

Upcycling agave and tortilla residues for the sustainable production of edible fungi and potential functional ingredients

Elisa Dufou-Hurtado^a , Marcela Gaytán-Martínez^b, Angel H. Cabrera-Ramírez^c, Emiro A. Leal-Urbina^a, Mario E. Rodríguez-García^d, Aurea K. Ramírez-Jiménez^{a,*}

^a Tecnológico de Monterrey, School of Engineering and Science, Av. Eugenio Garza Sada 2501 Sur, Monterrey, NL, C.P. 64849, Mexico

^b Facultad de Química, Programa de Posgrado en Alimentos del Centro de la República (PROPAC), Universidad Autónoma de Querétaro, Mexico

^c Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. Parque Científico Tecnológico de Yucatán, Km 5.5 Carretera Sierra Papacal-Chuburná, Chuburná, Mérida 97302, Yucatán, Mexico

^d Centro de Física Aplicada y Tecnología Avanzada, Departamento de Nanotecnología, Universidad Nacional Autónoma de México, Campus Juriquilla, Querétaro 76230, Mexico

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ABSTRACT

Agave bagasse, a lignocellulosic byproduct of mezcal production, remains underutilized due to its structural recalcitrance and environmental burden. This study explored the use of nejayote, an alkaline effluent from tortilla production, as a pretreatment to enhance delignification and improve the bagasse's suitability for solid-state fermentation (SSF) with edible fungi. Two nejayote samples from tortilla factories in Querétaro, Mexico, were physicochemically characterized, and a 3³ factorial design was employed to optimize pretreatment conditions. The nejayote maintained a high alkaline pH (~13), reduced furfural content by up to 98 %, and increased sugar availability. Pretreated bagasse was fermented with *Pleurotus ostreatus* and *Lentinula edodes*, enhancing its nutritional profile—soluble dietary fiber increased by 30–50 %, and antinutritional compounds like gallic acid and lignin were reduced by 96 % and 49 %, respectively. Scanning electron microscopy showed increased porosity and lignin degradation, particularly with *L. edodes*. Volatile compound profiles also improved, rising from 8 to 13 compounds post-fermentation. This bioprocess valorizes two agroindustrial residues—nejayote and agave bagasse—into a nutrient-rich substrate for edible mushroom cultivation. The fermented biomass offers dietary fiber and bioactives, contributing to circular economy models and sustainable protein and functional ingredient development in food systems.

1. Introduction

Lignocellulosic waste is the most abundant raw material globally, with an estimated annual production of 200 billion tons. However, only 8 billion tons are currently utilized, resulting in growing environmental concern due to accumulation and inefficient disposal practices (Inyang et al., 2022; Blasi et al., 2023). These residues, derived from inedible parts of plants, are widely produced in agricultural, forestry activities and industries, and represent a promising resource for valorization under circular economy principles (Cazier et al., 2024). One potential application is their use as substrate for edible mushroom through a solid-state fermentation (SSF), aligning with sustainable food systems and healthy diets (Zervakis and Koutrotsios, 2017; Letti et al., 2018).

Agave bagasse, is a lignocellulosic byproduct of mezcal production,

is abundant in Mexico with over 480,000 tons generated annually (Garzón et al., 2024; Álvarez-Chávez et al. 2024). This residue is composed of cellulose (41–44 %), hemicellulose (19–22 %), lignin (15–16 %), ash (4 %) and protein (3–4 %) (Álvarez-Chávez et al., 2025), it has a high carbon to nitrogen (C:N) ratio (50:1) and is structurally rich in carbohydrates but deficient in nutrients essential for fungal growth.

Pretreatments are essential for breaking down lignocellulosic complexity by disrupting lignin structure, reducing cellulose crystallinity, or increasing porosity. Methods include mechanical, chemical and biological strategies (Rajendran et al., 2018; Hernández-Beltrán et al., 2019). Among these, chemical alkali pretreatments are effective and scalable (Pellera and Gidarakos, 2018), and operating under mild conditions, such as low temperatures, atmospheric pressure, and the presence of oxygen, enhances the efficiency of the process while

* Corresponding author.

E-mail address: aramirezj@tec.mx (A.K. Ramírez-Jiménez).

minimizing the degradation of sugars into unwanted products such as furfural and hydroxymethylfurfural (HMF) (Chang and Holtzapple, 2000). NaOH and KOH are commonly used for alkaline pretreatment, but they pose environmental concerns due to high energy demand and generation of saline, caustic effluents (Jiang et al., 2020; Meléndez et al., 2021; Kang et al., 2018; Romero-Güiza et al., 2017; Jiang et al., 2016). In contrast, Ca(OH)₂ and CaO boost nutrient availability and fungal biomass production (Desisa et al., 2024; Larios-Ulloa and Ramírez-Muñoz, 2024; Velázquez-De Lucio et al., 2022; Do Carmo et al., 2021; Thomas et al., 2019; Gu et al., 2015) and offer sustainable alternatives, because they effectively break lignocellulosic bonds while producing waste with a lower environmental impact, making them safer to handle and sustainable options. Beyond their lower cost, these compounds do not generate saline residues, and their byproducts, such as calcium carbonate, can contribute positively to the physicochemical characteristics of the substrate. Specifically, they offer the added advantage of improving the post-cultivation value of the spent substrate when reused as a soil amendment, as they help neutralize acidity, supply bioavailable calcium, and enhance soil structure—characteristics not provided by NaOH or KOH (Rodrigues, Jackson and Montross, 2016; Kang et al., 2022).

A novel and underexplored alternative is the reuse of nejayote, an effluent from nixtamalization of corn, which is highly alkaline (pH > 11) and rich in Ca(OH)₂ (Fernandez-Muñoz et al., 2011). In Mexico, over 1.2 million m³ of nejayote are generated monthly (Campechano et al., 2012). Most of it is disposed untreated, affecting water and sewage systems (Gutiérrez-Uribe et al., 2010). Despite previous uses in animal feed and nutraceutical recovery (Ayala-Soto et al., 2014; Campechano et al., 2012; Gutiérrez-Uribe et al., 2010). However, the potential of this residue for the alkaline pretreatment of lignocellulosic waste, which offers an innovative and sustainable solution, has not yet been evaluated.

Therefore, this study aims to evaluate nejayote not only as a delignifying pretreatment for agave bagasse but also as a functional substrate modifier during solid-state fermentation (SSF) with edible macrofungi.

2. Materials and methods

2.1. Materials

Nejayote was obtained from two local tortilla factories located in Querétaro, México: "La Purísima" (Pedro Escobedo; NP) and "La Regia Inn" (Querétaro, NR). This byproduct is generated during the nixtamalization of maize, a process in which corn is cooked with hydrated lime (Ca(OH)₂) and left to steep overnight. Nejayote was collected the following morning by thoroughly mixing and sampling the contents of the cooking tanks. To preserve its composition, the samples were stored at -20 °C, and for characterization analyses, the samples were freeze-dried, milled, and stored at -80 °C until later use.

Agave bagasse (AB) was obtained from a mezcal distillery (La Cascada) in Malinalco, México. For characterization, the material was air-dried and ground to a uniform particle size of approximately 2 mm. However, for the solid-state fermentation (SSF) experiments, agave bagasse was used without prior drying or grinding.

All chemicals and solvents used for the analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA) and J.T. Baker (Mexico City, Mexico).

2.2. Methods

2.2.1. Proximate composition

The proximate composition of nejayote and agave bagasse before and after fermentation, including moisture content, lipids, protein, and ash, was determined using standard methods (Association of Official Analytical Chemists, 1987); 925.40; convection drying, 948.22; Soxhlet,

950.48; Kjeldahl method, 923.03; Oven, respectively).

