JBCT | Journal of BioProcess and Chemical Technology

ISSN: 2683-3271

Revista Científica de la Universidad Autónoma de Coahuila

ISSN: 2683-3271

Publicada desde 2009

JBCT-JOURNAL OF BIOPROCESS AND CHEMICAL TECHNOLOGY, año 2022 Volumen 14, No. 28, julio - diciembre 2022, es una publicación semestral editada por la Universidad Autónoma de Coahuila. Boulevard Venustiano Carranza s/n colonia República Oriente, C.P. 25280, tels.: (844) 4-14-85-82 y 4-10-02-78, http://www.biochemtech.uadec.mx, jbct@uadec.edu.mx. Editor Responsable Mónica L. Chávez González. Reserva de Derechos al uso exclusivo No. 04-2019-011112445500-203. **ISSN:** (2683-3271), ambos otorgados por el Instituto Nacional del Derecho de Autor. Responsable de la última actualización de este Número, Facultad de Ciencias Químicas de la Universidad Autónoma de Coahuila, Dr. Leonardo Sepúlveda, Saltillo, Coahuila, C.P. 25280, fecha de última modificación, julio de 2022.

JBCT | Journal of BioProcess and Chemical Technology

ISSN: 2683-3271

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Journal of Bioprocess and Chemical Technology

Revista Científica de la Universidad Autónoma de Coahuila

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Editorial julio-diciembre 2022

Al Equipo Editorial de JBCT nos complace en presentarles el más reciente número, el cual representa un avance importante para la revista ya que a partir de este número el Journal cuenta con registro **ISSN 2683-3271**. En esta edición se refleja el trabajo constante y comprometido por parte de quienes conformamos el equipo editorial y nos llena de alegría poder dar un paso en este proceso de consolidación de la revista. Agradecemos a todos quienes han contribuido en JBCT, particularmente a quienes han confiado sus trabajos para ser publicados en este espacio de divulgación de conocimiento y a todo el panel de revisores que con su apoyo desinteresado y su objetividad han permitido llevar a la revista a lo que es el día de hoy.

Esperamos que disfruten este número que cuenta con artículos de revisión, así como documentos originales. En este número encontrarán una revisión sobre el rambután y la posible extracción de elagitaninos a partir de este fruto; el efecto de la extracción por asistencia enzimática de aceite esencial y su efecto en cepas patógenas; el uso de sistemas fúngicos para llevar a cabo biotransformaciones sobre limoneno; una revisión sobre el efecto de la fermentación sobre las propiedades funcionales de masas libres de gluten y por último los resultados de bioprocesos aplicados a residuos agroindustriales para acumular taninos.

Dra. Mónica L. Chávez González

Editor

JBCT Journal of BioProcess and Chemical Technology

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2022 Volumen 14, No. 28
Revista Científica de la Universidad Autónoma de Coahuila

Generalities of Rambutan and Extraction of Ellagitannins

Generalidades del Rambután y Extracción de Elagitaninos

Cerda-Cejudo N.D.*, Estrada-Gil L.E., García-Méndez M.G., Flores-Gallegos A.C., Ascacio-Valdés J. A.

Departamento de Investigación en Alimentos, Facultad de Ciencias Químicas, Universidad Autonoma de Coahuila, Blvd. Venustiano Carranza y J. Cárdenas s/n, Col. Republica Oriente, 25280, Saltillo, México. Tel. +52(844)4161238

**Autor de correspondencia: nadia.cerda@uadec.edu.mx*

Recibido: 8 de abril de 2022
Aceptado: 15 de mayo de 2022

Resumen

Los polifenoles son metabolitos secundarios presentes en plantas y frutas, como el rambután, las fresas, o las uvas, los cuales pueden encontrarse en hojas, tallos, cortezas, flores o semillas. Los taninos hidrolizables, taninos condensados, flavonoides, fluorotaninos e hidroxiestilbenos se clasifican en polifenoles. Diversos métodos han sido implementados para obtener los polifenoles, como la extracción por ultrasonido, la extracción asistida por microondas, la extracción líquida presurizada o con la implementación de un bioproceso como lo es la extracción por fermentación en estado sólido. La importancia de obtener estas moléculas reside en las actividades biológicas que resultan ser benéficas para el ser humano, por ejemplo, la actividad antiviral, anti-inflamatoria, antitumoral, antioxidante y antimicrobiana. El presente artículo de revisión se enfoca en las generalidades del rambután como fuente de elagitaninos, principalmente, la geranina, corilagina y ácido elágico que el rambután puede proporcionar y en el método de extracción para adquirir éstos biocompuestos.

Palabras clave: corilagina, geranina, ácido elágico, elagitaninos, rambután.

Abstract

Polyphenols are secondary metabolites in plants and fruits as rambutan, strawberries, or grapes that can be found in leaves, bark, stems, flowers, or seeds. Hydrolysable tannins, condensed tannins, flavonoids, phlorotannins and hydroxystilbenes are classified in polyphenols. Several methods have been implemented to obtain these polyphenols, as ultrasound-assisted, microwave-assisted, pressurized liquid extraction or using a bioprocess as fermentation-assisted extraction. The importance to acquire these molecules lies in their biological activities that benefits the human health as antiviral, anti-inflammatory, antitumor, antioxidant and antimicrobial. In this review is focus on the generalities of rambutan fruit as source of ellagitannins, mainly, geraniin, corilagin and ellagic acid that rambutan fruit can provide and extraction method to obtain these biocompounds.

Keywords: corilagin, geraniin, ellagic acid, ellagitannins, rambutan.

INTRODUCTION

According to Beltrán-Ramirez et al. (2019) agro-industrial wastes are solid organic residues produced during fruit harvest and fruit preparation for its consumption. The non-edible parts of the fruits, such as peels and seeds, rich in bioactive compounds, have drawn the attention due to their potential to be applied to industrial applications (Beltrán-Ramirez et al., 2019; Kuan et al., 2008).

Agro-industrial wastes have long attracted attention of researchers globally, due to their ability to be implemented in products of interest, in a way to help the environment to be less polluted (Saval, 2012). According to Tang et al. (2015) agro-industrial waste bioconversion has increased due to the applications where they are being implemented, besides, industries must look up to the money invested and generated in terms of production and operation costs; reducing the water use in terms of ecology and allowing value-added products that improves the life costumers. It must be said that with the enhancement of the organoleptic properties of the products to

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avoid the environmental pollution, the agro-industrial wastes have come out as a transforming world of opportunities (Tang et al., 2015).

The problems that may arise are an environmental awareness for management, lack of technological capacity and economic resources, such as laws that promote the use of this type of waste and its final disposal (Tang et al., 2015). Subproducts are marketable, secondary, useful, and value-added products (Saval, 2012). Valdez-Vazquez et al. (2010), talked about the secondary residues that are obtained post-harvest, among which are sugarcane bagasse, corn, agave bagasse, coffee pulp and several types of fruits, such as grapes, pineapple, oranges and rambutan.

Rambután

Rambutan (*Nephelium lappaceum* L.) is a tropical fruit, from Asia, Southern America, Australia, and Africa; and the largest producer and exporters are Thailand, Malaysia, and Indonesia (Jahurul et al., 2020). According to reports by Maisanunt et al. (2016), mention that Thailand produces 318,000 tons between 2014 and 2015. This fruit is rich in minerals, sugar, proteins, and tannins. Its edible part is the pulp and consumed in its fresh form, in jams or juices. The increase in raw materials in industries means that there is an increase in agro-industrial waste (Akhtar et al., 2017).

Mahmood et al. (2018a) reported that the seed is a good source of vegetable fat due to the abundant fat content above 14-41% with oleic acid; the authors considered the possibilities of using seed fat in chocolate (30-wt % substitute) and personal care products are also on the focus. Authors also mentioned the nanostructured seed fat reported for encapsulation off soluble vitamins (like vitamin E) and additionally, most of the essential and non-essential amino acids that are concentrated as protein concentrate are contained in the seed. Rambutan seeds can be implemented in food industries for several applications due to their high content of nutritional compounds such as proteins, oil, and carbohydrates among others (Mahmood et al., 2018a).

Sérida et al. (2012) determined a proximal analysis of rambutan where author reported that the seed possesses 12.4% of protein, 2.26% ashes, 48% of carbohydrates, and 3.31% of moisture; and evaluated the fatty acids presents in the seedin which the results obtained were for arachidonic acid 36.36% and 40.45% of oleic acid.

Rambutan pulp is consumed fresh and is the only edible part of the fruit, where the major component is water (Den et al., 2021). Fila et al. (2013) determined the physicochemical analysis of rambutan pulp (bassed on (g/100g) in which the proximal composition of fresh rambutan was moisture (78.46 g), protein (0.66 g), crude fiber (0.38 g), aminoacids (19.66 g), carbohydrates (0.24 g), and ash (0.60 g). The fat content in the pulp is lower than in rambutan seed (Issara et al., 2014).

Rambutan peel is one of the main constituents of the fruit, which can reach up to 43 - 57% of total weight depending on the type of crop and its maturation. In a study reported by Mahmood et al. (2018b) authors describe the proximal composition of rambutan peel (bassed on (g/100g) in which the physicochemical analysis of rambutan peel were lipids (0.23), moisture (72.05), carbohydrates, ashes (1.2), (23.78), protein (2.04) and fiber (0.7).

Hernández et al. (2017) reported the mineral content of dry rambutan peel in mg/L with the following results: Fe (0.29), Mn (0.14), Cu (0.070), Zn (0.080), Na (0.04), Mg (0.15) and Ca (0.51). Also exhibits a chemical composition of fiber as hemicellulose with a reported content of 11.62 ± 2.31 (% w/w), cellulose with a value of 24.28 ± 2.30 (% w/w), and lignin with a value of 35.34 ± 2.05 (% w/w) (Oliveira et al., 2016).

Okonogi et al. (2007) reported a high content of phenolic compounds presents in rambutan peel; additionally, Palanisamy et al., (2008) reported in their research that it has a high antioxidant activity, besides, that it can be implemented in food products, pharmaceuticals or cosmetics due to its nontoxic capacity for normal cells and antioxidant activity content.

Compared to rambutan pulp or seed extracts, the peel is an interesting source of bioactive compounds. The supply of antioxidants compounds is greater which contains extent antioxidants compounds, which are primarily polyphenolic, being the hydrolyzed compounds that are in greater quantity. The major compounds present in rambutan peel are the ellagitannins corilagin, ellagic acid and geraniin (Hernández-Hernández et al., 2019).

This is not a recognized fruit, but several countries, such as southern Mexico, demonstrate favorable conditions for its cultivation (Solís-Fuentes et al., 2010). One factor that must be known is the polyphenol content in rambutan peel, in its cultivation and fruit stage development (Skerget et al., 2005). Nonetheless, some factors, such as extraction temperature, extraction technique, type of solvent, particle, size, temperature, pH, and solvent-to-solid ratio, contribute to the efficacy of polyphenol extraction (Kronholm et al., 2007).

The interest in taking advantage of agro-industrial waste, such as rambutan peel, has increased due to the presence of polyphenols and their importance derived from the attributed biological activities. Thitilertdercha et al. (2010) reported that rambutan peel extracts have a high antioxidant activity due to the presence of phenolic acid compounds and ellagitannins.

A study evaluated the antioxidant activity of an ethanolic extract of rambutan peels compared to grape extract and vitamin C. The results demonstrated that the extract of leaves and rind of rambutan fruit displayed the highest capacity to inhibit the radical HHDP with the ethanolic rind extracts, having the highest 1/IC50 value compared to a grape extract and vitamin C (Palanisamy et al., 2008).

The antimicrobial activity has been reported for extracts of rambutan peel. A study reported by Sektar et al. (2014) showed that bacterias Gram (+) had been sensitive to the actions of the methanolic compounds of rambutan peel. Thitilertdecha et al. (2008) reported the inhibitory effect of the growth of microorganisms (pathogenic bacteria like Staphylococcus aureus, *Staphylococcus epidermidis* and *Enterococcus faecalis*) was observed using extracts of rambutan (Thitilertdecha et al., 2008).

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Khaizil et al. (2013) reported the antiproliferative activity of the extracts of rambutan peel against MDA-MB-231 and MG-63 cell lines for breast cancer with IC50 values of 5.42 ±1.67 µ/mL, MG63 osteosarcoma cancer cell lines and HeLa cell lines for cervical cancer. Morshed et al. (2014) reported a study in which was demonstrated the anti-inflammatory (58.86%) and analgesic activity (51.27%) of methanol extract from rambutan seeds has.

Geraniin

Geraniin is part of the ellagitannins family, a subgroup of the tannins that show many health benefits with their consumption, geraniin was first discovered 40 years ago, and it is a secondary metabolite found in plants, and it is considered a hydrolyzable tannin. One of the earliest reports of tannins usage in history is in folk´s popular medicine as previous civilizations saw the potential of plants in the treatment of pain, bruises, gastrointestinal disorders, peptic ulcers, and alopecia in many countries like Japan, Mexico, and India, the plants used by these civilizations have one thing in common. It is the use of geraniin-containing herbs such as Geranium thunbergii and Geranium bellum, which are all well known for their high content of geraniin (Cheng et al., 2017a; Velázquez-González et al., 2014).

Ishimoto et al. (2012) reported that geraniin is very well known thanks to its excellent antioxidant properties like those of ascorbic acid (vitamin C). It demonstrated several biological activities of great interest, such as antihyperglycemic (Ling et al., 2012), apoptotic (Lee et al., 2008), antiviral (Yang et al., 2012), antidiabetic (Muhtadi et al., 2015) and liver-protective (Londhe et al., 2012). Properties that make a fascinating compound with many uses for the industry, some examples are food production, pharmaceutical, and the cosmetic industry.The use of the *A. donax* L. as fuel produces some problems related to the chemical composition, mainly the ashes quantity, that reduces the thermal conversion efficiency instead of the high yields; also, there is produced some harmful compounds as NOx, HCl, SO_2, CO, and fine dust (Payá et al., 2018; Corno, Pilu and Adani, 2014).

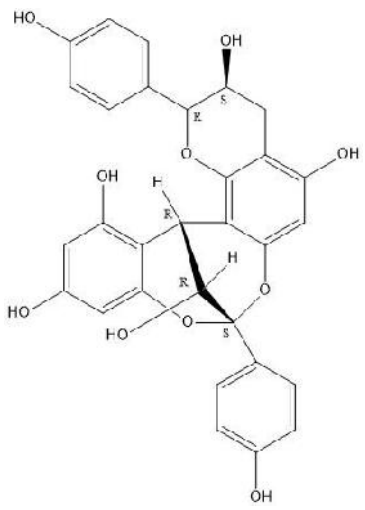

Figure 1. Chemical structure of geraniin.

Biological activities

Antioxidant activity

Geraniin's crude extract has been reported with high antioxidant activity, which can be comparable with that of vitamin C, for example, the crude extract of *N. lappaceum* (rambutan) peels have a powerful 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (Lin et al., 2008). In a study by Estrada-Gil et al. (2022) extract from *N. lappaceum* peel, antioxidant activities were evaluated and measured in IC50 values obtaining 0.098 ± 0.001, 0.335 ± 0.005, 0.034 ± 0.003 IC50 mg/mL for DPPH, ABTS and Lipid oxidation inhibition (LOI) respectively, compared to IC50 values obtained by Trolox of 0.207 ± 0.001, 0.512 ± 0.000, 0.026 ± 0.002 IC50 mg/mL for DPPH, ABTS and LOI. Based on the results rambutan peel possesses a great antioxidant activity which is greater than that of the reference antioxidant in 2 out of 3 assays.

Methanolic extracts from *N. lappaceum* peels tend to exhibit a tremendous antioxidant capacity as geraniin is the principal constituent found in the peel of the fruit. Ethanolic extract of this fruit contained 20% more geraniin, also with high antioxidant capacities and low pro-oxidant activity (Thitilertdecha et al., 2010), geraniin exhibited an IC50 value of Geraniin 0.38 ± 0.01 µM compared to that of the reference antioxidant 186 ± 3.00 Mol BHT/mol phenolics in LOI assay and for DPPH 0.79 ± 0.05 µM compared to that of the reference antioxidant of 87.1 ± 5.76 Mol BHT/mol phenolics. Furthermore, it has been reported that it has an excellent scavenging activity for Galvinoxyl and ABTS free radicals and lower pro-oxidant capacity compared to vitamin C and Phyllantus Emblica (Palanisamy et al., 2011).

Using a FRAP (ferric reducing/antioxidant power) assay has given light to the knowledge that geraniin is five to six times a more potent antioxidant than L-ascorbic acid and Trolox. Such properties are due to its free radical scavenging ability to many

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damaging radicals such as DPPH, superoxide radicals, hydroxyl radicals, and nitric oxide radicals. Geraniin is comparable to epigallocatechin gallate, a polyphenol with potent antioxidant activity that can be found in a wide variety of green teas (Cheng et al., 2017).

Geraniin can also inhibit xanthine oxidase, which is a reactive oxygen species (ROS) generating enzyme aside from the free radical scavenging activity. Geraniin also possesses an inhibitory effect on lipid peroxidation, which is caused by oxidative deterioration provoked by ROS. Also, explaining the low levels of malondialdehyde, which are the end products of certain lipid peroxidation, these effects shown are in animal models (Cheng et al., 2017).

In an in vitro study on human foreskin fibroblast Hs68 cells, rat pheochromocytoma PC12 cells and human neuroblastoma SH-SY5Y cells, was discovered the cytoprotective effect against apoptosis that geraniin obtained from *N. Lappaceum* has. The exposure to free radicals induced by peroxynitrite generator 3-morpholinosydnonimine (SIN-1) and peroxyl radical generator 2,2´-azobis dihydrochloride, attributed to geraniin´s capacity to scavenge peroxynitrite and peroxyl radicals (Ling et al., 2012).

