PRODUCCIÓN DE POLIFENOLES TOTALES POR FERMENTACIÓN LÍQUIDA DE *A. NIGER* UTILIZANDO COMO SUSTRATO RESIDUOS AGROINDUSTRIALES DE AGUACATE

PRODUCTION OF TOTAL POLYPHENOLS BY A. NIGER LIQUID FERMENTATION USING AGRO-INDUSTRIAL AVOCADO WASTE AS SUBSTRATE

VICTORIA CERVANTES RIVERA[1]

ROBERTO ARREDONDO VALDÉS[1]

MAYELA GOVEA SALAS[1]

ERIKA Y. ACOSTA CRUZ[1]

JULIA ANGUIANO CABELLO[2]

PATRICIA SEGURA CENICEROS[1]

ERIKA Y. ACOSTA CRUZ[1]

1 Grupo de Nanobiociencia, Facultad de Ciencias Químicas, Unidad Sureste, UAdeC.

Universidad La Salle, Saltillo, Coah.

Correspondencia
psegura@uadec. edu.mx
ORCID 000-0003-2744-4640

RESUMEN

Día a día se buscan alternativas para el tratamiento de las afecciones médicas, dentro de las bases que se buscan enfatizar en la producción de biocompuestos para su aplicación como tal alternativa, esto, el aprovechamiento de residuos agroindustriales para la reducción del impacto ambiental, pero también para la generación de subproductos dando valor agregado a dichos residuos; a su vez, la producción de biocomposites se apoya en la aplicación de microorganismos, así en el presente estudio se utilizó *A. niger*, debido a la rapidez y facilidad con la que se desarrolla, estableciendo una cinética de fermentación líquida utilizando como sustrato residuos agroindustriales de aguacate (cáscara, semilla, mezcla 1/1), de esta manera se analizó la capacidad de producción de compuestos bioactivos a partir del metabolismo de *A. niger* mediante la toma de muestras cada dos horas, obteniendo como resultado una alta producción de polifenoles, mayoritariamente en la variante peeling.

Palabras clave: microorganismos, polifenoles, nutrientes, hongos

ABSTRACT

Day by day, alternatives are sought for the treatment of medical conditions, within the bases that are sought to emphasize on the production of biocomposites for their application as such an alternative, this, the use of agro-industrial waste for the reduction of environmental *impact, but also for the generation of by-products giving added value to said waste; in turn, the production of biocomposites is supported by the application of microorganisms, thus in the present study A. niger was used, due to the speed and ease with which it develops, setting up a liquid fermentation kinetics using agro-industrial avocado waste as a substrate (peel, seed, 1/1 mixture), in this way the capacity for the production of bioactive compounds from the metabolism of A. niger was analyzed by taking samples every two hours, obtaining as a result a high production of polyphenols, mostly in the peel variant.*

***Keywords**: microorganisms; polyphenols; nutrients; fungus.*

INTRODUCCIÓN

Fermentation is a catabolic process that consists of a biological conversion, where complex substrates are used to transform them into simple compounds through various processes using different microorganisms, such as bacteria and fungi, as transformers (Caballero-Sanchez y col., 2023). This process can be carried out in three ways. Depending on the substrate and the product to be made, fermentation can be in a solid, liquid, or submerged liquid state. This process has been used for hundreds of years to make products beneficial to humanity (Ortega y col., 2016), these products range from foods such as bread, cheese, preserves, etc. (Rojas-García y col., 2022). Even many advances in medicine and the preparation of drugs for treating different diseases are thanks to fermentation with microorganisms (Kuang-Lung y col., 2022). These techniques have been perfected based on the control of important parameters for the standardization of fermentation, which can be light, temperature, humidity, time, substrate, etc. (Sergi, 2020). Two main compounds are produced largely apart from those expected based on fermentation. These are carbon dioxide and alcohol (Valencia-Hernández y col., 2021). For decades, fungi have gained relevance in science due to the wide variety of bioactive compounds that can be obtained through liquid/submerged fermentation (Montijo-Prieto y col., 2023). All compounds obtained through different processes are called "bioactive compounds, " from plants, microorganisms, or higher organisms to all compounds with biological activity (Ortega y col., 2016). Recently, it has been shown that several of these compounds may originate in the secondary metabolism of said microorganisms used in fermentation (Tamang y col., 2016). These secondary metabolites are important from an industrial and economic point of view, to improve the supply of the world population (Sergi, 2020). Currently, in most of these fermentations, different sources of nutrients are used so that microorganisms can take advantage of them; sometimes, these sources come from the application of agro-industrial waste for their use (Romero-Márquez y col., 2023). Using these residues contributes to caring for the environment as it reduces pollution. Most agro-industrial waste is underutilized, as it is reported to contain a large amount of nutrients, for example, citrus peels discarded from the juice industry, from which essences, colorants, etc. can be obtained. Likewise, another variety of waste is used to obtain polyphenols, which have biofunctional activity to treat various medical pathologies (SEGOB, 2013).