2.2.2. Determination of reducing sugars

An aqueous extract of the sample was prepared in a 1:2.3 ratio and Miller's DNS (3,5-dinitrosalicylic acid) method (Miller, 1959) was used to measure reducing sugars, and the results were compared with a fructose standard curve to obtain the concentration.

2.2.2. Physicochemical analysis of nejayote

Total solids content (TSC) was calculated after drying each sample at 105 °C, and the pH of the samples was measured following Hach methods (Company, 2018; Company, 2020).

2.2.3. Nutraceutical analysis and antioxidant capacity

2.2.3.1. Dietary fiber. Total dietary fiber was determined by using the Total Dietary Fiber Assay Kit "K-TDFR-200A" (Megazyme International, Bray, Wicklow, Ireland) and following the manufacturer's instructions, according to AOAC and AACC approved methods.

2.2.3.2. Phenolic compounds and antioxidant capacity. Maceration extraction was performed for free phenolic compounds (Cardador-Martínez et al., 2002) and determined according to the adapted methodology described by Singleton and Rossi (1965). Total flavonoids were quantified according to the method described by Oomah et al. (2005). Individual polyphenols were analyzed by high-performance liquid chromatography-diode array detection (HPLC-DAD) in an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) using a Zorbax Eclipse XDB-C18 column (Agilent technologies; 4.6 × 250 mm, 5 µm); experimental conditions were the same as previously reported (Ramírez-Jiménez et al., 2014) and quantification was carried out using the external standards of (+)-catechin, epigallocatechin gallate and gallic, protocatechuic and ellagic acids. Lastly, the antioxidant capacity was evaluated using two antioxidant systems: 2,2-diphenyl-picrylhydrazyl (DPPH) (Fukumoto & Mazza, 2000) and 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Nenadis et al., 2004). A standard Trolox curve was used to interpret the results, which were expressed as mM equivalent trolox/mg of dry matter.

2.2.4. Alkaline pretreatment

The agave bagasse underwent alkaline pretreatment using liquid nejayote from "La Purísima" (Pedro Escobedo, State of Queretaro, Mexico). Prior to application, the nejayote was homogenized to ensure consistency and added directly in its liquid form. Three concentrations were tested: 0 % (distilled water only, pH 7.4), 50 % (1:1 nejayote:water v/v, pH 10.9), and 100 % (undiluted nejayote, pH 12.9). In all cases, the liquid was poured over the agave bagasse until the substrate was completely covered. A completely randomized 3³ experimental design was implemented to select the pretreatment conditions that had the maximum effect at reducing the lignin content (%), varying nejayote concentration (0, 50, 100 %), temperature (25, 50, 100 °C), and incubation time (2, 12, 24 h).

Nejayote from "La Purísima" was selected because it has a higher content of fermentable carbohydrates, particularly reducing sugars (3.36 % compared to 0.76 % in nejayote from "La Regia Inn").

2.2.5. Solid-State fermentation (SSF)

Approximately 500 g (wet basis) of agave bagasse was subjected to alkaline pretreatment in a 2 L glass container, using 1.5 L of nejayote per container. After pretreatment under the selected time and temperature conditions, the excess liquid was drained to maintain adequate moisture for fungal growth (70 %) with a final pH of 6.2.

The pretreated agave bagasse was used as a substrate for SSF with edible macrofungi (*Pleurotus ostreatus* and *Lentinula edodes*). Fungi propagation was standardized for both substrates, under controlled

conditions (24 h darkness, 22–24 °C, 60 % humidity). Mycelial growth was observed for 2 to 3 weeks, followed by a fructification phase under natural indirect light (approximately 13.2 h of light), ambient temperature (22–26 °C), and 60–65 % humidity. Fruiting bodies were harvested after 3 weeks of fructification and further analyzed for their chemical and nutraceutical properties.

2.2.6. Cultivation system

The substrate was inoculated under aseptic conditions, cleaning the surfaces with ethanol and employing alcohol burners. 50 g of inoculum (sorghum seed with each fungal mycelium) was added to a plastic bag. Two independent experiments were conducted in triplicate for each treatment and strain. The inoculated bags were incubated at room temperature from 22 to 24 °C and a relative humidity range of 60 % in a dark room until the mycelium completely covered the bags. Each bag represented an experimental unit. Once the mycelium completely covered the substrate, they were moved to a growth area, consisting of a room with indirect natural light and a relative humidity of around 60 %. After the formation of the primordia, several cuts of approximately 2 cm were made on the bag's surface to allow the development of fruiting.

2.2.7. Identification of volatile compounds by gc-ms

To identify the volatile compounds in the samples, methanolic extracts were prepared and subsequently subjected to analysis by gas chromatography coupled with a mass spectrometry detector (GC-MS). The analysis was performed on agave bagasse that had been previously treated with nejayote-based alkaline pretreatment and then subjected to SSF with two edible fungi: *Pleurotus ostreatus* and *Lentinula edodes*. Both fermented and non-fermented (control) samples were analyzed.

Briefly, 1 g of the fermented sample with fungi and the non-fermented agave bagasse were weighed, and 10 mL of methanol was added. The samples were vortexed for 30 s, sonicated for 20 min, vortexed again for 30 s, and finally centrifuged at 6000 rpm for 10 min at 25 °C. Samples were then filtered through 0.45 µm membranes. Volatile compounds were identified on a gas chromatograph coupled to a mass spectrometer (GC-MS, model 7890A, US81829145, Agilent, USA). The injector was maintained at 280 °C, and the injected volume was one µL in splitless injection mode using a Combi PAL autosampler (CTC Analytics, G6500-CTC). The sample was separated using a DB-17ht column (30 m x 0.25 mm x 0.15 µm). Helium was used as a carrier gas at a 3 mL/min flow rate. The oven temperature was started at 50 °C and programmed to increase at 10 °C up to 240 °C and held at 240 °C for 10 min. Spectra were recorded in the mass/charge (*m/z*) range of 35 to 550 m/z. Peak identification was performed by comparing peak retention times to NIST/EPA/NIH Mass Spectra Library v. 08 standards (NIST, USA).

2.2.8. Determination of sugars by HPLC

For the determination of glucose by HPLC, the methodology of Ji et al. (2021) was followed, with some modifications. An Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) high-performance liquid chromatography equipment coupled to a refractive index (RI) detector was used, using a ZORBAX carbohydrate column (150 mm x 4.6 mm, 5 µm) and a mobile phase of acetonitrile and water in a ratio of 65:35 (v/v) with isocratic running conditions, the flow was maintained at 0.4 ml/min and the column temperature was maintained at 35 °C. A glucose standard was injected to construct the calibration curve [0.005 to 0.05 mg/mL] Fig. 1.