Antimicrobial activity

Geraniin extract bioactivities show the potential to be an excellent antimicrobial, due to in vitro tests where they show positive effects against common human pathogenic bacteria such as *Staphylococcus aureus* and *Vibrio cholerae* among other bacteria (Thitilertdecha et al., 2008). The values were measured in minimum inhibition concentration (MIC), and these were the results: The best results for inhibition on *Vibrio cholerae* were obtained by 15.6 mg/ml, for *Pseudomonas aeruginosa* the best results were obtained with 62.5 mg/mL, for *Enterococcus faecalis* the best results were granted by 15.6 mg/mL, for *Staphylococcus aureus* 31.2 mg/mL and *Staphylococcus epidermidis* with the best results by 2.0 mg/mL these were all obtained by methanolic extract.

Phyllanthus euphorbiaceae is a plant with around 750 species, and it is very well distributed among several tropical and sub-tropical countries making it ideal for the study against dengue. *Phyllanthus* is already used by conventional folk medicine due to its high geraniin content that has many biological activities previously mentioned. The species used to elaborate a cocktail to evaluate the antiviral potential were *P. uirinaria*, *P. niruri*, *P. amarus*, and *P. watsonii* obtaining maximum results of 83-95% virus inhibition against dengue virus type 2 (DENV2) (Lee et al., 2013).

A *Phyllanthus cocktail* from the various species was used in the in vitro trials and ran into an HPLC analysis were the first compounds found were corilagin, geraniin, trigalloylglucopyronside among others being geraniin the majoritarian compound in the composition. The trials were run on Vero cells with both aqueous and methanolic extracts of the *Phyllanthus cocktail* (Lee et al., 2013). Phyllanthus cocktail does have antiviral activities against DENV2 supported by differential regulation of various host and viral proteins. It is mostly the first inhibitor as its most activity was found before infection or during the infection. The plant extract targets 13 proteins in charge of different functions which were the cell-virus attachment, viral entry, viral polyprotein production, viral RNA replication as well as viral assembly and maturation (Lee et al., 2013).

Regarding antibacterial activities, it has been found that several hydrolyzable tannins have antibacterial activities against Helicobacter pylori causal to major chronic gastritis and, in some cases, peptic ulcers in the human body. In an experiment run by Funatogawa et al. (2004) polyphenols were isolated from their natural sources, selecting 36 compounds among them geraniin.

Antibacterial activity screening was carried out with 4 H. pylori strains. The hydrolyzable tannins used in the study all showed antibacterial activity against *H. pylori* without affecting intestinal bacterial flora. *H. pylori* membrane contains three kinds of cholesterol glucosides; cholesteryl-α-D-glucopyranoside (CGL), cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside (CAG), and cholesteryl-6-O-phosphatidyl-α-D- glucopyranoside (CPG), these membrane features not very common in bacteria may be related to the sensitivity to hydrolyzable tannins allowing them to disrupt the liposomal membranes releasing the liposomal content in a dose-dependent manner (Funatogawa et al., 2004).

Anticancer activity

Polyphenols have been widely studied for their anticancer and antiproliferative properties mostly in the prevention and the treatment of said disease; geraniin has been used in a variety of cancer cell lines in rat and human origins to stop the proliferation of the tumor cells.

A study carried out by Ko (2015), shows that geraniin inhibits epithelial-mesenchymal transition (EMT), which is an essential cellular process during which polarized epithelial cells become motile mesenchymal-appeared cells, that induce metastasis of cancer. This process is inhibited by inducing E- to N-Cadherin switching and vimentin expression in TGF-β1-activated A549 lung cancer cells. Additionally, it was demonstrated that geraniin reduced the TGF- β1 induced increase in migration, invasion, and anoikis resistance and inhibited the activation of Smad2 in A549 lung cancer cells.

Corilagin

Corilagin is an antioxidant compound of the polyphenol family, specifically of the gallotannin subgroup, many plants, and fruits such as *Phyllanthus ninuri* and *Nephelium lappaceum* contain this biological compound. It was first identified by Schmidt & Lademann in 1951 from the fruit of a plant called divi-divi, which grows in the shores of the beaches, hot and semi-arid zones of the world such as Mexico, Central, and South America.

ISSN: 2683-3271

Corilagin is well known for having many biological activities such as anti-tumoral, antioxidant, hepatoprotective, and anti-inflammatory properties, which make this a fascinating compound we can find in vegetable sources, in recent years the research of this compound has focused in the hepatoprotective, anti-inflammatory and anti-tumoral. (Li et al., 2018).

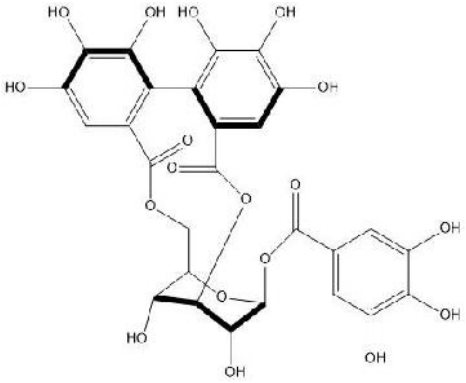

Figure 2. Chemical structure of corilagin.

Biological activities

Hepatoprotective

A study carried out in Japan by Kinoshita et al. (2007) was made to corroborate the hepatoprotective properties of Japanese medicinal herbs, specifically the leaves extract of a plant called *T. catappa* used in Japanese traditional medicine accurately in the region of Okinawa. The study evaluated the hepatoprotective properties using lipopolysaccharide (LPS) and D-galactosamine (GalN) induced liver injury in rats. Two extracts were made to compare the polyphenolic content, 50% ethanol and hot water, both identified corilagin and chebulagic acid as their majoritarian compounds, both compounds showed promise of having powerful scavenging action of various radicals such as O2- peroxyl radical or ROS from activated leukocytes and inhibiting lipid peroxidation.

To measure liver parameters aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured for serum activities. Glutathione S-transferase (GST) was used to determine activities in serum and liver cytosol. Lipid peroxidase in liver was evaluated measuring thiobarbituric acid reactive substances (TBARS) (Gyamfi & Aniya, 2002).

It was demonstrated that the extract of *T. catappa* and corilagin protect against GalN/LPS-induced liver toxicity evidenced by the reversed serum AST, ALT, and GST activities and by a decrease in lipid peroxide/nitric oxide levels and by preventing apoptosis. Corilagin prevents GalN/LPS-induced injury through the suppression of ROS generation and inhibition of apoptosis (Kinoshita et al., 2007).

Anticancer activity

The antiproliferative, anti-tumoral and anti-inflammatory effects of corilagin makes this compound a target for researchers that work in cancer cell lines to better comprehend the effects of this bioactive compound to be used in the possible treatment and prevention of cancer (Baliga & Dsouza, 2011; Li et al., 2018).

The anticancer and antitumoral activities of corilagin were investigated in a study by Attar et al. (2017) where all its efforts were aimed to demonstrate the apoptotic and genomic effects in ovarian cancer treatment. SKOV3 cells line were used in this study and to prove the effects a WST1 cell proliferation test, mitochondrial membrane potential JC1 assays and caspase 3 were realized in a time and dose dependent manner. Corilagin treatment in SKOV3 induced inhibition of cell proliferation and showed correlation with increased apoptotic cancer cells. Besides, corilagin stimulates the phosphatidylinositol signaling system and the MAPK to show its antitumoral effects on downstream pathways (Attar et al., 2017).

Ellagic acid

Landete (2011) reported that ellagic acid is a dilactone and a very potent bioactive compound that can be also found in pomegranate, walnuts, rambutan, berries and grapes; widely use in food industries as jams or beverages. In cosmetics area, Shimogaki et al. (2000) reported that this biocompounds, by chelating copper, could inhibit tyrosinase being used as an ingredient in skin whitening. Buenrostro-Figueroa et al. (2014) reported the biological activities that ellagic acid provides to human health such as antiviral, antiproliferative, and antitumoral.

In the past years, most of ellagic acid was obtained by acid hydrolysis and conventional methods with solvent extraction which turn out to be polluting to the environment. The use of a bioprocess like fermentation-assisted extraction have come out to obtain this biocompounds in a harmless recovery for the environment (Sepúlveda et al., 2018).

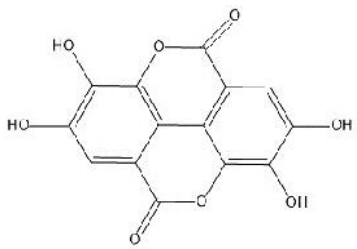

Figure 3. Chemical structure of ellagic acid.

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Biological activities

Anticancer activity

Ellagic acid is a bio compound reported as anti-proliferative agent in many cancers, especially breast cancer, in which it has a therapeutic and chemo-preventive role against cancers; besides, it´s anti-metastatic effects, antiproliferative and antiangiogenic are regulated by inhibiting the cell cycle inducing apoptosis (Ceci et al., 2018).

Kaur et al. (2021) reported a study on ellagic acid encapsulated Chitosan nanoparticles exhibiting a synergistic higher anti-breast tumor efficacy compared to the efficacy of ellagic acid alone in which the natural biomacromolecule chitosan was an excellent choice for formulating surfactant coated nanoparticles using an ionotropic gelation method with chitosan. The synergism among chitosan and ellagic acid combination showed a promising result to manage the cancer, more specifically the breast cancer.

Antioxidant activity

Polyphenols are in a several range of plants as second metabolites and they have attracted scientists for their strong antioxidant activity (Liu, 2013). Sun et al. (2017) compared the in vitro antioxidant activities of ellagic acid, punicalin and punicalagin from pomegranate by testing their free radicals scavenging capacities and their in vivo anti-oxidative stress effects against oxidative injury. The results showed that the three bio-compound exhibited strong in vitro antioxidant activities being the EA the best against oxidative injury in vivo.

Extraction of ellagitannins

The ellagitannins have been extracted mainly by conventional methods and recently the use of new technologies has increased. Organic solvents, such as methanol, water, acetone, ethanol, and ethyl acetate are use in conventional methods (Domínguez-Rodríguez et al., 2017). Ellagitannins can be obtained by a methanolic or ethanolic respectively (Hernández et al., 2017; Thitilerdecha et al., 2010).

A sample pretreatment before the extraction may be needed to guarantee the extraction efficiency of ellagitannins on the extraction process, in which, the pretreatment depends to the complexity of the sample matrix and to the moisture content. These factors may decrease the extraction of the analyte from inside the cell, if the sample gets dried (Plaza et al., 2013). The stability of the sample could decrease due to the water content because of enzymatic reactions that can happen due to the presence of water (Zhang et al., 2017). Some of the latest technologies are pressurized liquid extraction; ultrasound-assisted extraction, microwave-assisted extraction, and supercritical fluids extraction for mention some (Domínguez-Rodríguez et al., 2017). The use of new technologies is looking to reduce the use of contaminant solvents and getting better results in the extractions of these components.

Through the years, emergent technologies have come out as an alternative to standard extractions, with more significant advantages, because they are automated, with higher reproducibility and selectivity and they are faster than conventional methods. Moreover, the solvents used in these technologies are more sustainable and less polluting than the solutions used in conventional extraction (Hernández-Hernández et al., 2019; Ordoñez-Torres et al., 2021).

These techniques include ultrasound-assisted extraction, simultaneous distillation dual, pressurized liquid extraction, microwave-assisted extraction, and supercritical fluid extraction (Dominguez-Rodriguez et. al., 2017). However, one of the disadvantages of these techniques is the complexity and difficulty to be implemented in developing countries, as well as the use of high temperatures, which can damage the compounds. Currently, the use of other sustainable techniques for tannin extraction has been requested, so the bioprocess being one of them. Among the bioprocess used for these purposes, solid-state fermentation stands out (Torres-León et al., 2019).

Krishna (2005) describes two types of solid-state fermentations, in the first one, the microorganism grows in the natural material (agroindustrial waste like peel or seeds), and in the second, the substrate where the microorganism grows is inert, but this is impregnated with liquid culture medium to improve the growth.

Torres-Leon et al. (2019) reported that since it requires less capital, small equipment, and lower operating cost, developing this biotechnological process seems accessible to implement, additionally, has environmental advantages, since it allows the use of agro-industrial waste as a substrate. The disadvantages that this bioprocess has, is the microorganism that is going to be used, because it must grow at low water content (like filamentous fungus) since solid-state fermentation use low levels of water. Some other factors are the reactors used (like Petri dish in the laboratory) and to scale to be implemented in an industrial range and the time used in developing this bioprocess for obtaining the compound in interest.

CONCLUSIONS

It is necessary to evaluate new extraction ways to obtain these phenolic compounds and used them to provide the development of new scientific knowledge using the natural resources of our country. To obtain unique and different results, its necessary to find new ways to obtain ellagitannins like ellagic acid, geraniin or corilagin for a bigger picture in the studies that in the future could be compared, confirmed, and get to get a discussion with the current information.

The potential of ellagitannins and polyphenols, in general is such that more studies are needed to be able to identify how many bioactivities they can provide us, to enhance human health, like anti-inflammatory, anticancer, hepatoprotective, antioxidant, analgesic and many more bioactivities.

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This is just the tip of the iceberg as these compounds extracted by a biotechnological process, like SSF, can be used in many other applications. Some of these applications are the integration of the compound on a food matrix, the creation of products for use in the makeup and cosmetic industry and even to create nutraceuticals as there have been studies for the encapsulation of said compounds to maintain them for later use.

Further research on ellagitannins should come in handy to determine even more uses in the industry and to be able to take advantage of all the bioactivities contained within.

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JBCT Journal of BioProcess and Chemical Technology

ISSN: 2683-3271

2022 Volumen 14, No. 28
Revista Científica de la Universidad Autónoma de Coahuila

Gluten-Free Sourdoughs: Effect of Fermentation on their Nutritional and Physicochemical Properties, a Review

Masas Libres de Gluten: Efecto de la Fermentación en sus Propiedades Nutricionales y Fisicoquímicas, una Reseña

García-Flores L.A.[1], Torres-Maravilla E.[2*], Flores Gallegos A.C. [1*], Rodríguez-Herrera R.[1], Aguilar C.N.[1] Chávez-González M.L.[1]

[1]*Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Blvd. Venustiano Carranza S/N, Colonia República, 25280, Saltillo, Coahuila, México.*

[2]*MICALIS Institut, INRAE, Univ Paris-Saclay, Equipe Interactions des Micro-organismes Commensaux et Probiotiques avec l'Hôte (ProbiHôte), Jouy-en-Josas, France*

**Corresponding author: edgar.torres-maravilla@inrae.fr; carolinaflores@uadec.edu.mx*

Recibido: 29 de Abril de 2022
Aceptado: 30 de Mayo de 2022

Resumen

Los pseudocereales son granos pertenecientes a la familia Amaranthaceae; entre ellos se encuentran la quinua, el amaranto y el alforfón. Estos son buenas fuentes de macro y micronutrientes, con contenidos proteicos comparables a los de los cereales convencionales y excelentes propiedades nutricionales relacionadas con la proteína de alta calidad; también destacan como alimentos sin gluten.

Por otro lado, estos alimentos suelen contener cantidades mínimas de sustancias llamadas antinutrientes, como fenoles, fitatos, taninos, entre otros, que pueden ser responsables de la mala absorción de minerales y proteínas que poseen. La fermentación es uno de los procesos biotecnológicos antiguo, en el que tanto en alimentos como el pan se producen con la ayuda de levaduras y bacterias del ácido láctico. Este proceso transforma e influye en la biodisponibilidad de compuestos y nutrientes presentes en la masa, así como también en sus características organolépticas. Por lo tanto, la presente revisión explora una descripción de los pseudocereales, a su vez su contribución nutricional y algunos anti-nutrientes que suelen estar presentes, así de cómo es el efecto de la fermentación en masas sin gluten.

Palabras clave: Antinutrientes, fermentación, libre de gluten, pseudocereales, masas madre

Abstract

Pseudocereals are grains belonging to the family Amaranthaceae; among them are quinoa, amaranth and buckwheat. These are good sources of macro and micronutrients, with protein contents comparable to those of conventional cereals and excellent nutritional properties related to high quality protein; they also stand out as gluten-free foods.

On the other hand, these foods usually contain minimal amounts of substances called antinutrients, such as phenols, phytates, tannins, among others, which may be responsible for the poor absorption of minerals and proteins they possess.

Fermentation is one of the ancient biotechnological processes, in which both food and bread are produced with the help of yeasts and lactic acid bacteria. This process transforms and influences the bioavailability of compounds and nutrients present in the mass, as well as its organoleptic characteristics. Therefore, the present review explores a description of pseudocereals, in turn their nutritional contribution and some antinutrients that are usually present, as well as how is the effect of fermentation in gluten-free masses.

Keywords: Antinutrients, fermentation, gluten-free, pseudocereals, sourdoughs

JBCT Journal of BioProcess and Chemical Technology
ISSN: 2683-3271
2022 Volumen 14, No. 28
Revista Científica de la Universidad Autónoma de Coahuila

INTRODUCTION

Grain cereals are known to provide a source of dietary energy, which is generally considered important as an example: rice, wheat and corn are foods that provide about half of the energy source we require in our body (Sandez et al., 2020). However, because many cereals are known to contain gluten, the consumption of pseudocereals, which are those that come from the seeds of the flowers of some plants, is proposed. They differ from cereals in that they are the fruit of herb spikes. The name pseudocereals is because, although they belong to plants of different families, their appearance, culinary use as flour or grain, and great nutritional properties are similar to those of cereals. There are several types, the best known of which are amaranth, quinoa and buckwheat which are rich in bioactive and nutritional components (Fig. 1).

