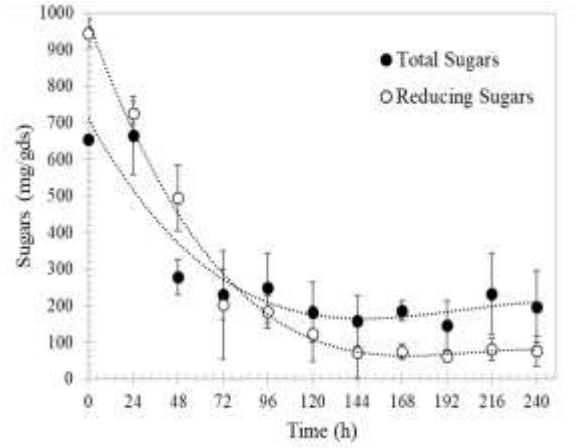

Figure 2. Kinetics of consumption of total sugars and reducing during the SmF process.

The microorganism has the enzymatic capacity to deplete glucose as an easily assimilated monomeric sugar and subsequently degrade complex onion residue substrates into simple compounds, thus obtaining greater energy for cell growth. These enzymes (cellulolytic, xylanolytic and pectinolytic) perform cell wall degradation functions and catalyze the breakdown of complex carbohydrates into their monosaccharide components (Choi et al., 2015b).

Total and reducing sugars appear to be a good source of raw material for use in SmF, allowing the release and recovery of potent phenolic antioxidants from the onion residue. The strain is able to metabolize carbohydrates

present in onion residues for growth and metabolic function (Tope et al., 2019).

3.3. Kinetics of Total Hydrolysable Phenols

THPs show a maximum concentration of 14.4 mg GAE/gds in 120 hours, (Figure 3). Based on the results described in the graphs, the microorganism allows the release of phenolic compounds. Nutongkaew et al., (2019), mentions that enzymes can degrade or transform complex compounds, into metabolites that can be used in various industries.

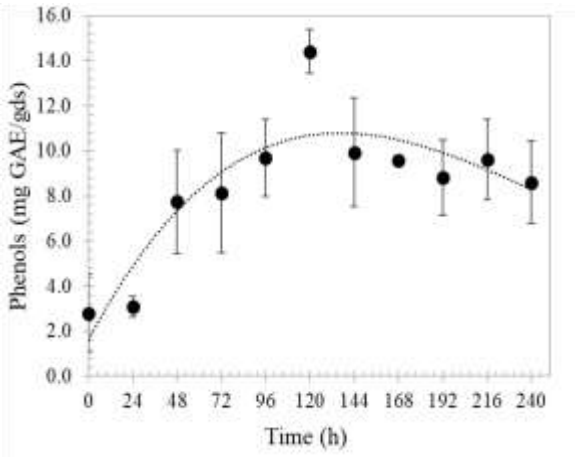

Figure 3. Kinetic behavior of total hydrolysable phenols during the SmF process.

Onion wastes seem to be a good source of raw material for SmF, allowing the release of phenolic compounds. This shows that the microorganism is able to degrade plant cell wall polysaccharides and as a result deliver phenolic compounds (Patyshakuliyeva et al., 2016). Obtaining the product of interest generally depends on the type of substrate and the microorganism used.

3.4. Kinetics of Total Flavonoids

Onion is among the richest plant sources of flavonoids, where its polyphenolic compounds consist of flavonols, characteristic of onion varieties (Cebin et al., 2020). The predominant flavonols are quercetin and its glycosides, which account for up to 80% of the total flavonoids (Pucciarini et al., 2019).

The results showed that from 0 to 240 hours, the total flavonoid content increased exponentially. The highest total flavonoid content was obtained at 240 hours, with a concentration of 4.1 mg QE/gds, (Figure 4). These values indicate that the microorganism has the capacity to produce bioactive compound through SmF and this is due to the release of enzymes from the microorganism, which help to break down the cell walls of the onion residues, resulting in a higher content of phenolic compounds.