To determine the sugar profile in non-fermented and fermented agave bagasse, 1 g of dried, ground, and sieved samples was dissolved in 9 mL of ultrapure water and placed in a water bath at 50 °C for 20 min. After heat treatment, the tubes were centrifuged at 10,000 x g for 10 min. The supernatant was collected and filtered through organic membranes with a mesh size of 0.45 µm. Then, 20 µL of the prepared samples were injected into the equipment under the previously described conditions. Finally, the glucose in the samples was identified by matching the corresponding retention time obtained in the standard

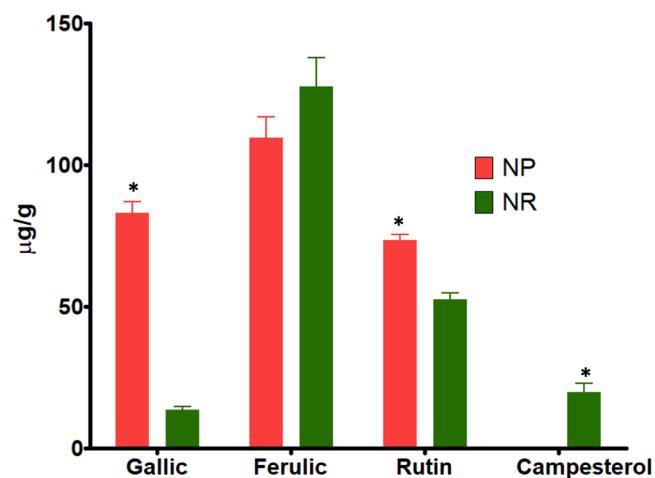


Fig 1. HPLC free-polyphenols identified in freeze-dried nejayote
NP: Nejayote La Purísima, NR: Nejayote La Regia Inn. Results are expressed as the mean ± standard deviation of two independent experiments on a dry basis.
* Indicates significant difference between samples using Student's t test for unpaired data $p < 0.05$.

chromatogram.

2.2.8. Vibrational analysis

The non-fermented and fermented agave bagasse substrates were subjected to vibrational spectroscopy to evaluate structural changes post-fermentation. A Perkin Elmer IR Spectrometer (Spectrum Two, Waltham-USA) equipped with ATR (Attenuated Total Reflectance) was used to obtain the IR spectra of the agave bagasse samples. The fine powder sample (ground and sieved No 60 US mesh, 250 µm) was scanned over a range of 600 to 4000 cm⁻¹, with 32 accumulations per sample.

2.2.9. Furfural derivatives quantification

The separation and quantification of furfural (FF) and 5-Hydroxymethylfurfural (5-HMF) was carried out using a high-performance liquid chromatography (HPLC) system (Agilent 1100 Series HPLC system; Agilent Technologies, Palo Alto, CA, USA), coupled with a diode array detector (DAD) and equipped with a quaternary pump, and an auto-sampler. The analysis was performed in isocratic elution mode, using HPLC water:acetonitrile (80:20), pumped through a Zorbax Eclipse XDB-C18 column (Agilent Technologies, 4.6 × 250 mm, 5 µm) at a constant flow rate of 1 ml/min. The injection volume was 20 µL. The DAD recorded the spectra in 277 and 258 nm for FF and HMF, respectively, for 15 min (Godoy et al., 2022).

2.2.10. Lignin quantification

Lignin content was quantified following the CASA method previously described by Lu et al. (2021) with slight modifications. Briefly, 5 mg of sample was weighed, and 1 mL of cysteine stock solution (0.1 g/mL in 72 % sulfuric acid) was added, which was stirred at room temperature using a magnetic stir bar (400 rpm) for 90 min. The solution was diluted with distilled water to 100 mL in a volumetric flask, and the absorbance was measured in a UV spectrophotometer at 238 nm using a 1 cm quartz cell; diluted stock solution was used as a blank, and lignin content was calculated based on the Beer-Lambert law.

2.2.11. Microstructure

The microstructure of previously dried and sieved fermented and non-fermented agave bagasse was evaluated. It was fixed with a carbon tape on a bronze sampler holder and mounted for measurement using a high-vacuum scanning electron microscope (SEM; JEOL, JSM-6060LV) with a resolution between 150x and 2500x and an electron

acceleration voltage of 12 kV.

2.3. Statistical analysis

The variables were implemented to optimize the conditions of pretreatment, including nejayote concentration (0, 50, 100 %), temperature (25, 50, 100 °C), and time (2, 12, 24 h). Response surface methodology (RSM) was employed to analyze factors' individual and interactive effects and optimize the delignification process conditions. Each experiment was performed in triplicate, and the standard deviations were calculated. Data were reported as mean \pm standard deviations. A three-way ANOVA was used to evaluate the effects of the independent variables and their interactions on the response variable. Since two-way and three-way interactions were not significant ($p > 0.05$), the analysis focused on the main effects. Differences among means for the bagasse composition were assessed using Tukey's HSD post-hoc test, while a Student's *t*-test was used to compare the nejayote samples. Statistical analyses were performed using JMP 10.0 software, and statistical significance was considered at $p \leq 0.05$.

3. Results and discussion

3.1. Effect of alkaline pretreatment of agave bagasse

Lignin removal is widely recognized as a critical step for improving the bioavailability of cellulose and hemicellulose. Thus, a lower lignin content in pretreated agave bagasse serves as a direct indicator of successful chemical disruption of recalcitrant structures, enabling more effective fungal biodegradation.

A full factorial (3^3) experimental design was used to evaluate the effects of temperature (°C), time (h), and nejayote concentration (%) on

the percentage of residual lignin in agave bagasse. A response surface analysis was applied to examine these factors' interactions and optimize the process conditions. The fitted model was statistically significant ($p < 0.05$), and all three factors had a significant influence on the response ($p < 0.05$), with nejayote concentration exhibiting the most important effect ($p = 0.000$).

The fitted regression equation was:

$$\text{Equation 1. } \% \text{ Lignin} = 13.763 - 0.01496 \text{ Temperature (} ^\circ\text{C}) - 0.0414 \text{ Time (h)} - 0.02561 \text{ Concentration (\%)}$$

Fig. 2 shows the contour and 3D plots of the regression analysis. The contour plots display a flat, diagonal surface, indicating that temperature and time interact linearly to reduce lignin content (**Fig. 2A**). It is also evident that increasing both temperature and concentration decreases lignin content in agave bagasse (**Fig. 2B**). Specifically, at temperatures between 90–100 °C and nejayote concentrations above 80 %, the lignin content drops below 10 %. **Fig. 2C** demonstrates that the gradient of concentration is steeper, confirming that this variable has the most significant impact, promoting greater lignin dissolution and facilitating access to cellulose and hemicellulose, which supports fungal growth and development during solid-state fermentation (SSF).

The 3D plots reveal flat surfaces with constant slopes without noticeable curvatures or saddle points (**Fig. 2D–F**), indicating that the increase of temperature and concentration within the experimental limits further reductions in lignin concentration.

We assessed two- and three-way interactions of the analyzed variables; however, these interactions were found to be insignificant. This allowed for the establishment of optimal conditions for the alkaline pretreatment of mezcal agave bagasse: a temperature of 100 °C using undiluted nejayote. Since time had the least effect on the sample pretreatment, the shortest evaluated duration of 2 h was selected, resulting in a 34.92 % reduction in the lignin content.