Pseudocereals do not contain gluten, this is one of the characteristics that differ from other foods. Therefore, in addition to these having many health benefits, integrating them into our diets could be a valuable contribution to improving the quality of existing gluten-free foods. On the other hand, it has also been found that certain in vivo studies showed that pseudocereals have hypoglycemic effects, so they have been suggested as an alternative to the usual ingredients in the production of gluten-free products. For such reasons, the use of pseudocereals has increased not only specifically in people with celiac disease or allergy to cereals, but also widely used to be part of healthy diets (Sandez et al., 2020).

Fermentation produced by lactic acid bacteria (LAB) or yeasts is one of the oldest and at the same time very economical biotechnological processes, in which both beer and bread make use of this practice. This process can be used in the processing of cereals and pseudocereals, where spontaneous fermentation simply activates the natural microbes in the milled grains, helping to produce and preserve food. In addition, sourdough fermentation can influence nutritional quality by decreasing or increasing compound levels and improving nutrient bioavailability. Pseudocereal sourdoughs, in addition to their nutritional value, would have a characteristic flavor, which would facilitate, also in combination with other flours, the production of new foods.

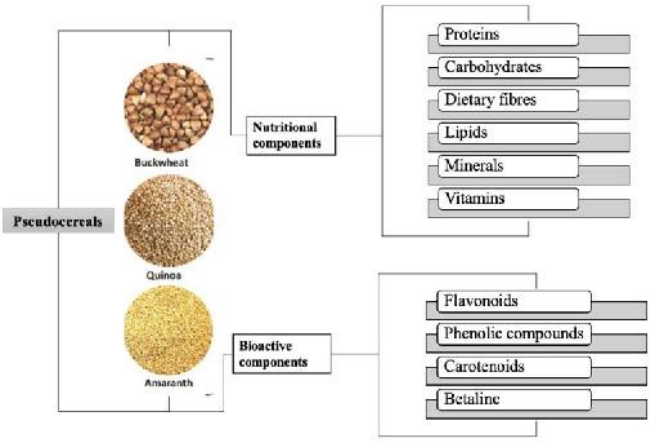

Figure 1. Nutritional and bioactive components of pseudocereals.

GLUTEN IN FOODS

Gluten is a protein present in cereals such as wheat, barley, oats, and rye, among others, which are commonly used to make flour. This protein is responsible for giving the characteristic elasticity to the flour and, because of fermentation, increasing the volume of the dough. However, gluten is not essential in the human diet. It can be easily substituted by some animal or vegetable proteins when a gluten-free diet is required (de la Calle et al., 2020). Moreover, nowadays, some foods, namely pseudocereals, have been studied to have the appeal of being naturally gluten-free (Ruiz et al., 2016).

One of the problems with the gluten content in a food is that many people today cannot digest it properly due to a disease called celiac disease (FACE, 2018). There are many foods that contain gluten naturally, such as bread, pasta, pastry, in addition to being added to other products such as sauces or even certain medicines, so it can be said that it is present in most products of the food chain. However, gluten is added to have good organoleptic characteristics, to satisfy the consumer, due to its moldable and elastic characteristics.

GLUTEN FREE FOODS, PSEUDOCEREALS

There are foods that have been found to be completely gluten-free, and in addition to this, they have a high content of nutrients that can bring benefits to our body in many ways. Each one has different characteristics; however, they are foods that help open horizons to the food industry to develop more nutritious foods and can satisfy the consumer.

Pseudocereals can be a great substitute for conventional flours because, in addition to being gluten-free, they provide a high nutraceutical content in our body. These are grains rich in

ISSN: 2683-3271

nutrients, belonging to the Amaranthaceae family; among them are quinoa (*Chenopodium quinoa*), amaranth (*Amaranthus caudatus*) and buckwheat (*Fagopyrum esculentum*).

Pseudocereals are composed of three important parts: 1) the pericarp, which consists of cellulose, hemicellulose, in addition to proteins, minerals and lignin; 2) the embryo, which contains lipids, proteins and minerals; and 3) the endosperm, containing starch and proteins. The three pseudocereals are good sources of macro- and micronutrients, are rich in proteins comparable to those of conventional cereals and possesses excellent nutritional properties related to high protein quality (Castro-Alba et al., 2019). These gluten-free pseudocereals, have attracted increased attention as foods worldwide, as they have high nutritional values (Sterr et al., 2009).

Quinoa (*Chenopodium quinoa* Willd)

Quinoa cultivation dates from about 5000 to 7000 years ago and was a source of food for the Tiahuanacota and Inca cultures (Angulo et al., 2019), and was considered a sacred food by many cultures (Delatorre et al., 2013). Quinoa was stigmatized as a "wild food" by the Spanish during the colonial period, who ended the cultivation and banned it according to their religious beliefs, so other crops were imposed (Nowak et al., 2016). Quinoa is a herb native to South America (Sezgin and Sanlier, 2019) and is a species of Chenopodiaceae, Chenopodiaceae. Chenopodiaceae is the main genus of Chenopodiaceae and is widely distributed worldwide with about 250 species (FAO, 1985).

Quinoa was selected by the Food and Agriculture Organization of the United Nations (2014) as one of the important crops for food security in the 21st century due to its ability to adapt to different agro-ecological levels, drought stress and withstand temperatures from -4°C to 38°C. Is a water-efficient plant, tolerant and resistant to lack of soil moisture and that allows an acceptable production with 100 to 200 mm of rain, considered perfect by the Food and Agriculture Organization of the United Nations (FAO, 1985; Jacobsen, 2003; Elsohaimy et al., 2015). Quinoa is nothing more than a seed, but with certain unique characteristics, since it can be consumed as a cereal, which is why it is also called a pseudocereal. Compared to other traditional cereals, quinoa has a high amount where it provides 16 g of protein per 100 g and offers about 6 g of fat in the same amount of food. The carbohydrates it contains represent 69% of its weight and provide 374 calories per 100 g. It is therefore an optimal source of energy that is released slowly because it is accompanied by a good dose of fiber (6 g out of 100). Likewise, quinoa is rich in essential minerals, such as iron (a serving of 60 g provides 46% of the daily requirement), but also others such as magnesium, phosphorus, manganese, zinc, copper and potassium. It also provides vitamin B2 (13%) and B3 (9%). In addition to this, it does not contain gluten, therefore, it is an easy-to-digest food. Quinoa is mainly used for breakfast, similar to corn and potatoes. Currently, this crop is still marginalized by more popular and cheaper foods such as rice and wheat (Repo-Carrasco et al., 2003; Nowak et al., 2016).

Chia (*Salvia hispanica L.*)

Chia is an herb of the Lamiaceae family native to southern Mexico and northern Guatemala (Ixtaina et al., 2008). Chia belongs to the genus Salvia, which consists of about 900 species, and takes its name from the Latin word "salvere", in reference to the healing properties of these plants (De Falco et al., 2017). The proximate composition of chia is 91- 96% dry matter, 20-22% protein, 30-35% fat, 25-41% carbohydrates, 18-30% crude fibre (mainly non-digestible cellulose, pentosans and lignin) and 4-6% ash (Angulo et al., 2019; Zettel and Hitzmann, 2018). Chia seed has the highest proportion of α-linolenic acid (60%) compared to other natural sources (Ayerza, 1995), as well as higher levels of protein compared to traditional cereals such as wheat (*Triticum aestivum L.*) (McKeon et al., 2016).

Chia is widely used in different foods such as snacks, cereal bars, breakfast cereals, cookies, fruit juices, cakes, and yogurt (López et al., 2019). The incorporation of chia in baked products not only improves the nutritional value but also acts as a hydrocolloid substitute for egg, fat, or gluten. Chia could represent an alternative source of isoflavones when incorporated into food, due to its high antioxidant capacity (Zettel and Hitzmann, 2018).

Amaranth

The word Amaranthus comes from the Greek "Anthos" (flower) whose meaning is eternal or soothing. Amaranth belongs to the Amaranthaceae family and is widely used in both vegetables and leaves throughout the world. It has long been cultivated in Central and South America for more than 5000 to 7000 years. The genus Amaranthus consists of more than 600 species, where the most important and used are *A. caudatus*, *A. cruentus* and *A. hypochondriacus* (Anubhuti, 2017, Ruiz et al., 2016).

The plant can reach approximately 2 to 2.5 meters in height. The leaves are elliptic or oval, with an entire edge and can measure 6.5 to 15 cm (Orosco, 2013). On the other hand, the seeds are small and lenticular in shape; each seed measures approximately 1 to 1.5 mm in diameter and 1000 seeds weigh approximately 0.6 to 1.2 g (Fig. 2). It is characterized by inflorescences of very showy colors such as yellow, orange, pink to purple; they can be axillary or terminal, which can vary from fully erect to decumbent (Orosco, 2013) and is composed of four parts: 1) seed coat, 2) endosperm, 3) embryo and 4) perisperm (Teutonico and Knorr, 1985).

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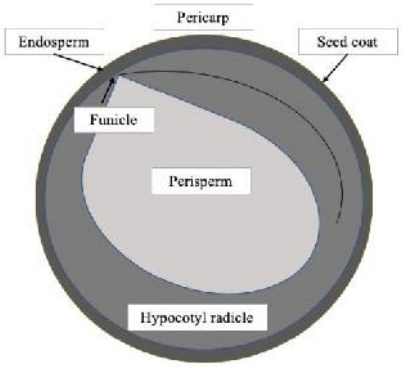

Figure 2. Parts of the amaranth seed.

Amaranth has been produced since ancient times, since the plant was used by ancient cultures such as the Aztecs in Mexico to celebrate the gods of the earth, fire, and rain, and in Peru by the Incas for the preparation of drinks associated with fertility. However, when the Spanish arrived in America, they decided to exterminate pseudocereals because of their religious complications (Rollán et al., 2019). In 2013, amaranth showed a great recurrence in many parts of the world such as South and Central America, Africa, India, China, and the United States (Aguilar et al., 2013). In recent years, China established itself as the main producer and with significant production to countries such as Russia, India, and Mexico. In China, approximately 2200-5500 kg ha^{-1} of grains and 90000-180000 kg ha^{-1} of silage are produced, mainly used for animal feed and in Latin American countries such as Peru, approximately 6000 kg ha^{-1} for human consumption (Corke et al., 2016).

In Mexico, 11 species of amaranth are cultivated, of which the species *Amaranthus hypochondriacus*, and *Amaranthus cruentus* are native to the national territory (Martínez, 2016). According to archaeological data, amaranth in Mexico was cultivated since 4000 years before Christ, as findings were found that *A. cruentus* in Tehuacán, Puebla and *A. hypochondriacus* was cultivated 500 years after Christ (Jacobsen, 2003). It later boomed during the time of the Aztecs and Mayans, who used it in beverages, medicinal treatments and mixed with corn to make "tortillas" (Orona et al., 2018). Currently, Mexico is one of the main amaranth producer countries, obtaining 6,804 tons in 2018. The state of Puebla stands out with 3,511 tons, making it the first place in production, followed by Mexico (1,298 tons), Tlaxcala (1,281 tons), Morelos (411 tons), Mexico City (182 tons), Oaxaca (108 tons) and finally Hidalgo (13 tons) (SIAP, 2018). The amaranth obtained is intended for human consumption, commonly to produce "superfoods" such as granola, cookies, and yogurts (Meyerding et al., 2018), but it is also applied in breads, pastas, and beverages. Traditional foods in Mexico formulated with amaranth are "Alegria" sweets, and it is found mixed in beverages such as "atole" (Corke et al., 2016). Consumption of this grain is estimated at 4.5 g per capita (SIAP, 2018).

Amaranth is considered low in nutrients, it is resistant or tolerant to extreme conditions, as well as to changes in temperature and radiation due to its high capacity for osmotic adjustment (Tamayo, 2017). Amaranth, considered a pseudocereal, has intensive growth and very accelerated and effective photosynthesis regardless of the quality of the soil where it is grown and can survive in areas with extreme climatic conditions "from dry to humid environments, from high regions to sea level and develops in soils of all types of qualities" (Martínez, 2016). Amaranth can be stored for a longer period than corn without altering its quality. For its consumption, a transformation process is required that consists of subjecting the amaranth grain to temperatures of up to 120ºC to inflate it. Once inflated, it is cooled and sieved to separate the grain that cannot be inflated. The already popped grain is the one that is sold as a cereal or used as an input for other food products (Ayala et al., 2016)

Amaranth is characterized by having a high agro-food potential since its seed, plant and leaves have nutritional values that surpass some commonly used cereals, so it is widely recommended for human and animal consumption (Martínez, 2016). Amaranth can be used to treat various degenerative diseases, thanks to the fact that it has both healing and protective properties attributed to its high antioxidant potential. In addition, another benefit of amaranth is that the actions of the peptides contained is that it helps lower blood pressure, so they are considered antihypertensive peptides, while another group of amaranth peptides has shown to have the ability to inhibit the enzyme dipeptidyl peptidase IV (DPPIV); this enzyme is considered the target to inhibit the disease of diabetes. Likewise, there is preliminary research in clinical studies, that amaranth could control obesity, since it is able to reduce hormones that are related to this disease (Barba, 2020). On the other hand, the protein contained in amaranth does not contain gluten, an important point for the consumption of this food in the diet of celiac (Anubhuti, 2017).

Its high content of flavonoids and dietary fiber has a covalent interaction, increasing its anti-inflammatory effects and its positive impact on the intestinal microbiota. In addition, pseudocereals such as amaranth are characterized by being rich in many health-promoting phytochemicals, such as polyphenols, dietary fiber that exhibit antioxidant and free radical scavenging activity (Rollán et al., 2019) (Fig. 3).

ISSN: 2683-3271

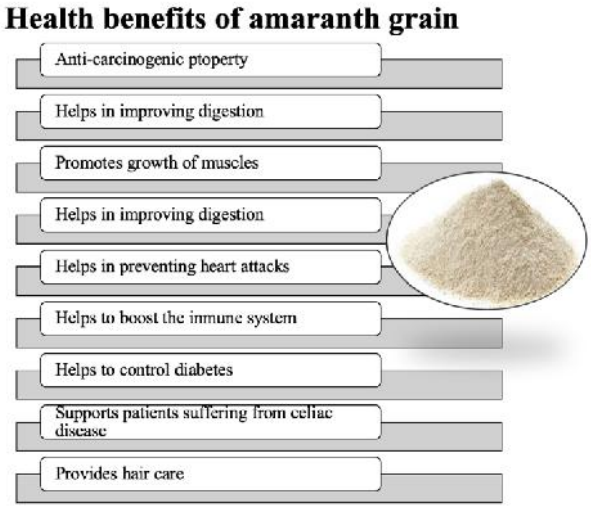

Figure 3. Benefits of amaranth grain.

The nutritional composition of amaranth is very similar to the characteristics of other cereals and legumes. Among the beneficial actions that amaranth has, it is worth mentioning that the small seeds contain considerable levels of high-quality proteins and low levels of saturated fatty acids in the oil fraction (González et al., 2015) so, amaranth is considered as a functional food or nutraceutical (Orona, 2018). Amaranth is a product of vegetable origin, where its main component of amaranth seed is starch, representing between 50 and 60% of its dry weight (González et al., 2015). This seed is also an important source of proteins, since for every 100 g of amaranth, there are approximately 13 to 25 g of proteins, and they are also very specific when digested by the gastrointestinal tract, where they exert their health benefit (Nardo et al., 2020). The essential amino acids it contains are alanine, valine, leucine, arginine, phenylalanine, tryptophan, isoleucine, serine, and it is also rich in lysine (Anubhuti, 2017). The protein components consist of 40% albumin, 20% globulins, 25-30% glutelins and 2-3% prolamins (Venskutonis and Kraujalis, 2013). It has high amounts of minerals such as calcium, magnesium, potassium, zinc, and iron. It is rich in vitamins such as riboflavin, also known as vitamin B2, vitamin C, vitamin E and folic acid, which have been found in amounts 2.5 times more than wheat (Anubhuti, 2017) and antioxidants (Rollán et al., 2019). Another benefit it has is that it has a digestibility of 93% (Ayala et al., 2016).

The lipid concentration of amaranth is 1.9 to 13%, highlighting linoleic, oleic, and palmitic acid (Ferreira and Gomes, 2010). Carbohydrates are the main constituent; starch is present in concentrations of 48-69% by weight (Bhat et al., 2015), followed by sucrose, maltose and raffinose. Dietary fiber consists in amounts of 8-11%; it contains high concentrations of minerals such as phosphorus, magnesium, iron and calcium, as well as vitamin C and A (Corke et al., 2016). The total dietary fiber content (soluble and insoluble) in the amaranth grains of *A. caudatus*, *A. cruentus* and *A. hypochondriacus* is between 7 and 16% (Corke et al., 2016).

Socio-economic impact

Gluten-free foods have improved over time thanks to the different ingredients, additives and technologies that have been developed and evaluated. However, as there is still no single raw material, ingredient or additive that can completely replace gluten, the production of gluten-free products remains a technological challenge (Carbó et al., 2019). One of the most studied gluten-free foods is bread. However, it still represents a very big challenge for the bakery industry as it does not satisfy most consumers, because these commercially gluten-free bread are characterized by a bad taste and an unpleasant flavor; they have a low content of dietary fiber, micronutrients. Therefore, it does not provide any nutrients to consumers and being high in starch, they experience faster aging due to starch retrogradation (Carbó et al., 2019).

Although gluten-free has given some serious problems to bakers, there is a great interest in the production of gluten-free flours, leading to the development of new food products (Ruiz et al., 2016). There are pseudocereal foods such as amaranth and quinoa that have been cultivated for a long time and today are recognized for their high nutritional value and potential health benefits, so these foods considered pseudocereals must be ideal candidates to produce gluten-free products and, for example, in this case, can be used as a good flour option for bread making (Rollán et al., 2019).