The present study was carried out to standardize and characterize the production of polyphenols from fermentation using *A. niger* enriched with avocado waste.

Materiales y Métodos

Obtaining and processing of agro-industrial waste

The agro-industrial avocado waste (seed and peel) was collected from the "Las Brazas" restaurant (Saltillo, Coahuila, Mexico). Subsequently, the samples were scraped with fiber to eliminate excess pulp and washed under running water to eliminate impurities. Once dried at room temperature, everything was cut into cubes and stored in a Ziploc bag in a domestic freezer at -15°C. The samples were freeze-dried for 48 h in equipment (FreeZone 4.5 LABCONCO) (Allauca, 2019). The waste was finally ground and sieved to a particle size of 0.3 microns, being stored in powder form, each separated in airtight and dark containers.

Strain reactivation

Before setting up the fermentations, the *A. niger* strain was reseeded on Petri dishes with PDA medium, sown in a streak manner. Next, a count of spores/ml was carried out in a Neubauer chamber to determine the concentration of the inoculum, being a concentration of 1×10^6 spores/ml of *A. niger*.

KINETICS OF EXPLORATORY FERMENTATION ENRICHED WITH AGRO-INDUSTRIAL AVOCADO WASTE.

The kinetics were assembled based on what was found in the literature (Cañadas y col., 2022). The fermentation was carried out in 250 ml Elenmeyer flasks, placing the three variants of concentration of substrate added in grams (seed, shell and mixture 1/ 1), which were 1.5 g, 2.5 g, 5 g plus PDA broth completing a total volume of 50ml plus the fungal inoculum (Garza, 2004), these were inoculated with 100 µl of *A. niger* at a concentration of 1×10^{10} spores/ ml, as a control only PDA broth and the inoculum were placed, this was carried out in triplicate for each of the substrates (seed, shell and 1/1 mixture). The kinetics were mounted on a shaker (Excella E24 – Labomersa) and taking as parameters for the kinetics a temperature of 36 °C, at 120 rpm, with a total duration of eight days, taking a sample to count total polyphenols (mg/ml) every 24 hours, and in turn the pH was monitored with a potentiometer (Hanna HI98103) (Sanchez-Perez y col., 2020).

POLYPHENOL DETERMINATION

To determine polyphenols (mg/ml), the Folin-Ciocalteu technique was carried out, first reading the substrates alone to have a comparison concerning the extracts obtained from the fermentation, then taking three readings per each of the triplicates of the fermentation flasks of each of the substrates, processing the data in Excel to determine which day the highest concentration of polyphenols was found (mg/ml).

DETERMINATION OF BIOMASS

At the end of the fermentation, biomass was determined by filtering the fermentations with a vacuum pump and medium-pore filter paper and then storing the remaining liquid for subsequent characterization tests. The filter paper with the biomass was placed in a brand oven (Kuang-Lung y col., 2020) at 65°C to be weighed until it reached a constant weight, thus determining the biomass obtained from each fermentation. This process lasted five days.

KINETICS OF FERMENTATION ENRICHED WITH AGRO-INDUSTRIAL WASTE LASTING 36 HOURS

For this fermentation, different factors were also monitored to determine the levels of total polyphenol production:

- Folin-Ciocalteu reading (Every 2 h)
- pH reading (Every 2 h)
- Biomass (Every 2 h)
- Reducing sugars (Every 2 h)

Table 1. Experimental design of kinetics of exploratory fermentation enriched with agro-industrial avocado waste.