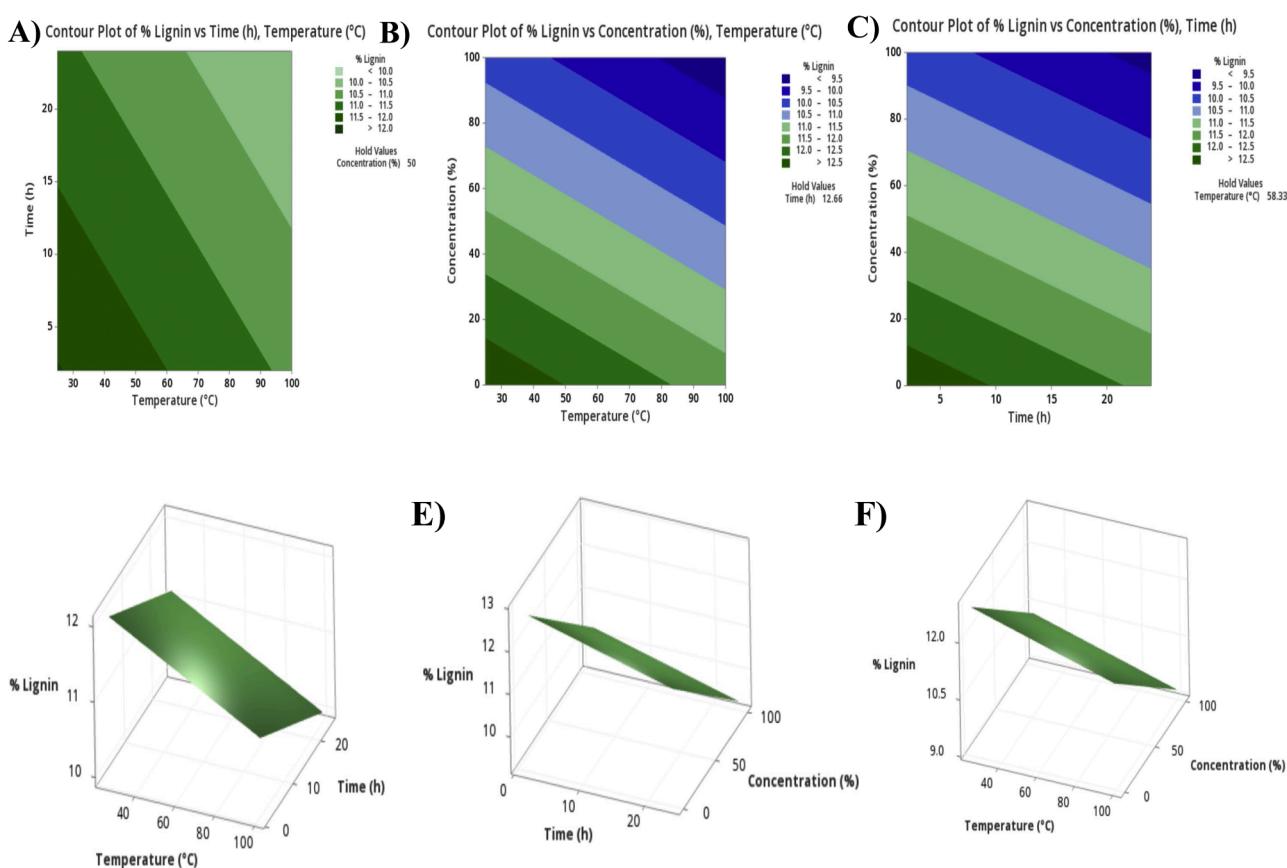


Fig. 2. 2D and 3D plots of the response surface analysis of the 3^3 full factorial design for reducing the percentage of lignin in agave bagasse using nejayote as an alkaline pretreatment.

Previous studies on agave bagasse, such as the work conducted by Ávila-Lara et al. (2015), explored the effects of alkaline pretreatment using sodium hydroxide (NaOH). They employed response surface analysis to optimize the process by examining treatment time, solids concentration, and NaOH concentration. They conducted the experiments under sterilized conditions (121 °C at 15 psi) and identified optimal pretreatment parameters based on the highest yield of fermentable sugars. The best-reported conditions from their research included 1.87 % NaOH, a treatment time of 50.3 min, and a solid concentration of 13.1 %.

In contrast, the present study utilized calcium hydroxide (Ca(OH)_2) as the alkaline agent, with an estimated 1–2 % and 2.2 % solids concentration. Unlike the previous study, sterilization was not applied, which may have impacted the reaction kinetics of the pretreatment and its overall efficiency. Prior research has evaluated residues from the specific agave variety *Agave sisalana* Perrine, used for fiber extraction, as a substrate for producing *Pleurotus ostreatus*. In that study, the substrate was prepared by soaking it in a 0.5 % hydrated lime solution (Ca(OH)_2) for 12 h (Do Carmo et al., 2021).

Recently, Morales-Huerta et al. (2021) investigated the effects of alkaline pretreatment with NaOH and mechanical pretreatment using twin-screw extruders on blue agave bagasse. Their findings indicate that the concentration of the alkaline agent, extruder residence time, and temperature are vital factors for the extraction of cellulose and hemicellulose from this valuable residue. These results align with our findings and highlight the potential of using other pretreatment technologies, such as ohmic heating and extrusion, on agave bagasse for mezcal, as previously explored by our research group (Álvarez-Chávez et al., 2025). Combining alkaline pretreatment and ultrasound has shown positive outcomes in removing lignin from *Agave sisalana* fibers, thus reducing pretreatment time (Brito et al., 2019).

Based on the response surface analysis of the experimental design, the combination of high nejayote concentration, moderate to high temperature (above 70 °C), and short treatment times (2–6 h) resulted in the lowest observed lignin percentages. These conditions represent the most effective delignification outcomes identified within the tested range. Therefore, to maximize lignin removal while minimizing processing time, we recommend using nejayote from tortilla factories, applying higher temperatures, and limiting the pretreatment duration. This approach ensures substantial lignin disruption without compromising efficiency, thus improving substrate accessibility for subsequent fungal colonization.

This structural modification is critical for enhancing the accessibility of cellulose and hemicellulose carbon sources for fungal metabolism. As a result, the improved availability of fermentable carbohydrates may explain the enhanced fungal colonization biomass development observed in subsequent solid-state fermentation (SSF) experiments.

Beyond its technical efficacy, this pretreatment strategy also offers practical and economic advantages. Nejayote is a readily available byproduct in maize-based food industries, particularly in Mexico, and its use aligns with principles of circular economy. Its application requires no specialized equipment, and it adds value to two agricultural residues while contributing to sustainable protein production through fungal biomass cultivation. This makes the approach especially appealing in rural or semi-industrial contexts where access to complex technologies is limited.

3.2. Mycelial growth and fructification

Both cultivated fungi showed rapid mycelial development, colonizing the pretreated agave bagasse in 7 to 10 days. Subsequently, *Pleurotus ostreatus* fruiting bodies were obtained and analyzed for their proximate composition. The results showed that, under equivalent alkaline pretreatment and cultivation conditions, *Pleurotus ostreatus* grown on agave bagasse produced fruiting with a higher nutrient content than those cultivated on wheat straw, which was used as a

comparative control substrate. Specifically, ash, fat, and protein contents were higher in mushrooms grown on agave bagasse (11.81 % vs. 9.51 %; 2.47 % vs. 1.99 %; 12.83 % vs. 10.45 %, respectively), while carbohydrate content was slightly lower (56.18 % vs. 58.53 %). This comparison demonstrates the nutritional potential of agave bagasse as an alternative substrate. Only *P. ostreatus* fruiting bodies were analyzed due to the consistent and abundant production observed in this strain, in contrast to limited or absent fruiting in the other tested species.

It is important to note that the agave bagasse was not washed after the alkaline pretreatment, allowing it to retain soluble compounds and residual alkalinity from nejayote. This decision may have influenced the final pH of the substrate during incubation, with potential effects on mycelial development and the nutritional composition of the fruiting bodies. The pH of the nejayote ranged from 10.5 (50 % dilution) to 12.9 (100 % nejayote), and the final pH of the pretreated, drained substrate was between 8.5–9.5, depending on the treatment. Previous studies have shown that mixing *Agave tequilana* bagasse with barley straw and adding calcium carbonate (5 g) creates a viable substrate for cultivating other mushroom species, such as *Pleurotus djamor*. These findings support the idea that both substrate composition and pH modification through alkaline agents significantly influence fungal performance and fruiting body composition (Gutiérrez-Antonio et al., 2023).