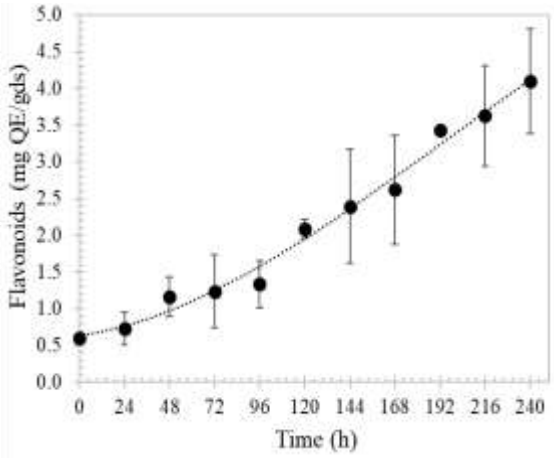

Figure 4. Kinetic behavior of total flavonoids during the SmF process.

That is, through enzymatic cleavage of the substrate, it allows the increase of flavonoid content by SmF (Lee et al., 2016). However, no previous studies have investigated the specific changes in flavonoid composition during fermentation comprehensively. Meanwhile, Choi et al., (2015a) observed that after enzymatic hydrolysis, the percentage of quercetin in onion skin extract reached up to 1.61 times, being higher than the value obtained by chemical extraction of an untreated sample.

This study shows that the use of submerged fermentation technology can increase the

concentration of total flavonoids. The outer layer of onion is considered a residue that can be used to obtain various compounds such as quercetin (Kimoto-Nira et al., 2019). It has also been shown that these wastes, especially in the external part, form a high flavonoid content, followed by a continuous decrease towards the inner part (Fredotović et al., 2021).

3.5. Kinetics of antioxidant capacity

Excess free radicals are one of the main risk factors for the development of various diseases. Onion skins are rich in phenolics with promising antioxidant and free radical scavenging activity and ability to provide protection against DNA damage caused by active oxygen (Lee et al., 2014).

Figure 5 shows the kinetics of antioxidant activity, initially at a concentration of 51.16 mM TE/gds. With time, the antioxidant activity decreased at 168 hours, which was attributed to the small number of biomolecules with antiradical capacity. It is also related that in the first hours *A. niger* undergoes an adaptation process hence a decreasing antioxidant capacity. Subsequently, an increase of 51.4 mM TE/gds was observed up to 240 hours, indicating that *A. niger* has the ability to produce phenolic compounds with antioxidant capacity in the degradation of onion residue, such as gallic acid.

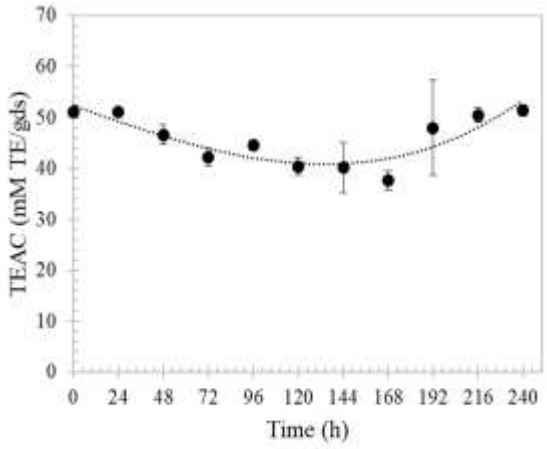

Figure 5. Kinetic behavior of antioxidant capacity during the SmF process.

In comparison to the results of Minh, (2019) the fermentation of red onion inoculated with *Lactobacillus plantarum*, presented a positive result. Other authors report radical scavenging activity in onion fermented by *A. kawachii*, however the result was not significant (Yang et al., 2012).

These studies clearly show that fermentation enhances the effect of onion antioxidant activity. According to reports, this effect is due to the increase of free hydroxyl group caused by hydrolysis during onion fermentation (Lee et al., 2016).

Consequently, submerged fermentation positively increased the antioxidant effect. In particular, onion residue extract is rich in phenolic compounds, with antioxidant function by scavenging free radicals, so it has high nutritional value and potential biological activity.

4. Conclusions

The fungus *A. niger* performs its metabolic function by using onion residues, at the same time it is considered to have a good supporting effect and is also an excellent substrate for microbial growth.

The fungal fermentation process allowed the

onion waste to be biotransformed into compounds with antioxidant activity, which will allow the fermentation extract to act, therefore, this waste material is a potential source of bioactive compounds, being an alternative for large-scale production and application in industries.

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