Kinetics conditions

Substratum	Half	Fungus	g/ml	Equipment	Temperature	rpm	Sampling
Control	Broth PDA	*A. Niger* (10 µL) (1 × 10 ^{6} ufc/mL)	5 mL (without added substrate)	Shaker	36 °C	120	2 h
Seed Shell Mix 1/1			4.5 mL - 0.5 g				

The kinetics were assembled to determine when the highest production of polyphenols mg/ml (highest concentration of polyphenols) was found (mg/ml). The fermentation was carried out in 30 ml flasks using the sacrificial flask technique, this time placing only the concentration of added substrate, which was selected based on the results obtained, being 5g for each of the different substrates analyzed (seed, shell, and 1/1 mixture). This kinetics was carried out at a volume of 1 to 10. Therefore, there were 0.5 g of substrate plus 4.5 ml of PDA broth, completing a total volume of 5 ml plus the fungal inoculum. These were inoculated with 10 µl of *A. niger* at a concentration of 1 × 10 ^{6} spores/ml, as a control (Astudillo y col., 2023). Only PDA broth and the inoculum were placed, and the procedure was carried out in triplicate for each of the substrates (seed, shell, and 1/1 mixture). The kinetics were mounted on a brand shaker (Excella E24 – Labomersa) (Balakrishnan y col., 2021) at 36 °C and 120 rpm, with a total duration of 36 hours, taking a sample to count total polyphenols (mg /ml) every 2 h, and in turn the pH was monitored with a brand potentiometer (Hanna HI98103) (Sanchez-Perez y col., 2020).

Resultados y Discusión

Obtaining and processing of agro-industrial waste

After finishing processing the avocado waste, a rule of three is carried out to determine the yield percentage per kilo of the seed and avocado peel at the end of the freeze-drying period. The seed yielded 78% since the seed does not contain a high water content. On the other hand, the peel yielded 63%. These results show the impact of processing and making proper use of agro-industrial waste, showing that the volume of these decreases, thus reducing their impact on the environment.

Strain reactivation

After reactivating the *Aspergillus niger* strain, an identification was carried out to corroborate the species using dichotomous keys and using the lactophenol blue technique to observe the samples under a microscope, thus determining that it is the *A. niger* fungus species.

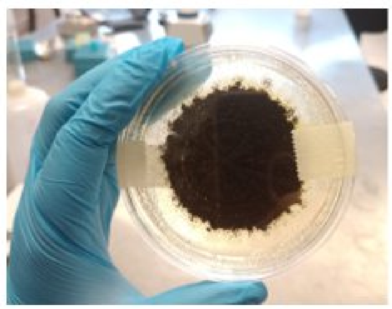


Figure 1. *Aspergillus niger*

Kinetics of exploratory fermentation enriched with agro-industrial avocado waste

The graphs with the data obtained during the fermentations carried out in this study are shown in Figures 2- 8. First, the results of the exploratory kinetics are shown, followed by the kinetics results, where a selection of the concentration of avocado substrate added to these fermentations is carried out.

Figure 2 shows the total polyphenols found in the agro-industrial residues of avocado. It is carried out to determine a comparative parameter between the polyphenols of the avocado itself and those produced thanks to fermentation. The levels were similar, although it is observed that the peel has a higher concentration of said polyphenols, being 2.5 mg/ml.

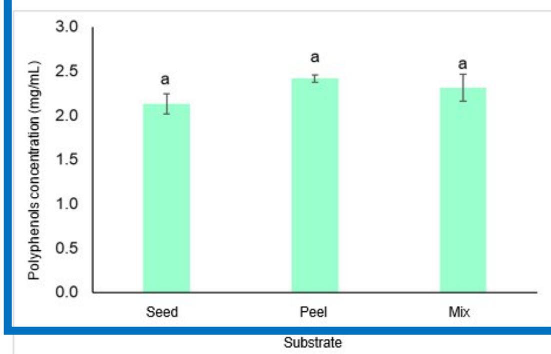
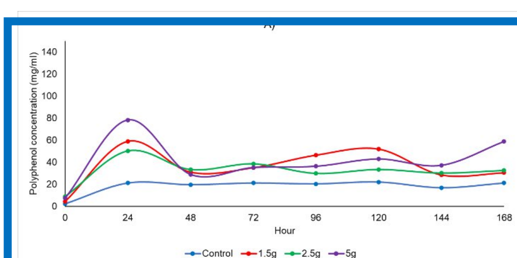


Figure 2. The initial concentration of total polyphenols (mg/mL) obtained using the Folin-Ciocalteu technique comparing the three substrate variants, seed, peel, and mixture (1/1).