ANTINUTRIENTS PRESENTS IN PSEUDOCEREALS

Nuts, cereals and pseudocereals are seeds that are considered very energetic, nutritious, and healthy, since as we have seen above, they are high in carbohydrates, proteins, fibers, vitamins, and minerals. However, in addition to this, these foods also contain substances called antinutrients. Antinutrients are natural or synthetic compounds that prevent the proper absorption of foods. Consequently, these antinutrients reduce the beneficial contributions contained in foods such as pseudocereals; that is, they decrease the digestibility of proteins, carbohydrates, minerals, and vitamins (Dyner et al., 2016).

One of the functions of antinutrients as an "advantage" to these grains is that they protect them against bacterial growth and predators and thus ensure that the species survives, but they are not beneficial to humans (Félix, 2018). Within these compounds are flavonoids, polyphenols and phytosterols, which contradictorily can also have beneficial effects when consumed. The high phytate content in these grains significantly reduces the retention of calcium, iron, and zinc, as well as decreases bioavailability in the digestive tract (Rollán et al., 2019). Amaranth contains a low concentration of saponins that produce low toxicity compared to other grains. These saponins are absorbed in small amounts and produce a detrimental effect in the intestinal tract. However, saponins are antimicrobial, anticancer, lower cholesterol, as well as

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modulate the immune system in addition to their anti-inflammatory effect (Anubhuti, 2017).

Some of the anti-nutrients that can be found in pseudocereals are:

1) Phytates. They are present in the fibrous part (Cruz, 2013), found in very high concentrations in foods such as cereals, legumes, nuts and whole seeds in general (López, 2019), they are located in the outer covers, hence the richness in phytates depends on the degree of extraction to which the corresponding cereal is subjected. They cause a reduction in the absorption of minerals, especially zinc, iron, and calcium (Félix, 2018).

2) Phenolic compounds or polyphenols. They are those that constitute a broad group of chemical substances, considered secondary metabolites of plants, with different chemical structures and activity. Phenolic compounds have traditionally been considered as anti-nutrients, due to the adverse effect of one of their main components, tannins, on protein digestibility (Martínez, 2016) leading to lower growth of livestock and lower egg laying by poultry (Lamuela-Raventós et al., 2004). Tannins are phenolic compounds found in abundance in many plants and fruits (Idict, 2019); these compounds are considered anti-nutrients, as they can form complexes with proteins, starch, and digestive enzymes, causing a reduction in the nutritional value of food (Melo and Ligarreto, 2010). It is not advisable to consume plants containing a high concentration of tannins for a prolonged period, as they inhibit the body's absorption of certain vitamins and minerals, such as calcium and iron (Idict, 2019).

Research on antinutrients has not only focused on their characterization, but also on the search for strategies to counteract their effect. One of these strategies is fermentation, which will be discussed in the following section.

SOURDOUGHS

The products of the bakery industry that are totally or partially composed of pseudocereals are of interest, since it is interested to create new sensory properties, in addition to developing substitutes for celiac patients. This type of mother dough, in addition to its nutritional value, would have a characteristic flavor, which would facilitate, also in combination with other flours of different foods and give a greater nutritional value to the production of new types of bread. A desired characteristic and a prerequisite for the commercial production of pseudocereal stem mass is that a potential starter crop must be able to ferment different pseudocereals as well as normal cereals, that is, it may have the same or even better organoleptic characteristics than the masses characterized by normal flours. The dough consists of flour and water and can ferment spontaneously with lactic acid bacteria, which come from the native microbiota of cereals, the ingredients of the dough or the environment (Sterr et al., 2009). The main contribution of sourdoughs is the inoculation of active microorganisms that, together with the products of their metabolism, create favorable conditions for the correct fermentation of the bread dough, in addition to providing a three-dimensional structural base constituted by the preformed gluten network, which facilitates the development of the dough and incorporates air and CO_2 nuclei, facilitating the best distribution of the gas produced during fermentation. The pH of a sourdough varies according to the nature of the process and is in the range of 3.5 to 4.3 (Conty, 2018).

The sourdough must represent a proportion equal to or greater than 5% of the total weight of flour in the final dough, not including additives. The following conditions must also be met:

FERMENTATION

In colloquial terms, we can define food fermentation as the natural preservation of food based on biological degradation, which consists of using one or more microorganisms to carry out this preservation. This process consists of converting sugars into acids, gas, or alcohol, depending on the type of food being fermented, so that they act as natural preservatives (Garre, 2020).

Fermentation is defined as a complex process, since the transformation of food, whether cereal, fruit or, eventually, milk, is produced by the action of microorganisms in the environment, which, by using the available components of the food, can reproduce by transforming the flavor, color, odor, texture and even the nutritional value of the product in which they are growing and fermenting (Limón et al., 2015).

Nowadays, traditional fermentation of cereals is used as a food source, containing both organoleptic characteristics and better preservation (Betancourt et al., 2013). Fermentation is a metabolic process in which carbohydrates are oxidized to release energy in the absence of an acceptor and electrons (Rollán et al., 2019). Fermentation refers to the conversion of complex organic substrates, mainly carbohydrates, into simple compounds by the action of microorganisms (bacteria, fungi, and yeasts) (Rudnitskaya et al., 2016). It can be spontaneous (microorganism grows in the raw material, without external manipulation) or induced (microorganisms are deliberately added to the raw material and conditions are manipulated to obtain this effect) (Batt, 2016; Wolfe and Dutton, 2015). Their use is common in the food or functional food industry, with approximately 5,000 varieties of fermented foods and beverages being consumed worldwide (Michalak and Chojnacka, 2014). Its importance in food lies in food preservation and in its transformation, by giving it a characteristic aroma, flavor and texture, as well as a better nutritional profile (Chen et al., 2013; Di Cagno et al., 2013). For the microorganism it is necessary to carry out fermentation mainly to obtain energy, through this process it also produces primary metabolites, which are essential for its growth: amino acids, nucleotides, lipids, and carbohydrates, and under adverse conditions, secondary metabolites such as peptides are obtained (Barrios-González, 2018; Limón et al., 2015).

Fermentation of cereals is one of the oldest and cheapest biotechnological processes, where both beer and bread were produced with the help of yeasts and lactic acid bacteria.

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Spontaneous fermentation simply activates the natural microbes in the ground grains (Poutanen et al., 2009), it helps to produce and preserve food as it can also be used in cereal processing (Rollán et al., 2019).

Fermentation process

One of the oldest biotechnological processes is the fermentation of the acid, it is considered a strategy for the food industry where it is imported by the bioactive components of pseudocereals, where they possess various antinutritional factors and at the same time increasing health benefits (Rollán et al., 2019). During the fermentation of cereals, which in addition to flour, water is also a prerequisite to develop a mother dough, where comparing with flour, water has not been included as one of the factors that may have significance to affect the microbiome of the mother mass, however it is considered as a requirement that could determine the consistency of the mass (Lau et al., 2021). When combined with LAB they produce lactic and acetic acids, which generally tend to reduce the pH below pH 5 while yeast produces carbon dioxide and ethanol. When there is an interaction between yeasts and lactobacilli the metabolic activity of the sourdough mass is activated where changing conditions during fermentation contribute to the activation of different metabolites such as the enzymes present, and pH adjustment selectively improves the performance of certain enzymes, such as amylases, proteases, hemicellulases and phytases (Poutanen et al., 2009). There are different types of fermentation, one of them being natural fermentation, i.e., when environmental conditions allow the interaction of microorganisms and susceptible organic substrates, and artificial fermentation, when humans favor the conditions and contact referred to. The best-known fermentations start from pyruvate, the main ones are four: acetic fermentation, alcoholic fermentation, butyric fermentation, and lactic fermentation.

Lactic acid fermentation is commonly considered in food production to preserve food, as well as to improve its safety, nutritional value, and sensory properties. During fermentation, the production of organic acids, mainly lactic acid, leads to acidification of the pH, which favors the increase of endogenous phytase activity. Lactic acid fermentations are carried out by microorganisms present in the raw cultures or by the starter culture itself. The starter cultures most used in food fermentation belong to the lactic acid bacteria. Some of the species involved in this type of fermentation are *Lactobacillus plantarum*, which is effective in reducing the antinutrients that these pseudocereals may present where it produces an effect on fermentation with its nutritional components (Fig. 4) (Castro-Alba et al., 2019). In Table 1, the main effects of fermentation in sourdoughs are presented.

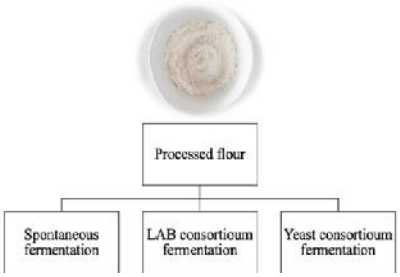

Figure 4. Process and effect of fermentation of sourdough.

Table 1. Investigation about effect of fermentation of sourdough

Effect of fermentation in dough	Reference
Microbial quality analysis	Cruz et al., 2021, Rudnitskaya et al., 2016
Improved in vitro starch digestibility	Poutanen et al., 2009, Sanchez, 2019
Improved in vitro protein digestibility	Poutanen et al., 2009
Increase in protein content	Bhat et al., 2015
Reduction in fat content	Rollán et al., 2019
Increase in ash	Bhat et al., 2015
Reduction in fiber	Bhat et al., 2015
Antioxidant activity	Anubhuti, 2017 Cruz et al., 2021; Tortora et al., 2020
Antihypertensive activity	Anubhuti, 2017
Immunology system	Cruz et al., 2021; Tortora et al., 2020

Lactic acid bacteria

Microorganisms are found in almost all ecological niches, pseudocereals are usually a good medium for microbial fermentations. Sourdough contains, according to this decree, "an acidifying microflora consisting essentially of lactic bacteria and wild yeasts" (Sanchez, 2019).

The term "lactic acid bacteria" (LAB) encompasses a heterogeneous group of microorganisms whose defining characteristic is the production of lactic acid from the fermentation of sugars (Cruz et al., 2021). They are usually in the form of cocci or bacilli, are gram-positive, non-sporulating, immotile, anaerobic, microaerophilic or aerotolerant, oxidase, catalase, and benzene negative. These microorganisms lack cytochromes and do not reduce nitrate to nitrite, their main carbohydrate fermentation product is lactic acid, so they are acid tolerant as they can grow at very low pH. Lactic acid bacteria use various sugars such as glucose and lactose to produce acetic acid by fermentation. Some bacteria are known as facultative anaerobes and others as anaerobes are forced to be able to temporarily the intestine and in turn, survive during intestinal transit; in addition, due to their adhesion to the

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epithelium, they modify the local immune response of the host (Cruz et al., 2021; Tortora et al., 2020).

Lactic acid bacteria are found in a wide variety of environments and can be used for multiple tasks such as food manufacturing and preservation as starter cultures, to control fermentation (Betancout et al., 2013). Lactic acid bacteria are gram-positive, non-sporulating, cytochrome-deficient, catalase-negative, aerotolerant, acid pH tolerant and strictly fermentative. They produce lactic acid as the main metabolic product of carbohydrate fermentation (Rollán et al., 2019). These types of bacteria are considered as a group of microorganisms with GRAS (generally recognized as safe) that have traditionally developed with fermented foods. Lactic acid bacteria are generally found in habitats characterized as nutrient-rich, such as in some foods, or they can also be found in the normal flora of the gut, in the mouth, or in the intimate part of mammals (Rollán et al., 2019). Currently, what is known as lactic acid bacteria groups in different genera, bacteria of the order Lactobacillales and bacteria of the order Bifidobacteriales (Cruz et al., 2021).

For the multiplication of lactic acid bacteria, nutrients are needed to support their growth, such as sugars like glucose and lactose, as well as amino acids, vitamins, and other growth factors. Milk is the typical and satisfactory medium for the growth of lactic acid bacteria. However, other foods are also excellent media for the growth and production of lactic acid bacteria metabolites; among them are cereal masses (such as wheat, quinoa, and amaranth), vegetables and meats. Therefore, these microorganisms are generally used as starter cultures in the production of dairy products, such as acidified milk, yogurt, butter, cream, and cheeses; as well as in the processing of meats, alcoholic beverages, and vegetables (Cruz et al., 2021), as it is also very common in the bakery industry, and even in some pharmaceutical companies.

Yeasts in the fermentation process

Yeasts have played an important role in the production of food and beverages by fermentation since ancient times. Yeasts are microscopic single-celled fungi; most reproduce asexually by budding and other species reproduce by multiple fission. True yeasts can reproduce sexually, this process involves the formation of ascopores. The shape of yeasts can range from spherical to ovoid, lemon-shaped, pyriform, cylindrical, triangular, and even elongated, forming a true mycelium or a false mycelium. They also differ in size, measuring from 1 to 10 um wide by 2-3 um long. Observable parts of their structure are the cell wall, cytoplasm, vacuoles, fat globules and granules, which can be metachromatic, albumin or starch (Méndez-Zamora et al., 2020).

They are currently used in different areas of biotechnology such as the production of beer, wine, sake and soy products, enzymatic products, flavorings, pigments, amino acids, organic acids. They are also used in the field of environmental biotechnology as an application of bioremediation and degradation of pollutants, as biocontrol for crop protection, probiotics, and food, for the production of pharmaceutical proteins, hormones, vaccines and toxins, for research in biology and molecules, they participate in the study of cell biology, genomics, pathway engineering, mechanisms of biological systems, among others.

Recently, there has been a growing interest in yeasts that are able to grow at high temperatures, since they present advantages in different industrial processes with respect to yeasts that do not have this quality. Yeasts that are characterized by resisting high temperatures are called thermotolerant yeasts; however, there is no absolute temperature value, since the limits from which thermotolerant yeasts are considered to vary in the literature (Mejía et al., 2016).

In lactic fermentation, lactate is produced as a waste product and less energy is achieved. Yeast has been known to be used for many years, and the Egyptians started using it to make bread and beer. Today, yeast is used in the production of all kinds of bakery and confectionery products, as well as in the production of fermented beverages. Yeast is a probiotic food as it offers many benefits to the organism (Acosta et al., 2020).

The yeast species with the most reports of thermotolerance are *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, the latter being the one that has shown the highest thermotolerance, besides being one of the most used in the food industry such as breweries or bakeries. Thanks to its high capacity to produce biomass, ethanol, heterologous proteins, in addition to the wide range of carbon sources it can metabolize, these characteristics make this species attractive for different industrial applications. Other important yeast species with thermotolerance, in addition to those mentioned, are *Hansenula polymorpha*, *Pichia pastoris* and *Kluyveromyces fragilis* (Mejía et al., 2016).

Microbiota of fermented foods

It is very common that the microorganisms in a food that has been fermented constitute a very complex microbiota, since it constitutes a large number and a great diversity of microorganisms. In modern fermented products produced today, it has been possible to determine those microorganisms that are key in the fermentation process, so it is possible to guarantee the microbiological safety of a product by pasteurization, thus eliminating most of the pathogenic microorganisms it may contain, and subsequently adding those key microorganisms, previously cultivated in the laboratory to induce the fermentation process. This also makes it possible to control the fermentation conditions, such as temperature and, eventually, aeration or pH. It is important to produce the food under controlled conditions, including packaging after the fermentation process, since it is sought, first of all, to ensure both the safety of the product, and that the characteristics of the food are homogeneous from batch to batch; if the color is yellow, there are no greens, they must all be yellow; as well as that the taste, smell and appearance are the same (Sánchez,

ISSN: 2683-3271

2019).

The microbiota of "conventional" cereal sourdoughs is composed of stable associations of lactic acid bacteria and yeasts. The wild microbiota of cereals and, consequently, of flour, will be affected by different factors. Mainly, it is influenced by the climate of the country, in particular temperature and humidity, storage conditions, insect conditions, and also the application of fungicides on the feed (De Vuyst et al., 2005). Numerous lactic acid type bacteria have been identified in sourdough, the most common of which is the genus Lactobacillus. The LAB (acidolactic bacteria) that usually occur in wheat and rye sourdoughs are *L. sakei Lactobacillus plantarum*, *L. pontis*, *L. acidophilus*, *L. alimentarius*, *L. fermentum* (Scheirlinck et al., 2008), *L. brevis* (De Angelis et al., 2002), *L. amylovorus* (Moore et al., 2007), *L. curvatus* (Fasano et al., 2003). During fermentation of wheat or rye, mainly lactic acid and acetic acid are produced from the LAB present, resulting in a low pH value. In addition, the production of ethanol, some bacteriocins, exopolysaccharides, aromatic compounds and enzymes has been observed (Leroy and Vuyst, 2004).

Benefits of fermentation in sourdoughs

Sourdough fermentation can influence nutritional quality by decreasing or increasing compound levels and improving nutrient bioavailability (Poutanen et al., 2009). Therefore, the nutritional and functional quality of pseudocereals can be improved by fermentation with lactic acid bacteria (Rollán et al., 2019). The use of sourdough is of increasing interest to improve the flavor, structure, and stability of baked products. Cereal fermentation also shows significant potential to improve and engineer the nutritional quality and health effects of foods and ingredients. In addition to improving the sensory quality of whole-grain, fiber-rich or gluten-free products, sourdough can also actively retard starch digestibility, resulting in low glycemic responses, modulate the levels and bioaccessibility of bioactive compounds and improve the bioavailability of minerals. Fermentation of cereals may produce non-digestible polysaccharides or modify the accessibility of the grain fiber complex to the gut microbiota. It has also been suggested that gluten breakdown may make bread more suitable for people with celiac disease (Poutanen et al., 2009). On the other hand, fermentation of pseudocereals by lactic acid bacteria has the ability for these bacteria to decrease anti-nutritional factors such as phytic acid, increase the nutritional value of phytochemicals such as phenolic compounds, as well as produce nutritional ingredients such as vitamin B (Rollán et al., 2019).