3.3. Physicochemical characterization of nejayote

The physicochemical characterization of nejayote from two local tortilla factories, La Purísima (PN) and La Regia Inn (NR), is shown in Table 1. The NR sample presented a significantly higher ash value (30.55 g versus 25.75 g in PN). This difference may be attributed to a lower mineral content in PN due to a previous washing of the corn, which could have reduced the minerals in the final nixtamalization, as the nixtamalization process involves several washes. Additionally, variations in ash content may be affected by the amount of lime used during processing and, to a lesser extent, by the type of corn that is processed. The higher mineral content in the NR sample is beneficial because it could enrich the agave bagasse, a key process during solid-state fermentation, modifying the chemical composition of the byproducts, enriching the substrates that contribute to the nutritional value and functional properties of the final product (Wang et al., 2022). In a study conducted by Dedousi et al. (2023) in which different calcium sources were tested in the growth of *Pleurotus ostreatus* on products such as wheat straw, barley, oats, and rice husk, it was shown that calcium sources can promote the development of mycelium and fruiting bodies of this strain. In addition, some calcium salts are added to the culture

Table 1
Physicochemical and nutraceutical composition of freeze-dried nejayote.

Proximal composition	La Purísima	La Regia Inn
Ash (g)	25.75 ± 0.32	30.55 ± 0.32*
Fat (g)	1.43 ± 0.00	1.47 ± 0.49
Protein (g)	7.61 ± 0.13*	7.15 ± 0.07
Total carbohydrates (g)	58.53 ± 0.28*	56.18 ± 0.62
Total dietary fiber (g)	16.48 ± 0.30	36.72 ± 0.92*
Soluble dietary fiber (g)	8.93 ± 0.74	22.15 ± 1.27*
Insoluble dietary fiber (g)	7.55 ± 0.43	14.57 ± 0.34*
Reducing sugars (%)	3.36 ± 0.11*	0.76 ± 0.05
Total solids (%)	2.77 ± 0.16*	2.22 ± 0.24
Total Phenolic Compounds (mg GAE)	19.17 ± 2.09	30.18 ± 3.19*
Total Flavonoids (mg RE)	1.87 ± 0.02	1.90 ± 0.07
TEAC (DPPH)	23,446.7 ± 14.85	13,570 ± 18.96*
TEAC (ABTS)	45,362.2 ± 29.4*	20,825.7 ± 51.41

Results are expressed as the mean ("units" per 100 g) ± standard deviation of three independent experiments per triplicate on a dry basis (d.b.). Protein content was calculated as nitrogen x 6.25. Gallic acid equivalents (GAE), Rutin equivalents (RE), TEAC (Trolox equivalent antioxidant capacity) measured using the stable radical DPPH. * Indicates significant difference between samples using Student's t-test for unpaired data $p < 0.05$.

media in SSF to improve the pH of the substrate and promote growth (Edo & Shareef, 2022; Melanouri et al., 2022). It has also been reported that adding calcium salts to the substrate in an SSF is beneficial for the agglomeration of the substrate for the cultivation of *Lentinula edodes* (Silva & Ferreira, 2023).

Regarding the protein content, the PN presented a significantly higher value (7.61 g versus 7.15 g in NR). Although the difference is slight, a more significant amount of protein in the substrate can provide benefits, especially in the growth of fungi, because proteins are the primary source of nitrogen, which could promote faster and more abundant growth since it is an essential element in the synthesis of organic compounds. In addition, it has been reported that fungi grown on protein-rich substrates usually have more and better-quality proteins (Mleczek et al., 2021; Heidari et al., 2022).

For total carbohydrates, NP presented a higher value (58.53 g versus 56.18 g in NR), which suggests a higher content of fermentable sugars that could be beneficial for using nejayote in fermentations and as substrate. Finally, the dietary fiber content is especially relevant. NR showed a significantly higher content, with 36.72 g of total fiber versus 16.48 g in NP and higher values of soluble and insoluble fiber; which is essential for biotechnological applications as the amount of dietary fiber, both soluble and insoluble, in the substrate aids in the retention of water, ensuring an ideal humid environment for the growth of microorganisms. This also improves the structure of the substrate by having a greater porosity and allowing a better gas exchange, which benefits the growth of fungi such as *Pleurotus ostreatus* and *Lentinula edodes* in SSF (Pandey et al., 2000).

The NR sample presented a significantly higher value of phenolic compounds, with 30.18 mg GAE compared to 19.17 mg GAE in NP. Phenolic compounds are essential for their antioxidant activity and protective properties. In mushroom cultivation, research shows that they inhibit the mycelial growth of phytopathogenic fungi at high concentrations, which can be beneficial in the post-harvest area (Rodríguez-Matutino et al., 2015). In the cultivation of basidiomycete fungi in SSF, other studies show that they contribute to the stability of the substrate and can enhance fungal growth by reducing the presence of free radicals using enzymes to transform them (Martínková et al., 2016; Torres-Farradá et al., 2024). Both samples showed similar values in total flavonoids, with no significant differences (1.87 mg RE for NP and 1.90 mg RE for NR); this suggests that, although flavonoids are part of the bioactive composition of nejayote, they are not the most relevant component in terms of variation between samples.

The antioxidant capacity, assessed using two different methods, revealed significant variations. In the DPPH assay, NP exhibited a noticeably higher antioxidant capacity (23,446.7 compared to 13,570 for NR). Similarly, in the ABTS assay, NP registered higher values (45,362.2 vs 20,825.7 for NR). The pH levels and dissolved salts in nejayote may contribute to these differences in antioxidant capacity. Since DPPH is more stable under neutral conditions, changes in the alkaline pH of nejayote could affect the electron-donating ability of phenolic compounds (Prior et al., 2005).

Furthermore, dissolved salts, especially divalent cations like Ca^{2+} , can influence radical stability and antioxidant interactions, impacting the measured scavenging activity (Rossi et al., 2003); this is important because a higher antioxidant capacity can enhance the quality of the substrate, thereby preserving the cultivation environment and mitigating oxidative stress on the growth of edible mushrooms (Varesi et al., 2022). These findings suggest that nejayote from both tortillerías have valuable antioxidant and nutraceutical properties. However, NP appears to excel in antioxidant capacity, while NR contains a greater quantity of phenolic compounds. These results direct us towards specific applications for each type of nejayote, either as an antioxidant medium or as a source of bioactive compounds in mushroom cultivation and other biotechnological uses.

Regarding individual phenolic compounds, ferulic acid was the most abundant individual phenolic compound found in both samples,

recorded at 109.63 $\mu\text{g/g}$ in NP and 127.87 $\mu\text{g/g}$ in NR (Figure 1). This result was expected, as ferulic acid is a well-known antioxidant in high quantities in corn, which aligns with previous studies on nejayote (Acosta-Estrada et al., 2014). Ferulic acid is typically found in the pericarp and other structures of the grain in a bound form and is associated with ferulated arabinoxylans. These compounds are sensitive to alkaline pH levels, which promote their hydrolysis, releasing free ferulic acid and the arabinoxylan component, explaining why ferulic acid is one of the most prevalent phenolic compounds in nejayote (Hussain et al., 2022).