TOTAL POLYPHENOL CONCENTRATION FROM FERMENTATIVE KINETICS LASTING 168 H.

A comparison was made between the production of polyphenols during the growth kinetics (Figure 3), comparing each substrate variant with its concentration variants in grams. It was observed that 24 hours is the highest point in the production of total polyphenols, with peel obtained levels above 125 mg/ml, followed by the seed and the mix with barely 80 mg/ml levels. In this way, it was determined that kinetics with a duration of 34 hours should be carried out to see better the consumption of nutrients, the production of polyphenols, and the decline of said production as time goes by.



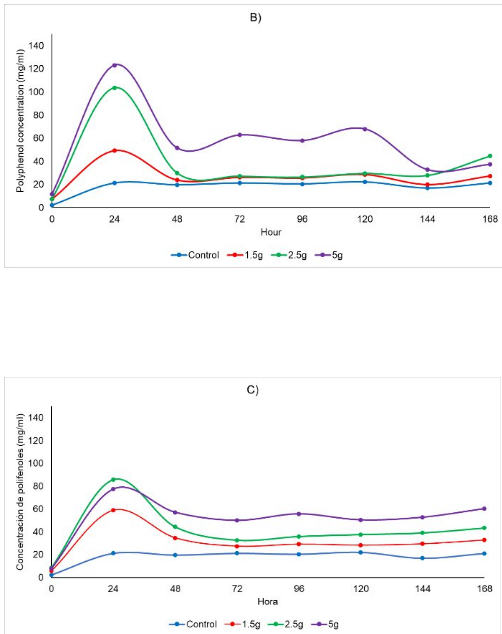


Figure 3. Results of the concentration of total polyphenols (mg/mL) for the 168 hours of *A. niger* fermentation: A) seed, B) peel, and C) mixture (1/1).

PH MONITORING OF FERMENTATION KINETICS

Figure 4 shows that the pH was maintained between six and eight during fermentation. It was monitored to see how the medium became acidic to evaluate if there was any contamination. It is known that bacteria growth affects the pH.

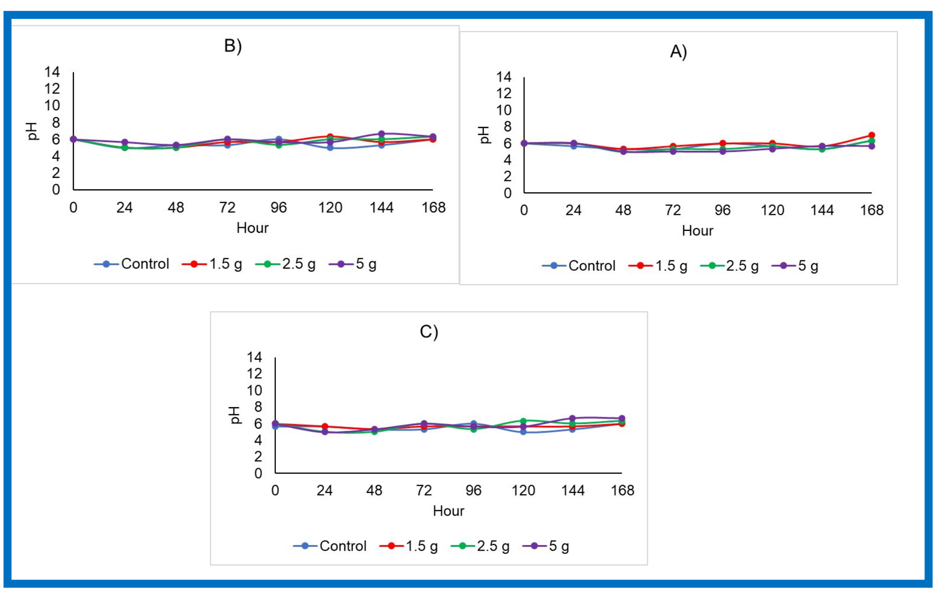


Figure 4. Comparative graphs of the pH of the *A. niger* liquid fermentation for 168

hours using avocado as a substrate, A) seed, B) peel, and C) mixure (1/1).