Fermentation degrades molecules to transform them into simpler ones. In the case of bread making, yeasts transform starch into glucose, which is made possible by the enzyme amylase. There are many changes in the cereal matrix that can lead to improved nutritional quality. Among them is the production of acid, suggested to slow down starch digestibility and adjust the pH to a range that favors the action of certain endogenous enzymes, thus changing the bioavailability pattern of minerals and phytochemicals. This is especially beneficial in bran-rich products to provide minerals and potentially protective compounds into the bloodstream. Enzyme action during fermentation also causes hydrolysis and solubilization of grain macromolecules, such as cell wall proteins and polysaccharides. This changes the texture of the product, which can affect the absorption of nutrients and non-nutrients. New bioactive compounds, such as prebiotic oligosaccharides or other metabolites, can also be formed in grain fermentations.
Thanks to the interaction of microorganisms present in the fermented masses, several properties are highlighted that provide several health benefits which are also listed in Figure 5 (Lau et al.,2021).

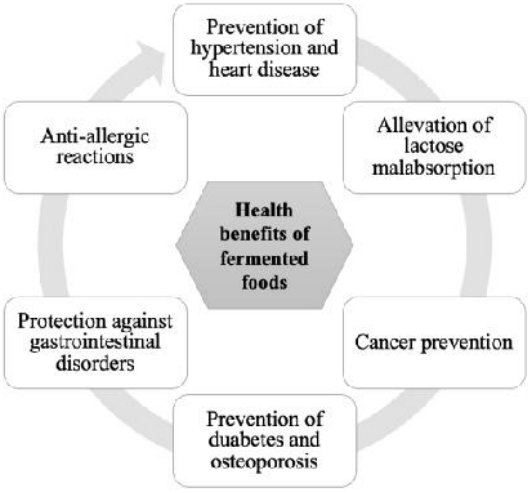

Figure 5. Health benefits of fermented foods.

CONCLUSIONS

Fermentation is known over many years as one of the biotechnological methods where it involves a lot of cost for the fermentation of certain foods, that as pseudocereals is found a biotransformation of its components and interacts with its nutrients to obtain a functional food with many beneficial properties for health. Amaranth is one of those pseudocereals, which at the root of time has been put in the spotlight since it is a very economical food, in addition to the fact that it does not cease to arise that it has high content of nutrients, in addition to having antioxidant, antihypertensive, antimicrobial properties. When fermented, either spontaneously or with the help of certain microorganisms such as LAB or yeasts, biotransformation occurs because these microorganisms compete with the nutrients that amaranth has, as has also been seen to decrease the content of those antinutrients that are characteristic of poor absorption of proteins and minerals. So, it leads to further research about the behavior of fermentation with different microorganisms to understand and procreate new alternatives for the creation of nutritional foods.

ISSN: 2683-3271

ACKNOWLEDGEMENTS

The authors thank the National Council of Science and Technology (CONACYT) of Mexico and the Department of Food Research of the Universidad Autónoma de Coahuila.

FUNDING

LAGF thanks the National Council of Science and Technology (CONACYT) of Mexico for its postgraduate scholarship grant [grant number: 001468].

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JBCT Journal of BioProcess and Chemical Technology

ISSN: 2683-3271

2022 Volumen 14, No. 28
Revista Científica de la Universidad Autónoma de Coahuila

Enzyme-Assisted Extraction of Mint Essential Oil and Evaluation of its Antimicrobial Activity Against Food-Borne Pathogens and *Fusarium* sp.

Extracción por asistencia enzimática de aceite esencial de menta y evaluación de su actividad antimicrobiana contra patógenos transmitidos por los alimentos y *Fusarium* sp.

Saldaña-Mendoza S.A.[1], Ramírez-Guzmán K.N.[2], Chávez-González M.L.[1] and Aguilar-González C.N.[1]*

[1]*Bioprocesses and Bioproducts Research Group. Food Research Department. School of Chemistry. Universidad Autonoma de Coahuila. Saltillo, 25280, Coahuila, México.*

[2]*Center for Interdisciplinary Studies and Research. Universidad Autonoma de Coahuila. Saltillo, 25280, Coahuila, México.*

**Corresponding author: cristobal.aguilar@uadec.edu.mx*

Recibido: 03 de mayo 2022
Aceptado: 30 de mayo 2022

Resumen

El presente trabajo evaluó el uso de asistencia enzimática para extraer aceites esenciales de menta mediante procesos de hidrodestilación y se evaluaron las propiedades antifúngicas y antibacterianas del aceite obtenido frente a patógenos transmitidos por alimentos. Se realizó un análisis exploratorio utilizando un modelo de Hunter & Hunter, evaluando el efecto del tiempo (90, 180 min), pH (4.5, 5), contenido de enzima (15, 30 mg), temperatura (40, 50 °C) y agitación (100, 150 rpm). Se encontró que el uso de asistencia enzimática contribuyó a un aumento de 70,4 % en los rendimientos de extracción con el mejor de los tratamientos (90 min, pH 4,5, 40 °C, 30 mg y 150 rpm). Asi mismo, el aceite esencial de menta obtenido por extracción con asistencia enzimática inhibió el crecimiento de *Fusarium* sp. en ensayos de microplaca, presentando además una notable actividad antimicrobiana frente a cepas de *S. aureus*, *E. coli* y *S. enterica* serovar Enteritidis, generando halos de inhibición de 19,45 ± 1,33, 17,12 ± 0,75 y 14,54 ± 1,17 mm, respectivamente, en ensayos de difusión en disco. De esta forma, se encontró que la asistencia enzimática contribuye a aumentar los rendimientos de extracción del aceite esencial de menta a través de procesos de hidrodestilación sin comprometer sus propiedades antifúngicas y antimicrobianas.

Palabras clave: Antimicrobiano, enzimas, aceite esencial de menta, patógenos, hidrodestilación.

Abstract

The present work evaluated the use of enzymatic assistance to extract mint essential oils through hydro-distillation processes and the antifungal and antibacterial properties of the obtained oil against food-borne pathogens. An exploratory analysis was performed using a Hunter & Hunter model, evaluating the effect of time (90, 180 min), pH (4.5, 5), enzyme content (15, 30 mg), temperature (40, 50 °C), and agitation (100, 150 rpm). It was found that the use of enzymatic assistance contributed to a 70.4 % increase in extraction yields with the best of the treatments (90 min, pH 4.5, 40 °C, 30 mg y 150 rpm). Likewise, the mint essential oil obtained by extraction with enzymatic assistance inhibited the growth of *Fusarium* sp. in tests of microplate, also presenting a remarkable antimicrobial activity against strains of *S. aureus*, *E. coli*, and *S. enterica* serovar Enteritidis, generating inhibition halos of 19.45 ± 1.33, 17.12 ± 0.75, and 14.54 ± 1.17 mm, respectively, in tests of disk diffusion assay. In this way, it was found that enzymatic assistance contributes to increased extraction yields of mint essential oil through hydro-distillation processes without compromising its antifungal and antimicrobial properties.

Keywords: Antimicrobial, Enzyme-assisted extraction, Mint essential oil, Food-borne pathogens, hydro-distillation.

JBCT Journal of BioProcess and Chemical Technology
ISSN: 2683-3271
2022 Volumen 14, No. 28
Revista Científica de la Universidad Autónoma de Coahuila

INTRODUCTION

Essential oils (EOs) are volatile substances of an oily nature present in all vegetables (Dutra et al., 2019). They are a product of the secondary metabolism of plants (Memarzadeh et al., 2020). They are composed of a mixture of aldehydes, hydrocarbons, terpenes, and phenols (Plant et al., 2019). EOs are found in different organs of the plants such as stems, leaves, seeds, flowers, and fruits; they can also be found in secretory cells, channels, epidermal cells, and glandular trichomes (Vásquez et al., 2001; Alzamora et al., 2014; Rezaei et al., 2021).

EOs have been part of traditional medicine and perfumery for many years (Das et al., 2021). They are currently used as preservatives and elemental substances by various industries, from food to pharmaceuticals, in the production of multiple products such as perfumes, cosmetics, flavorings, food, etc. (Castañeda et al., 2007; Popović-Djordjević et al., 2019). They have multiple effects, such as anti-parasitic, anti-virulent, insecticidal, fungicidal, and bactericidal (Radivojac et al., 2020). They are considered a safe, biodegradable, and natural alternative for controlling postharvest pathogens (Hasheminejad et al., 2019). Nowadays, EOs are considered GRAS (Generally Recognized as Safe) substances and are included in different food investigations in border areas (Das et al., 2021).

Conventionally, the EOs are extracted through hydro-distillation, steam distillation, and solvent extraction processes (Spadi et al., 2021), mainly based on the reaction temperature and the selection of solvents that allow for increasing the solubility of plant matrices (Gao and Liu, 2005). However, these methods have some disadvantages, such as the generation of low yields and traces of chemicals that are harmful to health (Shrigod et al., 2017). Currently, multiple assistive techniques friendly to the environment have been developed to improve extraction yields, including microwave assistance (Ghazanfari et al., 2020; Xiao et al., 2021; Yingngam et al., 2021), ultrasound (Liu et al., 2019), use of green solvents (Zhao et al., 2019) and enzymatic assistance (Zaizhi et al., 2021). Enzyme assists technology consists of using hydrolytic enzymes to degrade plant cell walls, composed of highly complex polymers such as hemicellulose, cellulose, and pectin, improving the release rate of intracellular components (Meini et al., 2019). It is a high-efficiency technology, has good environmental compatibility, and is easy to execute (dos Santos Reis et al., 2020; Zaizhi et al., 2021).

Mint (*Mentha* sp.) is a plant of the Laminaceae family widely distributed in regions of moderate temperatures. The genus has 19 species and 13 natural hybrids. It has multiple nutritional and pharmacological properties and a tremendous economic relevance due to the EO (Nazem et al., 2019; Lothe et al., 2021). In 2019, the world production of 74,200 tons of mint was reported. Morocco is the primary producer with 66,500 tons, and Mexico is in third place with 610 tons (FAO, 2021). The mint essential oil (MEO) is mainly composed of mentone (20-31%), mentofuran (6.8%), menthyl acetate (3-10%), and menthol (29-48%), which in addition to having a pleasant aroma, it has antiseptic, antispasmodic, and fungicidal properties (Mendoza et al., 2009; Singh et al., 2015). In addition, the MEO also contains some pharmacologically active ingredients such as caffeic acid, flavonoids, polymerized polyphenols, carotenes, tocopherols, betaine, choline, and tannins (Singh et al., 2015). Currently, food-borne pathogens represent a severe food health problem, generating considerable economic losses (Adley and Ryan, 2016). According to the surveillance of food-borne disease outbreaks during 2016-2019, pathogenic bacteria, such as *Escherichia coli*, *Salmonella* sp., and *Staphylococcus aureus*, were the most confirmed etiologies of many disease cases in the United States (Chuesiang et al., 2021).

In the present work, an exploratory analysis of the extraction of MEO was carried out through hydro-distillation processes using enzyme assistance extraction (EAE) technology using a pool of *Trichoderma harzianum* cellulases (SIGMA). In addition, the effect of extracted MEO against food-borne pathogens and an agriculturally important pathogenic fungus was evaluated. As far as the authors are aware, there is no history of the application of EAE for the extraction of MEO through hydrodistillation processes using cellulase enzymes from *T. harzianum*, with a subsequent evaluation of the maintenance of antimicrobial properties.

MATERIALS AND METHODS

Raw material and conditioning. The mint (*Mentha* sp.) was collected in "Ejido El Coyote" in the municipality of Villa Hidalgo; San Luis Potosí (Latitude 22 ° 51´ 34.6´, longitude 100 ° 35´ 59.9´´). The raw material was identified by its morphological characteristics. Later it was cleaned by removing foreign particles and ground for experimental development. The microorganisms used in the present work (*Fusarium* sp, *Escherichia coli*, *Salmonella enterica* serovar Enteritidis, and *Staphylococcus aureus*) were provided by the

strain collection of the Food Research Department of the Faculty of Chemical Sciences belonging to the Autonomous University of Coahuila.

Evaluation of the EAE of MEO. For the development of this stage was used an enzymatic pool of "lithic enzymes of *Trichoderma harzianum*" (SIGMA) composed of β-glucanases, cellulases, proteases, and chitinases, developed by Novozyme Corp. The experimental units consisted of Erlenmeyer flasks of 500 ml with 30 g of mint and 300 ml of citrate buffer 0.1 M following the conditions shown in Table 1. In addition, a Hunter & Hunter experimental design was used to evaluate five variables at two levels using the statistical program "STATISTIC 7". After enzyme treatment, the samples were subjected to a process of hydro-distillation at boiling temperature for 40 min.

Table 1. Evaluated conditions of enzyme pretreatment by Hunter & Hunter experimental design.

Treatment	Time	pH	°C	Enzyme (mg)	Rpm
MTx1	90	4.5	40	30	150
MTx 2	180	4.5	40	15	100
MTx 3	90	5	40	15	150
MTx 4	180	5	40	30	100
MTx 5	90	4.5	50	30	100
MTx 6	180	4.5	50	15	150
MTx 7	90	5	50	15	100
MTx 8	180	5	50	30	150

Recovery of essential oil. Subsequently, the extracts were treated with ethyl acetate in a 1:2 ratio to separate the organic phase. After the organic phases were rotavaporated using equipment (BÜCHI RE120) to 20 in Hg and 38 ° C. Finally, the quantities of oil recovered for the treatment were evaluated and stored in the dark for further tests.

Evaluation of antifungal activity. The effect of the MEO against *Fusarium* sp. was assessed using a technique in microplate reported by Mdee et al., (2009). First, 10 µl of potato dextrose broth were mixed in a microplate with 100 µl of the MEO obtained whit the treatment MTx1 undiluted and 10 µl of *Fusarium* sp. spore's solution at a concentration of 1 X 10^6 spores / mL. Then, the experiment was left in incubation at 30 °C for 48 h. A blank treatment was evaluated without MEO. Subsequently, samples of 10 µL were taken from the mixtures, inoculated in Petri dishes with PDA, and incubated for seven days at 30 °C. The growth was assessed every 24 h.

Evaluation of antibacterial activity. The MEO was evaluated using a disk diffusion assay against three bacterial strains, *E. coli*, *S. aureus*, and *S. enterica* serovar Enteritidis, using the methodology described by Carvalho et al., (2018) with some modifications. First, the bacteria were reactivated in nutrient broth and incubated at 37 ° C for 24 h. Subsequently, Petri dishes with nutrient agar were prepared and inoculated with 500 µl of the bacterial cultures using the pour-plate technique. Finally, four sterilized filter paper disks (7mm) were placed on each plate. Three were impregnated with 10 µL MEO produced by MTx1 treatment undiluted and one with MEO of industrial origin was used as a control. The plates were incubated at 37 °C and the inhibition halos were evaluated at 24 h. The results were analyzed by a Tukey means test using the InfoStat program.

RESULTS AND DISCUSSION

Extraction yields by EAE. The MEO yields obtained by the EAE technique are presented in Fig. 1. It is observed that the MTx1 treatment produced the highest yield, 0.213%, which is 70.4 % higher than the treatment without EAE. This result is associated with the action of cellulase enzymes from the enzyme pool on plant material. Endoglucanases, exoglucanases, and β-glucosidases work synergistically to break the β 1-4 bonds of cellulose that constitute cell walls and the oil glands. Finally, the release of intracellular components, among which EOs are present (Ovando-Chacón and Waliszewski, 2005; Kumar et al., 2019; Shimotori et al., 2020 Pradhan et al., 2021).

JBCT Journal of BioProcess and Chemical Technology
ISSN: 2683-3271
2022 Volumen 14, No 28
Revista Científica de la Universidad Autónoma de Coahuila

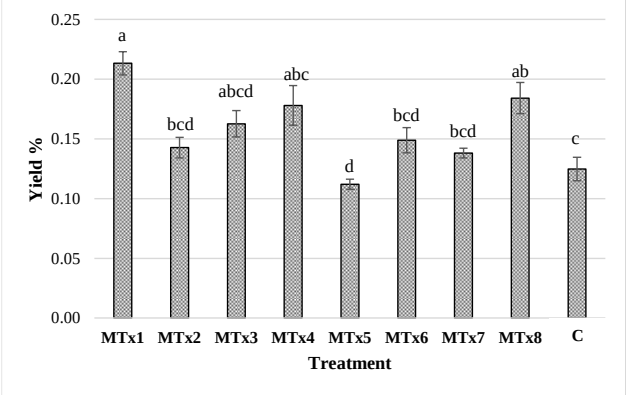

Figure 1. Percentage of MEO extraction yields obtained with the treatments evaluated in the exploratory analysis. (C) Treatment without enzyme assistance. Standard deviations are represented with bars and significant differences with letters.

Table 2 shows a comparison of the MEO extraction yields obtained in this study with those reported by other authors in the literature. It can be appreciated that the yields obtained were higher than those reported by de Sousa Barros *et al*., (2015) when working with species of *M. longifolia* and *M. arvensis*. However, it is also observed that there is a variation in the yields reported in the literature when working with different species of mint, and even within the same species.

In this case, the best treatment increased extraction performance by 70.4 % compared to the treatment without EAE. Therefore, the difference in extraction yields compared to some literature authors is not attributed to a deficiency of the methodology in question. It has been reported that the content of EOs depends on several factors, among which are the plant species, the cultivation conditions (such as the nutrients available in the soil, fungal colonization of the rhizosphere, periods of light), and harvest (Gupta et al., 2002; Tabatabaie and Nazari, 2007). Likewise, it is known that the temperature at which plants develop considerably influences their content of EO, favoring their production in cold climates (Heydarizadeh et al., 2013). The raw material used in this work comes from the state of San Luis Potosí, located in the central region of Mexico, characterized by its temperate climate with an average annual temperature of 21 ° C (INEGI, 2018) which could influence the low oil content presented by the raw material.