Other significant results include the levels of reducing sugars and total solids. The NP sample showed significantly higher values of reducing sugars (3.36 %) compared to NR (0.76 %), indicating that NP contains more fermentable sugars. That is advantageous for its use as a substrate in fermentation processes, as microorganisms can utilize these sugars as a carbon source, suggesting a higher potential for promoting fungal growth during solid-state fermentation. Therefore, selecting a nejayote sample with a higher carbohydrate content could enhance alkaline pretreatment and optimize fungal metabolism, leading to a more efficient bioconversion process and a higher-value final product. Additionally, the NP sample exhibited a higher total solids value (2.77 %) than NR (2.22 %), indicating more usable dry matter.

Finally, the pH of both samples remained alkaline, with 12.90 in NP and 13.01 in NR, slightly higher in NR, which supports its use as an alkaline treatment for lignocellulosic waste.

3.4. Physicochemical characterization of agave bagasse after solid-state fermentation

The composition of the agave bagasse was evaluated before and after the cultivation of *Pleurotus ostreatus* and *Lentinula edodes* to observe its physicochemical, nutraceutical, and structural changes, as shown in Table 2.

Generally, a decrease in the content of ash, fat, reducing sugars, total

Table 2
Physicochemical and nutraceutical composition of unfermented and fermented agave bagasse with *Pleurotus ostreatus* and *Lentinula edodes*.

Proximal composition	Pretreated agave bagasse	Agave bagasse <i>Pleurotus ostreatus</i>	Agave bagasse <i>Lentinula edodes</i>
Ash (g)	7.80 \pm 0.09 ^a	8.43 \pm 0.04 ^b	9.09 \pm 0.53 ^b
Fat (g)	0.21 \pm 0.02 ^a	2.47 \pm 0.09 ^c	1.98 \pm 0.05 ^b
Protein (g)	3.10 \pm 0.06 ^c	5.02 \pm 0.10 ^b	5.97 \pm 0.08 ^a
Total carbohydrates (g)	79.47 \pm 0.18 ^a	63.77 \pm 0.24 ^b	66.11 \pm 0.26 ^b
Total dietary fiber (g)	26.10 \pm 0.11 ^b	21.75 \pm 0.18 ^a	22.10 \pm 0.19 ^a
Soluble dietary fiber (g)	1.22 \pm 0.04 ^a	2.80 \pm 0.07 ^c	2.33 \pm 0.06 ^b
Insoluble dietary fiber (g)	24.88 \pm 0.09 ^b	18.95 \pm 0.16 ^a	19.77 \pm 0.17 ^a
Reducing sugars (%)	4.80 \pm 0.53 ^a	1.56 \pm 0.30 ^b	2.19 \pm 0.11 ^b
Glucose (%)	3.67 \pm 0.04 ^c	1.54 \pm 0.02 ^b	1.31 \pm 0.03 ^a
Total phenolic compounds (mg GAE)	12.95 \pm 0.85 ^a	1.45 \pm 0.02 ^c	5.11 \pm 0.23 ^b
Gallic acid (mg/g)	5539.4 \pm 86.2 ^a	ND	213.9 \pm 41.0 ^b
Total Flavonoids (mg RE)	1.04 \pm 0.01 ^a	0.48 \pm 0.00 ^c	0.80 \pm 0.06 ^b
Furfural (mg/g)	14.19 \pm 0.20 ^a	0.29 \pm 0.05 ^c	1.77 \pm 0.05 ^b
Lignin (%)	13.69 \pm 0.51 ^a	7.01 \pm 0.12 ^b	7.22 \pm 0.22 ^b

Results are expressed as the mean ("units" per 100 g) \pm standard deviation of three independent experiments per triplicate on a dry basis. The columns for *P. ostreatus* and *L. edodes* represent the fermented substrates including fungal biomass, as no separation of mycelium or fruiting bodies was performed. Protein content was calculated as nitrogen \times 6.25. Gallic acid equivalents (GAE), Rutin equivalents (RE). Different letters indicate significant differences between samples using the Tukey test $p < 0.05$. ND: Not detectable.

phenolic compounds, flavonoids, furfural, lignin, and gallic acid was observed. These reductions can be attributed to the metabolism of the organism, which uses these compounds as sources of carbon and energy during its growth. Both fungi are white-rot basidiomycetes, known for their ability to degrade recalcitrant compounds such as lignin and other aromatic compounds (including furfurals and some phenolic compounds) through oxidative enzymes such as laccase and peroxidase, which can transform these compounds into less toxic ones such as furfuryl alcohol or furoic acid, being useful as detoxifiers of specific agro-industrial residues (Caselli et al., 2015). These findings align with the previous reports for another white-rot fungus, *Trametes versicolor*, which metabolized and grew in the presence of all inhibitors, such as phenols and furfurals (Nilsson et al., 2016).

A notable increase in protein content can also be observed, reflecting the metabolic activities of both fungi on the substrate observed through protein synthesis. SSF with *Pleurotus ostreatus* using Carob pulp as a substrate has been shown to triple the total protein and fiber in the substrate (Iqbal et al., 2024). Fungi possess some enzymes of interest, such as lipases, which hydrolyze lipids into simpler compounds, resulting in reduced post-fermentation fat content, in both fungi.

The most noticeable changes in dietary fiber composition occur after solid-state fermentation (SSF), leading to increases in both insoluble and soluble fiber fractions. The increase in soluble fiber is likely due to the enzymatic breakdown of the lignocellulosic components of the substrate. In contrast, the rise in insoluble fiber appears to stem from the fungi's synthesis of polysaccharides. Specifically, the production of fungal exopolysaccharides and components of the cell wall contributes to the enhanced fiber content in the residual solid matrix.

Fungi generate various polysaccharides, including β -glucans and heteropolysaccharides, which become incorporated into the substrate, boosting fiber content. The enzymatic processes involved in this transformation include the breakdown of lignocellulosic components during SSF, facilitated by various fungal enzymes. Key enzymes, such as lignin peroxidase (LiP) and manganese peroxidase (MnP), play a critical role in

degrading lignin, allowing access to cellulose and hemicellulose (Dashtaban et al., 2010). Additionally, cellulolytic enzymes like endoglucanases and exoglucanases hydrolyze cellulose into oligosaccharides and monosaccharides, further altering the fiber composition of the substrate (Mardetko et al., 2021).

Studies have reported an increase in soluble fiber in certain byproducts, such as SSF of corn husk (Ban et al., 2024) and oilseed cakes for poultry (Sousa et al., 2024). This increase can benefit human health, and SSF can serve as a valuable strategy for treating byproducts and revalorizing them by producing ingredients that enhance food product characteristics.

Interestingly, an increase in glucose concentration has been observed, indicating efficient hydrolysis of polysaccharides in the substrate. In contrast, the concentration of reducing sugars decreases, suggesting that other reducing sugars are likely being consumed before glucose in both solid-state fermentation (SSF) processes. A similar finding was reported by Iqbal et al. (2024) during the fermentation of carob pulp with *Pleurotus ostreatus*, where a reduction in sugar content was noted alongside changes in antioxidant properties. This phenomenon may be linked to the hydrolytic activity of fungal enzymes, which break down polysaccharides into monosaccharides, such as glucose (Mardetko et al., 2021).