DETERMINATION OF BIOMASS OBTAINED FROM FERMENTATION DETERMINED BY CONSTANT WEIGHT

The total biomass produced during the kinetics was determined (Figure 5), and in this way, it was also determined which substrate variant generates more biomass of the fungus. The substrate variant with the highest biomass production was the peel, with more than 30 g/ml, followed by the seed, with almost equal production. In the end, the mixture only reached 20 g/ml.

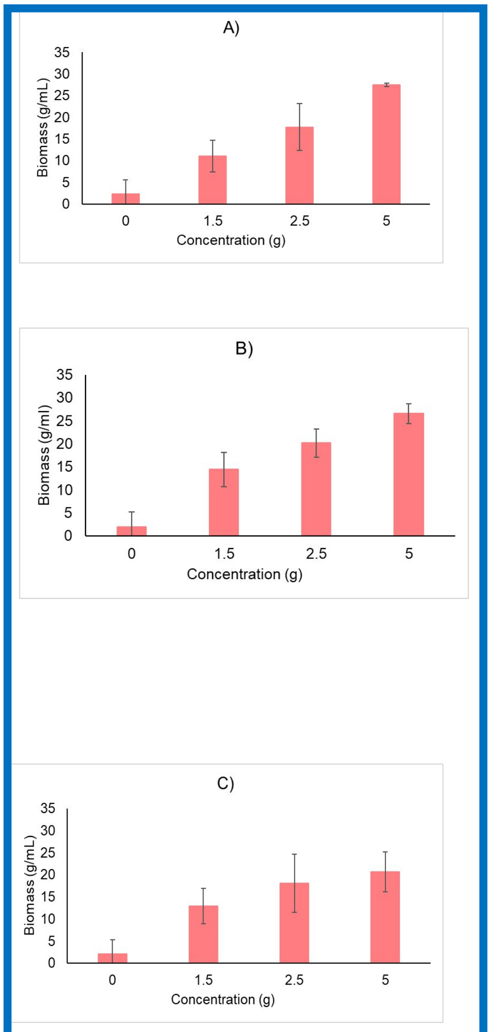


Figure 5. Biomass obtained from liquid fermentation lasting 168 hours of *A. niger* enriched with the substrates A) seed, B) peel, and C) mixture (1/1).

TOTAL POLYPHENOL CONCENTRATION FROM FERMENTATIVE KINETICS

LASTING 34 H

Figure 6 shows the kinetics of polyphenol during *A. niger* fermentation with avocado fractions. The levels detected in fermentations with avocado were higher than in the control assay.

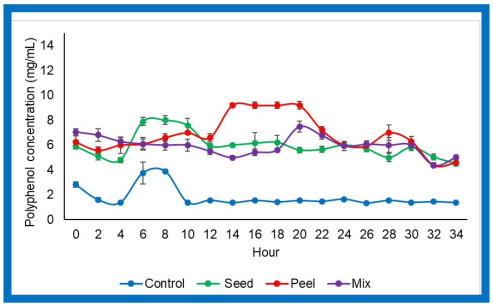


Figure 6. The concentration of total polyphenols (mg/mL) from the 34-hour fermentation of *A. niger* with seed substrate, avocado peel, and a mixture (1/1).

PH MONITORING DURING FERMENTATION KINETICS

For this kinetics, the pH was also monitored to rule out any contamination and, even more importantly, to determine the medium's vinegar point and the endpoint of the fermentation. In this fermentation, we observed how it became acidic since it dropped from an average of six to levels of 4 (Figure 7).

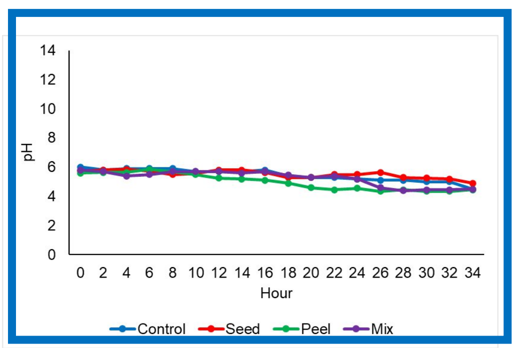


Figure 7. Graph of the pH of the liquid fermentation from the 34-hour fermentation of *A. niger* enriched with seed substrate, avocado peel, and a mixture (1/1).