Table 2. MEO yields presented by different authors through hydro-distillation processes.

Yield %	Species	Reference
0.213 ± 0.02	*Mentha* sp.	Present work
2.29 ± 0.16	*M. piperita*	(Gavahian et al., 2015)
1.7 0.26 0.05 0.1 0.54	*M. spicata* *M. aquatica* *M. longifolia* *M. arvensis* *M. canadiensis*	(de Sousa Barros et al., 2015)
0.64	*M. piperita*	(Singh et al., 2015)
0.8 1 1.25	*M. spicata* *M.longifolia* *M. piperita*	(Heydarizadeh et al., 2013)
0.6	*M. arvensis*	(Gupta et al., 2002)

In the Fig. 2 the Pareto diagram obtained from the exploratory analysis of the MEO extraction by EAE is presented. It can be appreciated that only the stirring and temperature factors had a significant effect on the yields obtained. In the case of agitation, it shows a tendency to higher levels. It is known that an increase in agitation increases the enzyme-substrate interaction, thus favoring its action and therefore the structural lysis of the raw material, improving the release rate of intracellular components (Quirasco-Brauch and López-Mungía, 2006). Regarding the temperature, this presents a tendency to lower values which correspond to that indicated by the manufacturer of 37 °C. Although the optimal cellulase activity varies depending on the producing microorganism, some researchers report an optimal temperature of 30 °C working with *T. reseei* cellulases (Silas et al., 2017), while other reports determined an optimal temperature of 45 ° C working with *T. longibrachiatum* cellulases (Pachauri et al., 2020) and even 60 ° C in work with mutant strains of *T. reseei* (Silva et al., 2020).

Antifungal activity. Table 3 shows the results of the evaluation of the antifungal effect of MEO obtained by EAE using the MTx1 treatment, which was performed in triplicate with two repetitions. It is appreciated that there was no growth of *Fusarium* sp. throughout the trials in comparison with the blank which showed growth at 48 h of incubation.

ISSN: 2683-3271

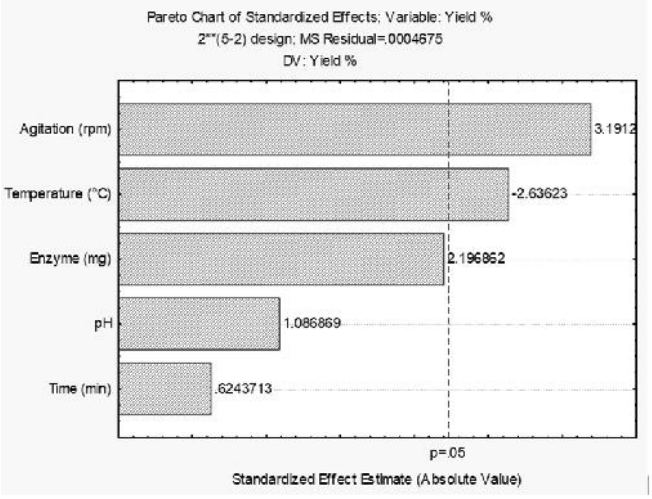

Figure 2. Pareto diagram obtained from the exploratory analysis of MEO extraction by EAE

Table 3. Evaluation of the antifungal activity of MEO extracted by EAE using the treatment MTx1

Replica	Repetition	24 h	48 h	72 h	96 h	120 h	144 h	168 h
1	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	OF	-	x					
2	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	OF	-	x					

(-) - no growth x- growth OF- Oil-free

These results agree with the research carried out by Sharma et al., (2017), who reported the inhibitory effect of *M. piperita* EO against *F. oxysporum* at a concentration of 500 ppm. Vilaplana et al., (2018) also reported the fungistatic effect of MEO on a strain of *F. verticillioides*. The MEO has multiple components with fungicidal activity, such as limonene, menthone, and menthol. The last one is lethal for *Fusarium* spp strains and other fungi like *Mucor* spp., *Aspergillus flavus*, and *Rhizopus* (Kamatou et al., 2013). Menthol, one of the main components present in mint, has been reported to reduce the growth of *F. ventricoloides* by 75% at a concentration of 200 ppm in works developed on semisolid agar (Kamatou et al., 2013). In addition to their chemical composition, the lipid nature of EOs contributes to the inhibition of microorganisms. It allows them to penetrate the lipid bilayer of fungal membranes, facilitating their disruption (Sharma et al., 2017).

Antibacterial activity. Table 4 shows that the inhibition halos produced by the EO obtained by EAE presented a wider diameter than those produced by the EO obtained industrially against the different evaluated microorganisms. It is also appreciated that *S. aureus* is the microorganism most susceptible to the inhibitory effects of the EO obtained by EAE. The halos obtained in this work were similar to those reported by Chrysargyris et al., (2017), who got halos of 9.3 mm (*E. coli*) and 18.3 mm (*S. aureus*), and greater than those reported by Singh et al., (2015), who obtained halos of 5.1 ± 0.4 mm (*E. coli*) and 17.2 ± 0.9 mm (*S. aureus*) when working with EO of *M. piperita* and 19.7 ± 0.3 (*E. coli*) and 14.7 ± 0.4 (*S. aureus*) when testing the inhibitory effect of the antibiotic gentamicin, all within a 24-hour period. The halos obtained in this experiment showed a larger diameter than the previous cases, except in the case of *E. coli*, which showed greater sensitivity against gentamicin than to the MEO evaluated in this work.

Table 4. Results of inhibition halos produced by mint essential oil against food-borne pathogens

Assay	Halo (mm)
S aureus	19.45 ± 1.33 [a]
S aureus control	11.27 ± 1.03 [d]
E coli	17.12 ± 0.75 [b]
E coli control	12.13 ± 0.02 [cd]
S. enterica serovar Enteritidis	14.54 ± 1.17 [bc]
S. enterica serovar Enteritidis control	10.41 ± 0.28 [d]

Controls: Tests carried out using industrially obtained essential oil

The MEO is effective against gram-positive and negative bacteria, presenting a more significant effect on gram-positive bacteria. This is due to the lipopolysaccharides that are present in the membranes of gram negatives (Işcan et al., 2002), these prevent the passage of hydrophobic molecules due to the double lipid layer and the external capsule they possess, which makes it difficult to access the cell wall and membrane (Scherer et al., 2013; Chrysargyris et al., 2017; Carvalho et al., 2018).

The antimicrobial effect reported in this work may be associated with some of the compounds that different authors have reported as components of MEO, among which are menthol, limonene, and linalool, among other phenolic compounds (Singh et al., 2005; Verma et al. al., 2010; Zaidi and Dahiya, 2015; Herman et al., 2016; Chouhan et al., 2017). It has been associated that these compounds produce the

deterioration of some enzymes and structural components present in bacterial cells that intervene in the energy production for the survival of microorganisms, altering the mechanisms of electron transport, the synthesis of nucleic acids, assimilation of nutrients, and the plasma membrane causing cell lysis (Tassou et al., 2000; Kamatou et al., 2013; Kang et al., 2019).

CONCLUSION

The application of EAE technology in hydro-distillation processes to extract MEO increased by 70.4% of the yield compared to the extraction by conventional hydro-distillation. The EO obtained showed antimicrobial activity against important food-borne pathogens and an antifungal effect against *Fusarium* sp. In this way, the present work presents an environmentally friendly extraction alternative that allows increasing extraction yields of the MEO without the usage of solvents harmful to health to increase yields and guarantees the preservation of the integrity of its components, allowing its use as an alternative for the control of food-borne pathogens and pathogenic fungi of agricultural importance.

ACKNOWLEDGMENTS

The authors thank the National Council of Science and Technology (CONACYT, Mexico) and the Autonomous University of Coahuila for the support granted to carry out this work.

Conflict of interest

The authors declare that there is no conflict of interest.

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JBCT Journal of BioProcess and Chemical Technology
ISSN: 2683-3271
2022 Volumen 14, No. 28
Revista Científica de la Universidad Autónoma de Coahuila

Fungal Biotransformation of limonene into high added value derivates

Biotransformación fúngica del limoneno en derivados de alto valor agregado

Villarreal Quintero, C.P.[1,2]; Chávez González, M.L.[1,2]*; Iliná, A[2]; Martínez Hernández, J.L.[2]; Aguilar González, C.N.[1].

[1]*Bioprocesses and Bioproducts Research Group, Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila, 25280 Saltillo, Mexico.*
[2]*Nanobioscience Research Group. Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila, 25280 Saltillo, Mexico.*
**Corresponding author: monica_chavez@uadec.edu.mx*

Recibido: 14 de mayo de 2022
Aceptado: 23 de mayo de 2022

Resumen
El uso de la biotransformación microbiana para producir cambios químicos sobre compuestos en particular es una opción para la obtención de compuestos de diverso interés industrial, con la ventaja de que estos cambios químicos pueden ser llevados a cabo bajo condiciones de reacción con baja severidad. Dentro de los compuestos naturales susceptibles a transformaciones se encuentran los monoterpenos que pueden dar origen a una amplia diversidad de compuestos saborizantes y medicinales. En el presente trabajo se evaluaron diversas condiciones de cultivo para lograr la biotransformación del limoneno (monoterpeno) a través del uso de cepas fúngicas. Se determinó el mejor medio de cultivo, pH, que permitió el mayor crecimiento microbiano, así como la caracterización de los compuestos obtenidos por biotransformación del limoneno a través de CG-MS. Los principales productos de biotransformación de limoneno fueron cis-carveol, trans-carveol, limonen-1,2-diol, limonen-4-ol, mentadienol, carvona, entre otros. El proceso de biotransformación de limoneno es una alternativa viable para la producción de compuestos químicos con aplicaciones en diversas áreas del quehacer humano así mismo constituye una alternativa ecológica de producción de metabolitos específicos.

Palabras clave: Biotransformación, cepas fúngicas, fermentación, limoneno.

Abstract
The use of microbial biotransformation to produce chemical changes on compounds is an option for obtaining compounds of diverse industrial interest, with the advantage that these chemical changes can be carried out under low severity reaction conditions. Among the natural compounds susceptible to transformation are monoterpenes, which can give rise to a wide variety of flavouring and medicinal compounds. In the present work, different culture conditions were evaluated to achieve the biotransformation of limonene (monoterpene) using fungal strains. The best culture medium, pH, which allowed the highest microbial growth was determined, as well as the characterization of the compounds obtained by biotransformation of limonene through GC-MS. The main biotransformation products of limonene were cis-carveol, trans-carveol, limonen-1,2-diol, limonen-4-ol, mentadienol, carvone, among others. The limonene biotransformation process is a viable alternative to produce chemical compounds with applications in various areas of human activity, as well as an ecological alternative to produce specific metabolites.

Keywords: Biotransformation, fungus strain, fermentation, limonene.

INTRODUCCIÓN

La biotransformación, proceso por el cual ocurren cambios químicos estructurales llevados a cabo por sistemas biológicos, pretende ser una alternativa y una herramienta en la biotecnología para obtener derivados con alto valor agregado a partir de compuestos de origen natural (Chávez-González, 2015). Uno de los grupos más susceptibles a sufrir biotransformaciones son los terpenos, que se encuentran ampliamente distribuidos en diversas plantas y frutos (Molíns,

2015).

El limoneno es un monoterpeno cíclico de 10 carbonos que puede ser obtenido a partir de la extracción de los aceites esenciales de plantas, frutos, flores, verduras y hierbas y se encuentra principalmente en la cáscara de los frutos cítricos, como la naranja, limón, toronja (Evageliou y Saliari, 2017). El limoneno tiene aplicaciones en el área de perfumería, farmacia, alimentos, también es utilizado como insecticida y antifúngico y, en diversos estudios, se ha demostrado tiene efectos anticarcinógenos (Bacanli et al. 2015).

El limoneno posee una estructura química que puede dar origen a un gran número de compuestos derivados, por lo que la biotransformación es una alternativa a la síntesis química convencional, teniendo como ventajas la reducción de costos y tiempos, además de ser una alternativa verde para la producción de dichos compuestos (Bier et al. en 2017; Chávez-González, 2015). Algunos compuestos derivados del limoneno más comunes, obtenidos por síntesis química orgánica alcohol perílico, perilaldehído, ácido perílico, carveol, carvona, a-terpineol y mentol. Una de las desventajas de la síntesis química son los bajos rendimientos de los productos debido a la baja enanteo y estereo-especificidad de las reacciones. Estos derivados son importantes para diversas industrias ya que poseen actividades tales como anticancerígena, antiinflamatoria, antimicrobiano, insecticida, desinfectante, además de poseer olores apreciados en la industria de la perfumería (Madeiros et al 2021; Ren et al 2020).

A través del uso de microorganismos, como hongos filamentosos, se logrará la biotransformación del limoneno, ya que se ha visto que estos microrganismos poseen la capacidad de poder transformar estos compuestos naturales en otros, durante los procesos metabólicos propios del microorganismo (Molina et al. 2015). Los hongos son capaces de crecer sobre un amplio rango de substratos orgánicos gracias a la resistencia que le confiere su maquinaria enzimática, por lo que se les puede encontrar creciendo en entornos climáticos diversos.

En el presente trabajo se definieron las condiciones de cultivo idóneas para lograr una biotransformación del monoterpeno limoneno a través de sistemas fúngicos

MATERIALES Y MÉTODOS

Evaluación de las condiciones de cultivo para biotransformar limoneno

Se evaluaron cuatro medios minerales con diversas composiciones (Tabla 1) en matraces Erlenmeyer de 250 mL, posteriormente fueron inoculadas con 6 diferentes cepas fúngicas (Aspergillus niger ESH, *A. niger* Aa20, *A. niger* GH1, *A. ustus* PSS, *Fusarium oxysporum*, *Penicillium* sp.) todas ellas pertenecientes a la colección DIA-UAdeC. Una vez inoculados se incubaron en agitación constante a 180 rpm a 30 °C durante 48-72 h. De los matraces anteriormente descritos se tomó una alícuota de biomasa para pasar a un medio mineral fresco y estéril (pH 7). El medio inoculado se incubó por 96 horas en agitación constante a 180 rpm a 30 °C. A las primeras 24 horas de incubación se agregó un pulso de limoneno y etanol al 0.1 % (1:1 limoneno-etanol) y se continuó la fermentación hasta las 96 horas con muestreo cada 24 horas. En cada muestreo, se recuperó la biomasa producida y se llevó a secado a 60 °C durante 48 h, para determinar por peso seco. Al sobrenadante se le midió el pH y el contenido de azúcares totales (Carranza-Mendez et al 2022); al resto del sobrenadante se le adicionaron 25 mL de acetato de etilo, se agitó la mezcla y se recuperó la fase orgánica.

Tabla 1. Composición de los cuatro medios de cultivo evaluados.

Medio	Composición (g/L)
MI	Extracto de levadura (1), sacarosa (50), $NaNO_3$ (2), K_2HPO_4 (1), KCl (0.5), $MgSO_4 \cdot 7H_2O$ (0.5).
MII	Extracto de malta (10), peptona (5), glucosa (10), extracto de levadura (5).
MIII	Extracto de levadura (2), $NaNO_3$ (5), KH_2PO_4 (1), $MgSO_4$ (1), $CaCl_2$ (1), sacarosa (50)
MIV	Extracto de malta (10), peptona (5), glucosa, (10), extracto de levadura (5), $FeSO_4$ (1)

Evaluación de las condiciones de cultivo para biotransformar limoneno

Las cepas seleccionadas por su capacidad para crecer en presencia de limoneno (0.1 %) (*A. niger* Aa20 y ESH) fueron sometidas a diferentes formas de estrés con la finalidad de que le estrés causado promoviera la biotransformación del limoneno.

Estrés con solventes orgánicos

El medio MI fue preparado (pH =7) y esterilizado una vez enfriado se inoculó con 1 mL de cada una de las cepas de *A. niger* Aa20 y ESH se incubó durante 72 h en agitación constante a 180 rpm a una temperatura de 30° C. Después de las 72 h de crecimiento, se filtró el medio y la biomasa se transfirió a matraces que contenían 30 mL del solvente a evaluar (etanol, metanol y cloroformo). La biomasa en solvente fue incubada durante 1 h en condiciones de 30 °C y agitación de 180 rpm. Después de la hora de tratamiento se volvió a filtrar la biomasa y ésta se pasó a un medio mineral nuevo y se colocaron los matraces en un agitador orbital con las condiciones anteriormente mencionadas durante 96 h. A las primeras 24 h de la fermentación se le adicionó un pulso de limoneno/etanol al 0.1 % y se continuó la fermentación. Se muestreó una alícuota de 1 mL cada 24 h hasta finalizar las 96 h, en donde se tomaron alícuotas del medio después del pulso de limoneno.

Estrés térmico

El medio y las condiciones de cultivo fueron las mismas que en la sección anterior, después de las 72 h de crecimiento, se ajustó la temperatura del agitador orbital a 20, 30 y a 40° C y se corrió el tratamiento durante 1 h. Después de la hora se filtró la

biomasa y esta se pasó a un medio basal nuevo y se colocaron los matraces en el agitador orbital con las condiciones normales (180 rpm/30° C) durante 96 h. A las 24 horas de la fermentación se le aplicó un pulso de limoneno/etanol al 0.1 % y se continuó la fermentación. Se muestreó cada 24 horas, en donde fueron tomadas alícuotas del medio después del pulso de limoneno.