3.5. Volatile profile of agave bagasse after solid-state fermentation

The volatile compounds present in the agave bagasse after solid-state fermentation with *Pleurotus ostreatus* and *Lentinula edodes* were evaluated (Fig. 3). It is essential to highlight that a more significant number of compounds were identified in the sample fermented with *Lentinula edodes* (13 compounds) than in the sample fermented with *Pleurotus ostreatus* (8 compounds) (Supplementary Table 1). Among the metabolites detected in both samples, fatty acids such as oleic, linoleic, and palmitic stand out, which can be derived from the primary metabolism of fungi, play a key role in the biosynthesis of cell membranes, especially

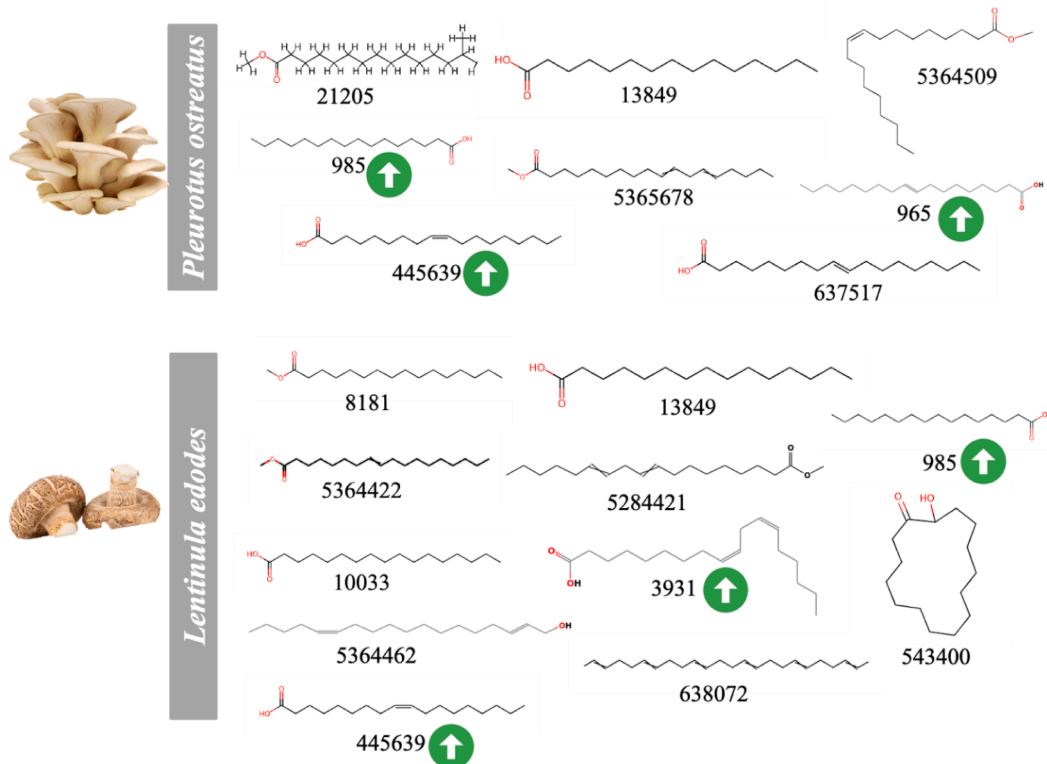


Fig 3. Main volatile metabolites produced during SSF of agave bagasse with *Pleurotus ostreatus* and *Lentinula edodes*. The number below each compound belongs to their PubChem CID.

in the regulation of fungal growth and as precursors of characteristic aromas of edible mushrooms (Ribeiro et al., 2009; Mokochinski et al., 2015). It has been shown in *Lentinula edodes* that linoleic acid serves as a precursor to the compound 1-octen-3-ol, which imparts a specific aroma to mushrooms (Ribeiro et al., 2009). In this study, the presence of Z,E-2, 13-Octadecadien-1-ol was identified in the agave bagasse with mycelial growth of *Lentinula edodes*, a sample in which a characteristic odor was perceived. More recently, Mykchaylova et al. (2024) reported that linoleic, oleic, and palmitic acids were the primary fatty acids found in the mycelium of *Lentinula edodes* grown in submerged fermentation and exposed to irradiation with different types of light (red, green, and blue lasers). Other compounds, such as α -linolenic acid and cis-10-heptadecenoic acid, were also found. These compounds coincide with the volatile compounds identified in this study.

In the agave bagasse fermented with *Pleurotus ostreatus*, one of the primary fatty acids identified was octadec-9-enoic acid, which coincides with what was previously reported by Barman et al. (2021), who reported the presence of this compound in the ethanolic extract of a fruiting body and different stages of growth of the common mushroom (*Agaricus bisporus*) grown in a rice straw-based substrate. The analysis of alcoholic extracts of *Pleurotus sajor-caju* and *Pleurotus ostreatus* by GC-MS identified 20 different compounds, highlighting the presence of hexadecanoic acid, linoleic acid, octadecanoic acid, 2,3-hydroxypropyl ester, and palmitic acid in a submerged fermentation using potato dextrose medium (PDA), which coincides with the compounds identified in the mycelium of *Pleurotus ostreatus* grown in mezcal agave bagasse, demonstrating the potential to find these compounds in the fruiting body of this edible mushroom and thus its potential use as antimicrobial, anticancer, antioxidant and antiaging agents, food supplements or functional ingredients (Gülmез et al., 2021).

The presence of volatile compounds suggests an active transformation of the structural components of the substrate, which also influences the sensory profile and functionality of the fermented agave bagasse as an ingredient and the fruiting bodies obtained. The variation in the composition of these volatile compounds between both samples indicates differences in their metabolism, which can impact the bioconversion process's efficiency and the fermented substrate's applicability in the food or biotechnology industry.

3.6. Vibrational analysis of agave bagasse after solid-state fermentation

A vibrational analysis was performed to observe the changes in the agave bagasse before and after fermentation with *Pleurotus ostreatus* and

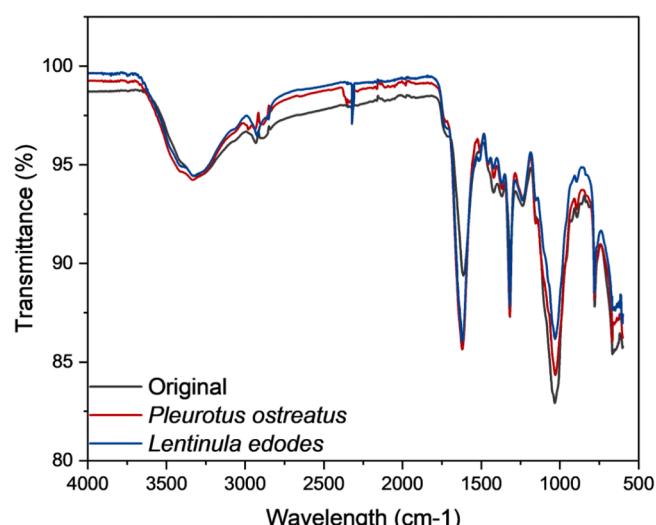


Fig 4. Vibrational analysis spectra of agave bagasse before and after solid-state fermentation.

Lentinula edodes, following the characteristic carbohydrate bands shown in Fig. 4. Regardless of the agave bagasse treatment, consistent bands are observed in the 3200–3600 cm^{-1} region (O—H groups), mainly from cellulose and hemicellulose. Another region of interest is the 1600–1750 cm^{-1} region, associated with the stretching vibrations of the carbonyl groups ($\text{C}=\text{O}$) of reducing sugars, lignin, and lipids, which increase after fermentation with both fungi, suggesting a more significant presence of reducing sugars and lignin degradation. However, it is essential to mention that there is a particular increase in the 1650 cm^{-1} band (conjugated carbonyl groups, $\text{C}=\text{O}$) after fermentation with both fungi; which could also be related to the presence of fungal proteins since the carbonyl bonds of the amide groups in proteins absorb in this region (Wickramasinghe et al., 2023). Other bands of interest are those at 1100 cm^{-1} (crystalline cellulose) and 898 cm^{-1} (amorphous cellulose), which show a significant decrease in the fermented samples, an effect that is more pronounced in the sample fermented with *Lentinula edodes*. This suggests a more effective degradation of cellulose, coinciding with the most evident changes observed in the SEM micrographs (Fig. 4C) (Klaai et al., 2022). In this sense, nejayote as an alkaline pretreatment may have contributed to the exposure of cellulose, facilitating the action and growth of both fungi (Olugbemide et al., 2018; Batista et al., 2022).