DETERMINATION OF BIOMASS OBTAINED FROM FERMENTATION DETERMINED

BY CONSTANT WEIGHT

In the same way, as for the previous kinetics, biomass production was also evaluated (Figure 8). In this kinetics, values close to 4.6 g/ml were determined. The biomass produced in the peel presence and with the mixture were very similar throughout the experiment. The seed also generated values close to 4 g/ml, while the control values did not exceed 2 g/ml.

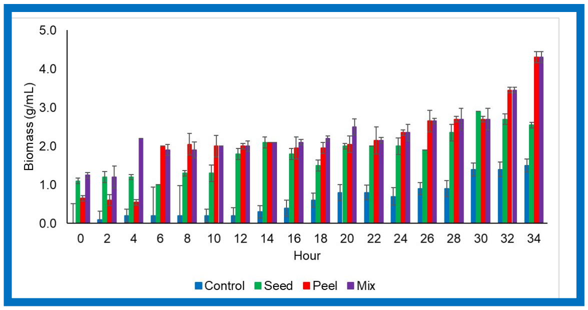


Figure 8. Biomass obtained from liquid fermentation lasting 34 hours of *A. niger* enriched with seed substrate, peel, and a mixture (1/1).

Fermentation in a liquid state has great potential for obtaining bioactive compounds in an easy and fast way (Wei y col., 2022); since microorganisms such as fungi naturally produce enzymes that degrade the cell wall, generating hydrolysis that facilitates the obtaining of bioactive compounds from the substrate that is added (Montijo y col., 2023), in this case, the agroindustrial waste of the avocado. As mentioned above, this biotechnological process is economical and easy to implement since it requires only a liquid medium and a shaker to control the environmental variants, requiring less capital and low operating costs (Mora y col., 2021). Using agroindustrial waste also provides several environmental advantages since it allows the use of solid waste destined to be garbage, which would generate a negative environmental impact, which is why taking advantage of it as a nutrient-enriching substrate reduces pollution. (Šelo y col., 2021). Monitoring the pH of fermentations contributes to the ease with which contamination or the end of available nutrients in the liquid medium can be determined since a very acidic pH indicates contamination with other microorganisms (Sanchez-Perez y col., 2020).

CONCLUSIONS

The kinetics of the *Aspergillus niger* growth allowed us to obtain graphs that demonstrate the performance and productivity of producing biomass and total phenols. The accelerated metabolism of *A. niger* leads to the high production of polyphenols thanks to the fermentation process. These polyphenols can be applied in subsequent works to treat various diseases related to cellular deterioration, such as cancer, due to the great antioxidant capacity of polyphenols.

REFERENCIAS

Astudillo, Á., Rubilar, O., Briceño, G., Díez, M.C., Schalchli, H. (2023). Avances en los residuos agroindustriales como sustrato para la obtención de productos microbianos ecoamigables. *Sostenibilidad*, *15*: 3467. https://doi.org/10.3390/su15043467

Caballero-Sanchez, L., Lázaro-Mixteco, P.E., Vargas-Tah, A. et al. (2023) Pilot-scale bioethanol production from the starch of avocado seeds using a combination of dilute acid-based hydrolysis and alcoholic fermentation by Saccharomyces cerevisiae. *Microb Cell Fact.*, *22*: 119. https://doi.org/10.1186/s12934-023-02110-5

Cañadas, A., Gualle, A., Vizuete, K., Debut, A., Rojas-Silva, P., Ponce, S., Orejuela-Escobar, L.M. (2022). Green Synthesis of Antibacterial Silver Nanocolloids with Agroindustrial Waste Extracts, Assisted by LED Light. Colloids I*nterfaces*, *6*: 74. https://doi.org/10.3390/colloids6040074

Cerda-Cejudo, N. D., Buenrostro-Figueroa, J. J., Sepúlveda, L., Torres-Leon, C., Chávez-González, M. L., Ascacio-Valdés, J. A., & Aguilar, C. N. (2022). Recovery of ellagic acid from mexican rambutan peel by solid-state fermentation-assisted extraction. *Food and Bioproducts Processing*, *134*: 86-94.