Estrés ácido/básico

Se preparó el medio basal MI y se ajustó a pH de 2, 6 y 8, el medio fue esterilizado, una vez a temperatura ambiente se inoculó con las cepas *A. niger* Aa20 y ESH y se incubó durante 72 h en agitación constante a 180 rpm a una temperatura de 30 °C. Después de las 72 h de crecimiento, se filtró el medio y la biomasa se pasó a un medio basal nuevo y se colocaron los matraces en un agitador orbital con las condiciones ya mencionadas durante 96 h. A las 24 horas de la fermentación se le aplicó un pulso de limoneno/etanol al 0.1 % y se continuó la fermentación. Se muestreó cada 24 horas, fueron tomadas alícuotas del medio después del pulso de limoneno.

Tratamiento de muestras

Al final de las diferentes fermentaciones, se filtró la biomasa y se recuperó el medio. La biomasa se colocó en estufas para su secado. El medio recuperado se trató con 25 mL de acetato de etilo para separar la fase orgánica, la fracción fue llevada a análisis por cromatografía de gases acoplada a masas (CG-MS).

Cinética fermentativa bajo las mejores condiciones de biotransformación de limoneno

Se preparó el medio mineral MI y fue inoculado con las cepas *A. niger* Aa20 y ESH previamente reactivadas, los matraces fueron incubados durante 72 h a 30 °C con agitación constante a 180 rpm. Después de 72 h de cultivo la biomasa se sometió a un tratamiento con 30 mL de etanol a 30 °C y con agitación constante a 180 rpm durante 1 h. Transcurrido el tiempo la biomasa fue recuperada a través de filtración y se pasó a un medio mineral MI nuevo con un pH de 7 y se inoculó con las condiciones ya antes descritas. A las primeras 24 h de la fermentación se tomó el primer muestreo que fue el control y a los demás reactores se procedió a aplicarles el pulso de limoneno/etanol 0.1%. La biomasa de cada uno de los reactores se les recuperó biomasa y se colocó en una estufa para su secado, se recuperó sobrenadante y se extrajo fase orgánica con acetato de etilo para posteriormente inyectar en el CG-MS. Se realizó el mismo procedimiento a las 36, 48, 72 y 96 h de la fermentación, realizando toma de muestra cada 12 horas dentro de las primeras 24 h, y después cada 24 h hasta terminar a las 96 h de la fermentación.

Análisis de cromatografía de gases acoplada a espectrometría de masas

Los extractos obtenidos con acetato de etilo fueron inyectados en a un equipo CG-MS CP3800 (Varian ®) acoplado a un espectrómetro de masas Saturn 200 (Varian ®). Las inyecciones se hicieron en una columna de mediana polaridad Varian VA 5-MS (30 m x 0.25 mm x 0.25 micron/ -60 a 325/350 °C). Las condiciones del análisis fueron las siguientes: temperatura de inyector 250 °C, temperatura de detector 240 °C, temperatura de horno: 60 °C mantenido durante 5 minutos, una rampa de calentamiento hasta 150 °C a una velocidad de 2 °C/minuto, una segunda rampa de calentamiento hasta 280 °C a una velocidad de 6 °C/ minuto. Gas acarreador Helio, flujo de 1 mL/min.

RESULTADOS Y DISCUSIÓN

Evaluación de las condiciones de cultivo para biotransformar limoneno a través de sistemas fúngicos

En la figura 1 se muestran los diferentes crecimientos alcanzados por cada una de las cepas evaluadas en los cuatro medios de cultivo mineral, todos los medios de cultivo fueron adicionados con limoneno para lograr biotransformar este compuesto. Puede notarse que las cepas más sobresalientes en cuanto a crecimiento, y por lo tanto a tolerancia a la presencia del monoterpeno, fueron las cepas de *A. niger* ESH y *A. niger* Aa20 con crecimientos de 2.10 ±0.785 g/L y 1.993 ±0.154 g/L, respectivamente. La cepa con menor tolerancia a la presencia del limoneno fue *A. ustus* PSS con crecimientos que iban de 0.239 ±0.113 g/L a 0.735 g/L ±0.333 g/L en los cuatro medios de cultivo evaluados.

Respecto a la comparación entre los medios de cultivo, en la figura 1 se muestra que el medio de cultivo que más favoreció el crecimiento de las cepas fúngicas fue el Medio MI, el cual permitió el mejor desarrollo de *A. niger* ESH, Aa20 con crecimientos de 2.1 ±0.785 g/L, y 1.99 ±0.154 g/L respectivamente. Las cepas que presentaron los mejores desarrollos fueron *A. niger* ESH y Aa20 empleando el Medio MI. Chávez-González (2015) reporta crecimientos superiores de *A. niger* TAAN15, *A. niger* TAAN1, *A. niger* Aa20, *A. niger* ESH con 6.82, 6.46, 6.33 y 6.28 g/L respectivamente, empleando concentraciones de limoneno al 2 % y un medio de cultivo compuesto por: sacarosa, $NaNO_3$, K_2HPO_4, KCl, $MgSO_4.7H_2O$ y extracto de levadura. Maróstica Jr. y Pastore (2007) obtuvieron mayores crecimientos de *Fusarium oxysporum*, utilizando 0.1 % de aceite esencial de naranja en presencia de decano obteniendo 27.0 g/L de biomasa.

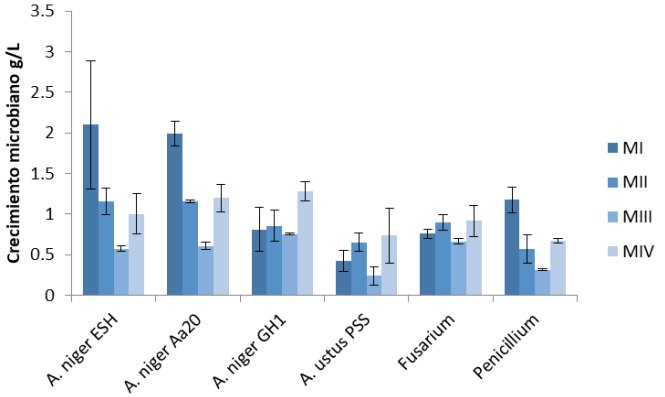

Figura 1. Crecimiento microbiano de las seis cepas fúngicas en los cuatro medios de cultivo adicionados con limoneno.

Los resultados de crecimiento microbiano en presencia de limoneno concuerdan con los resultados obtenidos de pH final de la fermentación (Figura 2), ya que las cepas que mostraron los cambios más drásticos en cuanto a pH son aquellas que presentaron la mayor producción de biomasa fúngica; el pH inicial para todas las cepas fue de 7 en todos los medios de cultivo. Se muestra una línea punteada indicando el pH inicial de 7, además se muestra como *A. niger* ESH, Aa20 y GH1 son las cepas que bajaron su pH de 7 a 3 aproximadamente en todos los medios de cultivo, indicando que existe una actividad metabólica importante lo que puede ser un indicativo de adaptaciones por parte de la cepa al ambiente extremo al cual fueron sometidos. Para el resto de las cepas (*A. ustus* PSS, *Fusarium* y *Penicillium*) no hubo una modificación importante de pH.

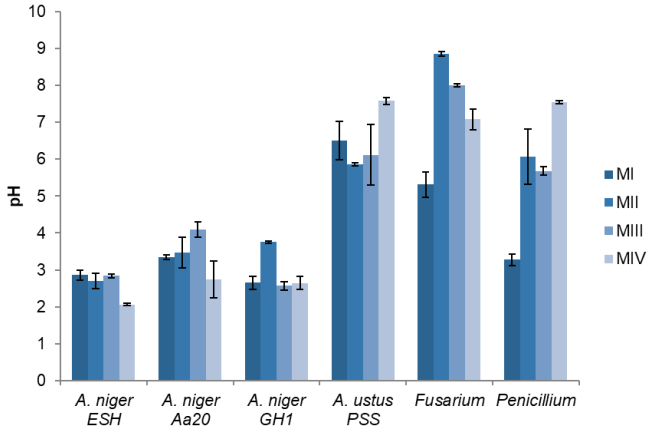

Figura 2. Cambios de pH presentados durante los procesos fermentativos de las cepas fúngicas estudiadas en los diferentes medios de cultivo.

Además, fue posible cuantificar los azúcares totales de los extractos de fermentación, demostrando que los microrganismos utilizan como primera fuente de carbono a los azúcares de los medios para posteriormente utilizar al limoneno como sustrato para lograr su crecimiento y la biotransformación del limoneno. En la figura 3 se observa que las cepas consumieron entre un 90 y 95% de azúcares fueron Aa20 y GH1 en los cuatro medios de fermentación utilizados. En la cepa Aa20 el medio que presentó mayor rendimiento fue el medio IV, el cual la fuente de carbono usada en este cultivo fue sacarosa. En el caso de la cepa GH1, el medio que presentó mejor rendimiento fue el MIII, utilizando como fuente de carbono la glucosa. ESH tuvo rendimientos similares en los medio MIII y MIV.

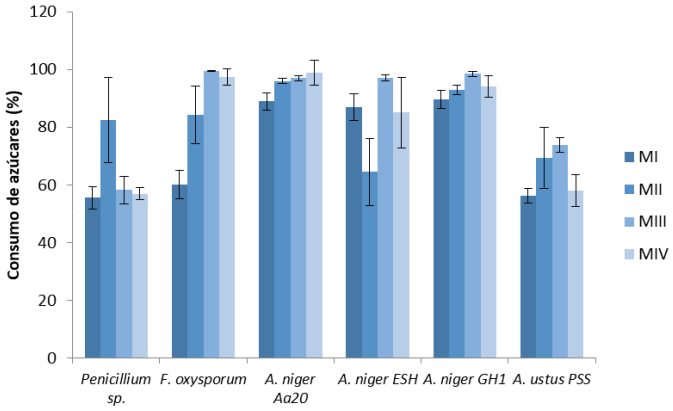

Figura 3. Porcentaje de consumo de sustrato presentado en los medios de cultivo a las 96 h de fermentación.

Evaluación factores abióticos sobre el crecimiento de cepas fúngicas en presencia de limoneno

Estrés con solventes orgánicos

Durante el tratamiento con solventes orgánicos se evaluó el crecimiento microbiano que tuvieron ambas cepas. La cepa Aa20, fue la que presentó el mayor crecimiento microbiano en el tratamiento con cloroformo alrededor de los 21 g/L de biomasa producida (Figura 4), el etanol favoreció el crecimiento de Aa20 (16.5 g/L), mientras que con el tratamiento de metanol fue el que menor crecimiento mostró con alrededor de 12 g/L. Para el caso de la cepa ESH el comportamiento del crecimiento en presencia de los diferentes solventes fue similar.

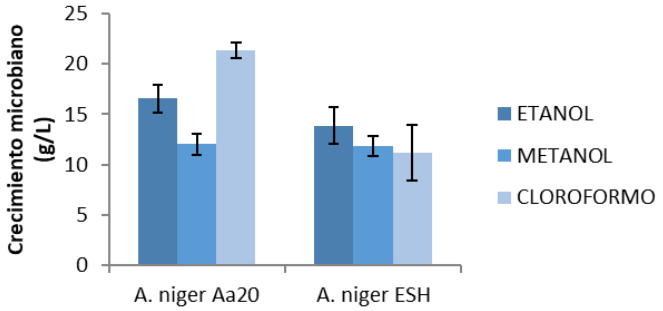

Figura 4. Crecimiento microbiano de las cepas *A. niger* Aa20 y *A. niger* ESH al final de las 96 h de fermentación en el tratamiento con los solventes orgánicos.

Trytek et al. en 2016 demostró que la actividad biocatalítica de los microorganismos en la biotransformación de un monoterpeno mejora a la exposición a estrés con solventes

orgánicos. Otro hallazgo fue que dicho efecto dependía particularmente del disolvente utilizado y el momento de su contacto con el biocatalizador. Fue reportado que la exposición de una cantidad mínima de micelio a solventes orgánicos lograba una disminución en la concentración total de productos de biotransformación hallados en sus estudios. Por lo tanto, la presencia de solventes orgánicos en el medio de crecimiento de los microorganismos ejerce una influencia directa sobre las estructuras celulares, aumentando la permeabilidad y la fluidez de las membranas comparado a un medio sin la adición de solventes orgánicos, por lo tanto, obteniendo resultados mayores en cuanto a crecimiento y productos de biotransformación.

Estrés térmico

El efecto del tratamiento térmico sobre el crecimiento microbiano en ambas cepas fue diferente en los tres tratamientos, siendo la temperatura 20 °C la que permitió un mayor crecimiento microbiano, con un valor de 31.06 g/L en *A. niger* Aa20 y 30.13 g/L para *A. niger* ESH (Figura 5). En tanto la temperatura de 30 °C fue la que se obtuvo el menor crecimiento de las 3 temperaturas con crecimientos de 18.4 y de 17.7 g/L para *A. niger* Aa20 y *A. niger* ESH respectivamente.

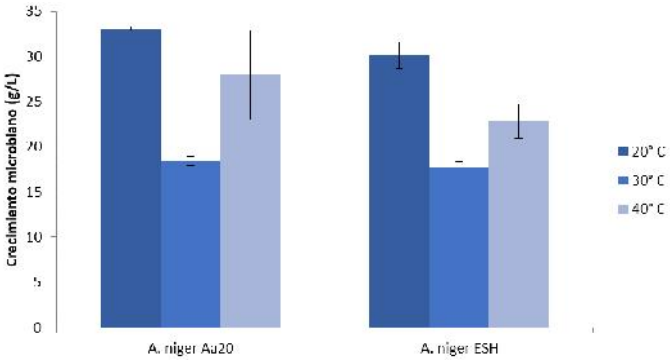

Figura 5. Crecimiento microbiano de las cepas *A. niger* Aa20 y *A. niger* ESH al final de las 96 h de fermentación en el tratamiento térmico.

Estrés ácido/básico

Durante el tratamiento ácido/básico en ambas cepas se observó que al ajustar el pH a 2 era posible alcanzar un mayor crecimiento de los hongos, comparado con los otros dos pH evaluados (Figura 6). Para el caso de Aa20, el mayor crecimiento fue de 31 g/L, seguido de 22.5 g/L para el pH 6 y por último con crecimientos de hasta 18.8 g/L para el pH 8. El efecto de pH sobre el crecimiento de *A. niger* ESH fue diferente ya que no hubo una diferencia significativa entre el pH 2 y el 6 en donde se tuvieron los mayores crecimientos con 29.7 y 31.1 g/L respectivamente, el tratamiento a pH 8 fue el que tuvo los menor crecimientos con 20.91 g/L.

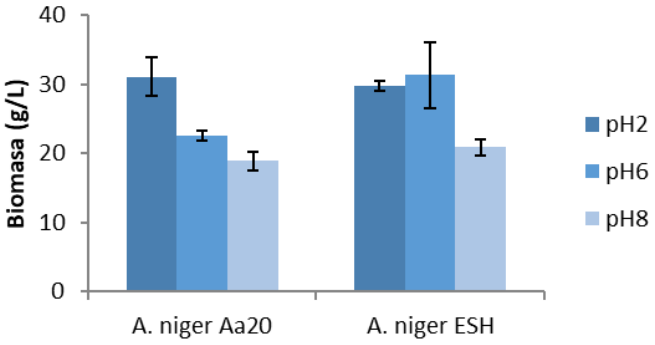

Figura 6. Crecimiento microbiano de las cepas *A. niger* Aa20 y *A. niger* ESH al final de las 96 h de fermentación en el tratamiento ácido-básico.

Trytek et al. 2016 logró obtener productos de biotransformación, en medios con pH ácido (pH 2) y medios con pH alcalino (pH 10). Teniendo como control un medio a pH óptimo de 5.6, y comparando con su control que obtuvo más del 50% de incremento en sus productos en ambos pH. En tanto al presente trabajo, no se observó en alguno de los tres pH (2, 6, 8) ajustados a los medios, productos de biotransformación del limoneno.

Molina et al. (2015) evaluaron la bioconversión de limoneno a α-terpineol a través del hongo *Fusarium oxysporum*, las condiciones del proceso fueron a 30 °C y un pH de 6.5 alcanzando rendimientos de hasta 4 g/L de α-terpineol.

Productos de biotransformación caracterizados por cromatografía de gases acoplada a espectrometría de masas

En la Tabla 2 se muestran los productos de biotransformación identificados por CG-MS en cada uno de los extractos de fermentación recuperados. Puede observarse que, para ambas cepas, el pretratamiento con solventes orgánicos favoreció el proceso de biotransformación de limoneno. *A. niger* Aa20 y ESH mostraron diferentes y variados productos de biotransformación bajo el pretratamiento de los diferentes solventes.

En el caso particular de *A. niger* Aa20 la biotransformación se vio mayormente influenciada con el pretratamiento con cloroformo, en donde se lograron detectar e identificar 6 compuestos diferentes, todos ellos compuestos derivados del limoneno; cis-p-menta,-2,8-dien-1-ol; 1,8-mentadien-4-ol (Limonen-4-ol); 2-metil-5-(1-metiletil)-Ciclohexanona, trans (D-dihidrocarvona); Limonen-1,2-diol; trans-p-menta-2,8-dienol; cis-p-menta,2,8-dien-1-ol y el trans-carveol. Con el pretratamiento de metanol se identificaron 4 compuestos derivados de limoneno (2R,4R-)-p-menta-6,8-dieno, 2-hidroperóxido, ácido 4-pentenoico, 3-hidroxi-2,4-dimetil, éster metílico, (R, S), trans-p-Menta-2,8-dienol y 2-ciclohexen-1-ona, 2 metil-5-(1-metiletil) y por último con el etanol se lograron

identificar solo dos compuestos 1-metileno-3-(1-metiletil)-R ciclohexano y 2-metil-5-(1-metiletil) 2-ciclohexen-1-ona. Para el caso de *A. niger* ESH el solvente que más favoreció el proceso de biotransformación fue el etanol con 5 compuestos derivados de limoneno p-menta-e-2,8(9)-dien-1-ol; 1,8-mentadien-4-ol (Limonen-4-ol); (2R,4R)-p-menta-g, 8-dieno 2-hidroperóxido; el ácido 2-hidroxitetradecanóico; 2- ciclohexen-1-ona 2-metil-5-(1 metiletenil), seguido de metanol con dos (trans-p-menta-2,8-dienol y el p-menta-e-2,8(9)-dien-1-ol) y por último el cloroformo con tan solo un compuesto identificado, el 2-pentanona, 3-cloro-4-4,4-dimetoxi.

Tabla 2. Compuestos químicos encontrados en cada uno de los tratamientos de estrés con solventes mediante CG-MS.

Cepas	Etanol	Metanol	Cloroformo
A. niger Aa20	-1-metileno-3-(1-metiletil)-R Ciclohexano 2-metil-5-(1-metiletil) 2-ciclohexen-1-ona	(2R,4R-)-p-menta-6,8-dieno, 2-hidroperóxido Ácido 4-pentenoico, 3-hidroxi-2,4-dimetil, éster metílico, (R, S) trans-*p*-Mentha-2,8-dienol 2-ciclohexen-1-ona, 2 metil-5-(1-metiletil)	Limoneno cis-*p*-mentha,-2,8-dien-1-ol Limonen-4-ol D-dihidrocarvona 2-ciclohexen-1-ona, 2-metil-5-(1-metiletil) Limonen-1,2-diol trans-*p*-mentha-2,8-dienol cis-*p*-mentha,2,8-dien-1-ol trans-carveol
A. niger ESH	*p*-mentha-e-2,8(9)-dien-1-ol 1,8-menthadien-4-ol (Limonen-4-ol) (2R,4R)-*p*-mentha-g, 8-dieno, 2-hydroperóxido Ácido tetradecanoico, 2-hidroxi 2-ciclohexen-1-ona, 2-metil-5-(1 metiletil)	trans-*p*-mentha-2,8-dienol *p*-mentha-e-2,8(9)-dien-1-ol	2-pentanona, 3-cloro-4,4-dimetoxi

Los compuestos derivados de limoneno encontrados durante los procesos fermentativos para ambas cepas han sido reportados como componentes de aceites esenciales tal como el reporte hecho por Zhu et al en 2018 en donde encuentra compuestos como el 2-ciclohexen-1-ona, 2-metil-5-(1-metiletenil), cis-carveol, trans-carveol, carvona, terpineol, mirceno y otros monoterpenos en aceite esencial de lavanda. El aceite esencial de lavanda como el aceite esencial de los cítricos tienen propiedades similares farmacéuticas, cosméticas y antimicrobianas. Trans-p-menta-2,8-dienol, p-menta-e-2,8(9)-dien-1-ol, cis-p-menta,2,8-dien-1-ol conocidos como cis y trans-mentadienol, son compuestos principales del aceite esencial de plantas originarias del Sudán, *Cymbopogon nervatus*. Estos compuestos han demostrado que presentan actividad antimicrobiana, antioxidantes y tiene efectos antiespasmódicos (Omar et al. 2016). Jakab et al. en 2018 reporta que, a partir de una descomposición termo-oxidativa de los aceites esenciales de la lima, bergamota y de cardamomo, la extracción de compuestos, entre ellos se encuentran los terpenos como limoneno, limonen-1,2-diol, cis-carveol, trans-carveol, terpineol, p-cimeno, pineno, mirceno, geraniol, y en el aceite esencial de la lima se halló 1,8-mentadien-4-ol comúnmente llamado limonen-4-ol. Trans-ciclohexanona, 2-metil-5-(1-metiletenil) o D-dihidrocarvona, es uno de los compuestos encontrados en el aceite esencial del eneldo, planta de la región oriental del Mar Mediterráneo. Las actividades biológicas de estos compuestos son antimicrobianas, antioxidantes, también como tratamientos contra Leishmaniasis y malaria (Sintima et al. 2015). Reportes que ponen de manifiesto el potencial de aplicación de todos los compuestos obtenidos a través de biotransformación microbiana.

Cinética de biotransformación de limoneno con *A. niger* Aa20 y *A. niger* ESH

El crecimiento fúngico que tuvo la cepa Aa20 se mostró de forma acelerada durante las primeras 24 h logrando un crecimiento de 13.48 g/L, a las 24 h de cultivo un pulso de limoneno fue adicionado, en la curva de crecimiento puede notarse como entre las 24 y 36 h se hace notar una fase lag en la que presumiblemente *A. niger* Aa20 se adapta a la adición del limoneno. Posteriormente existe un incremento en el crecimiento, mostrando el máximo de crecimiento a las 48 h de cultivo con una producción de biomasa de 16.04 g/L para luego permanecer constante hasta el final de la fermentación a las 96 h (Figura 7). Es bien sabido el carácter antimicrobiano del limoneno, el uso de este en el medio después de 24 h de incubación, en cantidades pequeñas no ocasionó la muerte de la

cepa, lo que demuestra que la cepa posee características para crecer en presencia de este potente antimicrobiano y la curva de crecimiento muestra que *A. niger* Aa20 logró utilizarlo como fuente de carbono, el empleo de este sustrato como fuente de carbono nos indica que este hongo es capaz también de biotransformar el sustrato (limoneno) para seguir llevando a cabo sus reacciones metabólicas.

En la cinética de biotransformación de *A. niger* ESH observamos un crecimiento durante las primeras 24 horas de cultivo alcanzando una producción de biomasa de 13.29 g/L (Figura 7). El crecimiento de ESH disminuyó a las 48 h, posteriormente al igual que *A. niger* Aa20, ESH mostró un comportamiento similar al tener un incremento en su crecimiento entre las 48 y las 72 h lo cuál puede ser un indicativo de que ESH emplea el limoneno adicionado como fuente carbono.

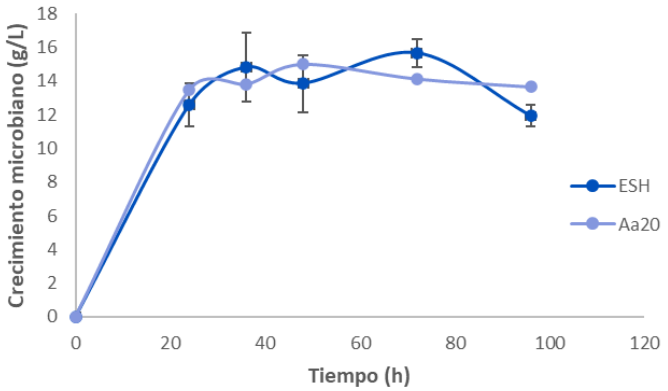

Figura 7. Cinética de crecimiento microbiano de la cepa *A. niger* Aa20 después del tratamiento con etanol y con la adición de limoneno.

En la tabla 6 se muestran los productos de biotransformación durante la cinética realizada durante las 96 h de fermentación con ambas cepas fúngicas.

A las 36 horas *A. niger* Aa20 generó 4 productos de biotransformación y ESH también 4 productos. El único compuesto que coincidió en producción para ambas cepas fue el 2-cyclohexen-1-ol, 2-metil-5-(1-metilletil)- trans (trans-carveol). Mientras que Aa20 produjo carvacrol ESH produjo timol. A las 48 horas la presencia de carvacrol continuó en la cinética de Aa20; para el caso ESH no se pudo determinar la composición del extracto. A las 72 horas de la adición del pulso de limoneno, se registraron 5 compuestos diferentes para Aa20 y 4 para ESH; ambas cepas fúngicas mostraron la presencia del compuesto 2-cyclohexen-1-ona, 2-metil-5-(1-metiletil), timol y de isómeros de p-menta-E-2,8(9)-dien-1-ol (trans-mentadienol). A las 96 horas tanto Aa20 como ESH mostraron 3 productos, coincidiendo en la producción de isómeros de 2-cyclohexen-1-ol, trans-carveol.

Tabla 3. Compuestos encontrados durante la cinética de biotransformación de limoneno por *A. niger* Aa20 y A. *niger* ESH mediante CG-MS.

Tiempo (h)	*A. niger* Aa20	*A. niger* ESH
36	2-pentanona, 3-cloro 4,4-dimetoxi ciclohexano, 2-metileno-5-(1-metiletil)-, (1S-Trans) acetic acid ethyl ester	4,6,6,-trimetillbiciclo [3.1.1]hept-3-en-2-ol *cis*-carveol *trans*-carveol Timol
48	trans-carveol Carvacrol Carvacrol	No determinado *cis*-mentadienol acetato de hidroxi-alfa-terpenilo
72	*trans*-mentadienol *cis*-carveo D-carvona Timol Limonen-1,2-diol	2-ciclohexen-1-ona, 2-metil-5-(1-metiletil)-(s) Timol
96	1,3,8-*para*-mentatrieno 2-metilprop-1enil)-ciclohexa-1,5 dieno *trans*-carveol	acetato de crisantenilo *cis*-mentadienol D-carvona

Carvacrol y timol son monoterpenos fenólicos que se encuentran en el aceite esencial del orégano y el tomillo. Poseen actividades farmacológicas, con efectos anticancerígenos, antiinflamatorios, antioxidantes, también con actividades antimicrobianas, antibacteriales, antifúngicos e insecticidas (Brotzman et al. 2018). Moussa y Almaghrabi en 2016 obtuvieron del aceite esencial de la planta *Peganum harmala* diferentes compuestos, específicamente ácidos grasos, y entre un grupo identificado como ácidos no-grasos, el compuesto eicosano, que, con los demás compuestos estudiados, poseen propiedades bioactivas, empleadas en la fisioterapia y cosmética. El-Zaeddi et al. (2016) utilizaron la técnica de hidrodestilación para la extracción del aceite esencial del perejil, teniendo como resultados la identificación de compuestos por medio de CG-MS y CG-FID, b-felandreno, miristicin, mirceno, terpinoleno, limoneno, α-pineno, α-felandreno y 1,3,8-p-mentatrieno. Crisantenil acetato es uno de los compuestos principales en el aceite esencial de la flor *Tanacetum vulgare*, los cuales presentan actividad antibacteriana (Móricz et al. 2015).

Los compuestos como cis y trans-mentadienol, carveol, limonen-1,2-diol fueron los productos de biotransformación con mayor importancia. Fueron identificados durante la

biotransformación de la etapa número dos. Siendo derivados de gran valor agregado, ya que han sido reportados por sus beneficios y actividades biológicas, empleados en la industria farmacéutica, cosmética y alimenticia. 4,6,6,-trimetilbiciclo [3.1.1]hept-3-en-2-ol; 2-pentanona, 3-cloro 4,4-dimetoxy; ciclohexano, 2-metilen-5-(1-metiletenil)-,(1S-Trans); hidroxi-a-terpenil acetato; 2-ciclohexen-1-ona, 2-metil-5-(1-metiletenil); (2-metilprop-1enil)-ciclohexa-1,5 dieno) son compuestos aun no reportados y con propósito de ser estudiados posteriormente.

CONCLUSIONES

En el presente proyecto se logró encontrar las condiciones de cultivo idóneas para llevar a cabo la biotransformación de limoneno. De las cepas evaluadas destacaron *A. niger* Aa20 y *A. niger* ESH por tener los mayores crecimientos en presencia de este monoterpeno, así mismo el medio de cultivo mineral MI fue el que permitió un mejor desarrollo para dichas cepas. Respecto de los tres diferentes factores abióticos evaluados (temperatura, pH y solventes) sobre cada una de las cepas solo el tratamiento con solventes mostró un efecto sobre los procesos de biotransformación limoneno. Por último, de las cinéticas de biotransformación llevadas a cabo se pudieron identificar diversos compuestos que han sido reportados por poseer interés de aplicación en diversas áreas industriales debido a las actividades biológicas tales como antimicrobianas, fungicidas, insecticidas, efectos farmacológicos así también como anticancerígenos, y propiedades organolépticas deseables para la producción de diversos alimentos. Lo que convierte a cada uno de estos derivados de limoneno en compuestos de gran valor agregado para la industria alimenticia, agrícola, cosmética y farmacológica.

Los resultados encontrados en esta investigación constituyen una fuente de información relevante para el desarrollo de bioprocesos encaminados a la producción de metabolitos con valor considerable.

AGRADECIMIENTOS

Este proyecto contó con el apoyo financiero del Programa para el Desarrollo Profesional Docente en la modalidad de Incorporación como Nuevo Profesor de Tiempo Completo (UACOAH-PTC-421).

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JBCT Journal of BioProcess and Chemical Technology
ISSN: 2683-3271

2022 Volumen 14, No. 28
Revista Científica de la Universidad Autónoma de Coahuila

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.

Esmeralda-Guzmán, Maricela[1], Buenrostro-Figueroa, Juan J[2]., Aguilar, Cristóbal N[1]., Ascacio-Valdés, Juan A[1]., Sepúlveda, Leonardo[1]*

[1]*Bioprocess and Bioproducts Research Group, Food Research Department, Facultad de Ciencias Quimicas, Universidad Autonoma de Coahuila, 25280, Saltillo, México.*

[2]*Research Center in Food and Development A.C., 33089, Cd. Delicias, Chihuahua, México*

**Corresponding author: leonardo_sepulveda@uadec.edu.mx*

Recibido: 16 de Mayo 2022
Aceptado: 27 de Mayo 2022

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.

ISSN: 2683-3271

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

ISSN: 2683-3271

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.

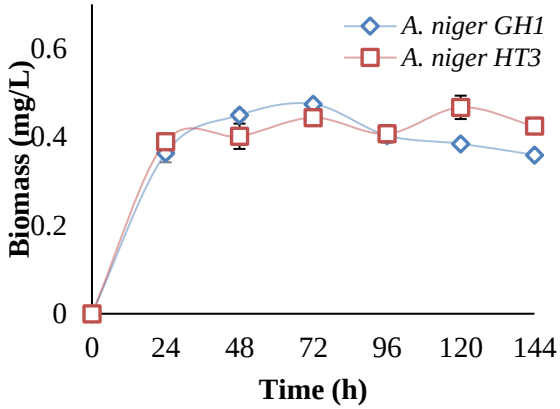


Figure 1. Biomass production in submerged fermentation.

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.

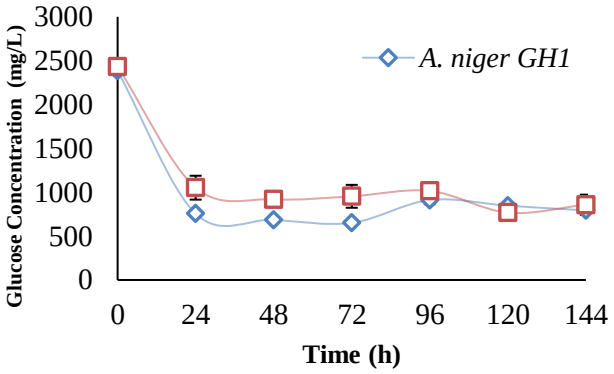


Figure 2. Decrease of total sugars in submerged fermentation.

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,

ISSN: 2683-3271

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.

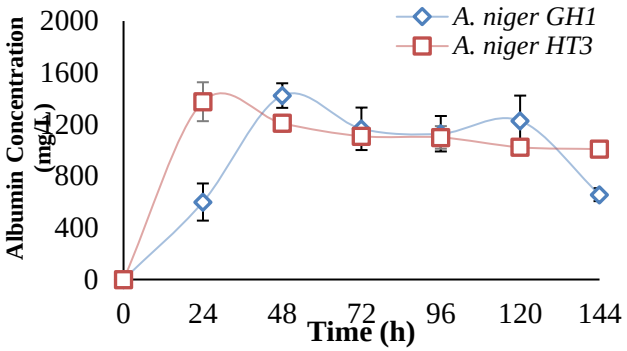


Figure 3. Determination of soluble proteins in submerged fermentation.

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).

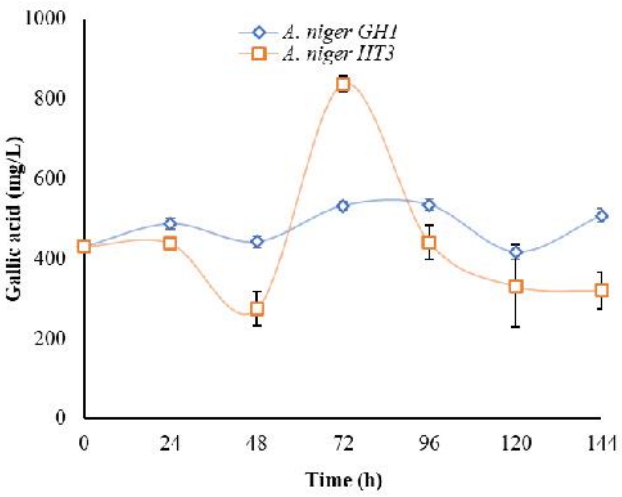
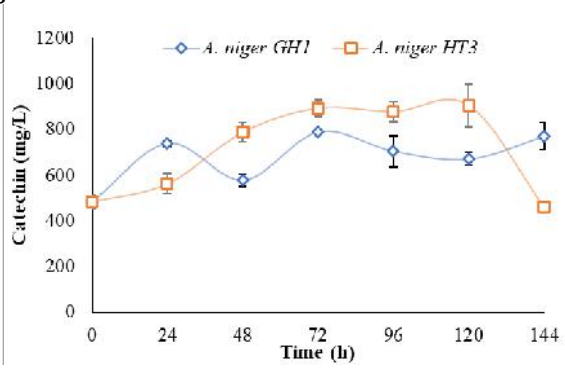


Figure 4. Accumulation of hydrolyzable tannins in submerged fermentation.

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

ISSN: 2683-3271

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.

ACKNOWLEDGEMENT

The authors thank the Universidad Autonoma de Coahuila for the facilities provided in this research. The student Esmeralda-Guzmán thanks CONACYT for the master's scholarship awarded.

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