De Montijo-Prieto, S., Razola-Díaz, MdC., Barbieri, F., Tabanelli, G., Gardini, F., Jiménez-Valera, M., Ruiz-Bravo, A., Verardo, V., Gómez-Caravaca, A.M. (2023). Impact of Lactic Acid Bacteria Fermentation on Phenolic Compounds and Antioxidant Activity of Avocado Leaf Extracts. Antioxidants, *12*(2):298. https://doi.org/10.3390/antiox12020298

Kuang-Lung, H., Lin, T.-Y., Lee, M.-T., Hsiao, Y.-Y., Gu, Y. (2022). Diseño de

Experimentos para la Modelación de la Caracterización del Proceso de Fermentación en la Producción de Fármacos Biológicos. *Procesos*, *10*(2): 237. https://doi.org/10.3390/pr10020237

Mora-Sandí, A., Ramírez-González, A., Castillo-Henríquez, L., Lopretti-Correa, M., Vega-Baudrit, J.R. (2021). *Persea Americana* Biorrefinería de Residuos Agroindustriales para Productos Sostenibles de Alto Valor Agregado. Polímeros, *13*(11):1727. https://doi.org/10.3390/polym131117 27

Ortega, F.A., Pérez, O.A., & López, E.A. (2016). Modelo Semifísico de Base Fenomenológica del Proceso Continuo de Fermentación Alcohólica. *Información tecnológica*, *27*(1): 21-32. https://dx.doi.org/10.4067/S0718-07642016000100004

Rojas-García, A., Fuentes, E., Cádiz-Gurrea, M.d.l.L., Rodriguez, L., Villegas-Aguilar, M.d.C., Palomo, I., Arráez-Román, D., Segura-Carretero, A. (2022). Biological Evaluation of Avocado Residues as a Potential Source of Bioactive Compounds. *Antioxidants*, *11*: 1049. https://doi.org/10.3390/antiox1106 1049

Romero-Márquez, J.M., Navarro-Hortal, M.D., Orantes, F.J., Esteban-Muñoz, A., Pérez-Oleaga, C.M., Battino, M., Sánchez-González, C., Rivas-García, L., Giampieri, F., Quiles, J.L., et al. (2023) In Vivo Anti-Alzheimer and Antioxidant Properties of Avocado (Persea americana Mill.) Honey from Southern Spain. *Antioxidants*, *12*: 404. https://doi.org/10.3390/antiox12020404

Sanchez-Perez, J.A, Béjar Castillo, V., Villanueva Cotrina, F., & Guevara-Granados, J.M. (2020). Actividad antimicrobiana de metabolitos secundarios de Aspergillus fumigatus sensu stricto sobre cepas clínicas de Staphylococcus aureus y Streptococcus pneumoniae. *Anales de la Facultad de Medicina*, *81*(2): 180-185. https://dx.doi.org/10.15381/anales.v81i2.1617 0

SEGOB (2013) NORMA Oficial Mexicana NOM-161-SEMARNAT-2011, Que establece los criterios para clasificar a los Residuos de Manejo Especial y determiner cuáles están sujetos a Plan de Manejo; el listado de los mismos, el procedimiento para la inclusión o exclusión a dicho listado; así como los elementos y procedimientos para la formulación de los planes de manejo. DOF - Diario Oficial de la Federación

Šelo, G., Planinić, M., Tišma, M., Tomas, S., Koceva Komlenić, D., Bucić-Kojić, A. (2021). A Comprehensive Review on Valorization of Agro-Food Industrial Residues by Solid-State Fermentation. Foods, *10*(5):927. https://doi.org/10.3390/foods10050927

Sergi, M. (2020). El papel de las levaduras en los procesos de fermentación. *Microorganismos*, *8* (8): 1142. https://doi.org/10.3390/microorganisms8081142

Tamang, J.P., Watanabe, K., Holzapfel, W.H. (2016) Review: Diversity of Microorganisms in Global Fermented Foods and Beverages. *Front. Microbiol*., *7*:377. doi: 10.3389/fmicb.2016.00377

Valencia-Hernández, L.J., Wong-Paz, J.E., Ascacio-Valdés, J.A., Chávez-González, M.L., Contreras-Esquivel, J.C., Aguilar, C.N. (2021). Procianidinas: de los residuos agroindustriales a los alimentos como moléculas bioactivas. *Al imentos*, *10*: 3152. https://doi.org/10.3390/foods10123152

Wei, C., Luo, C.-Y., Yao, X.-Z., Jiao, Y.-J., Lu, L.-T. (2022). Optimization of the Theabrownins Process by Liquid Fermentation of *Aspergillus niger* and Their Antioxidant Activity. Applied Sciences. *12*(19):9720. https://doi.org/10.33 90/app12199720