3.7. Morphological analysis

SEM was used to examine the surface morphology of agave bagasse fibers at magnifications ranging from 150 to 2500x; micrographs (Fig. 5) of agave bagasse show structural changes in lignocellulosic fibers after biological treatment with *Pleurotus ostreatus* and *Lentinula edodes*. In the unfermented agave bagasse sample, an intact, compact, fibrous structure can be observed on a relatively smooth surface, without apparent pores (Fig. 5A), characteristic of materials with high lignin content, which acts as a physical barrier protecting cellulose and hemicellulose. This aligns with what was reported by Xie et al. (2024), who observed fibrous and smooth structures with a uniform particle size in okara, a byproduct of soy milk and tofu.

On the other hand, the micrographs obtained after fermentation with both fungi show alterations in the fiber structure. In the agave bagasse sample fermented with *Pleurotus ostreatus*, the porosity of the fibers and the beginning of the disintegration of the lignin fibers can be observed, indicating a partial degradation. The treatment with *Lentinula edodes* shows deeper cracks and pores, evidenced by a greater separation between fibers, suggesting that this fungus is more efficient in lignin degradation. The mixed solid-state fermentation of orange peel using *Aspergillus niger* and *Trichoderma reseii* produces a sparse and porous structure with a network appearance. At the same time, the untreated sample presents a relatively smooth structure and intact peel fibers (Cheng et al., 2023). These results agree with those previously reported by Liu et al. (2023), who observed the lignocellulosic degradation of sawdust using *Lentinula edodes* during the mycelial colonization of the fungus, agreeing with the results obtained from agave bagasse with the appearance of cracks and small holes in the sawdust residue, as a large number of mycelia invaded the sawdust. The treatment of residues such as corn sawdust and xyloma straw with *Pleurotus ostreatus* and *Lentinula edodes* show changes similar to those found in this study in the structure of the fibers of these byproducts characteristic of the appearance of small pores, eroded areas and cracks between the fibers that increase in number and size as the fermentation progresses, allowing said fibers to come into contact with the mycelia and be degraded by the evaluated fungi (Lu et al., 2023).

The results presented in this study consistently demonstrate that the effect of alkaline pretreatment of agave bagasse in different species leads to the dissolution of lignin, altering the structure and morphology of the fibers, in addition to an increase in the number of pores observed by SEM microscopy (Ávila-Lara et al., 2015; Brito et al., 2019); illustrating that nejayote is helpful as an alkaline pretreatment agent for lignocellulosic materials.

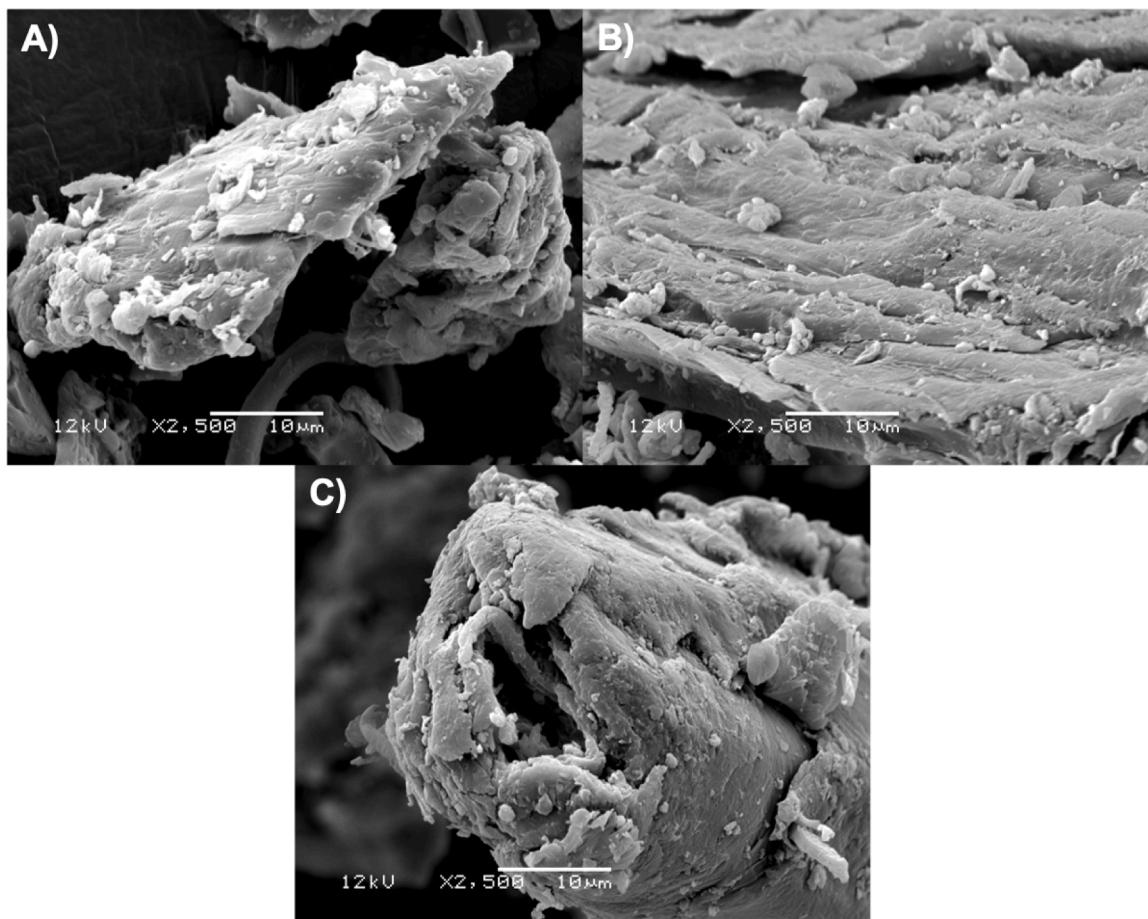


Fig 5. Scanning electron microscopy (SEM) images of the unfermented agave bagasse (A), agave bagasse fermented with *Pleurotus ostreatus* (B), and agave bagasse fermented with *Lentinula edodes* (C) taken at 2500x resolution.

4. Conclusions

This study demonstrated that nejayote exhibits variable proximate and nutraceutical composition depending on corn type and the nixtamalization conditions, and possesses high alkalinity, phenolic content, dietary fiber, and calcium. These properties make it a promising agent for modifying lignocellulosic residues like agave bagasse.

Nejayote-based pretreatment significantly reduced lignin content and improved the physicochemical characteristics of agave bagasse, which in turn supported fungal colonization and transformation through solid-state fermentation. *Pleurotus ostreatus* showed superior fruiting and nutritional enrichment of the substrate. Although no direct comparisons with other alkaline agents or untreated controls were included, these results suggest that nejayote can serve as both a delignifying and nutritive input for upcycling agro-industrial residues. It is important to note, however, that preliminary unpublished data indicate limited fruiting when fungi are cultivated on untreated bagasse alone, highlighting the intrinsic recalcitrance of this material and underscoring the relevance of chemical pretreatment. Future studies should incorporate untreated substrates and conventional alkalis to validate nejayote's comparative performance.

Overall, the use of nejayote and agave bagasse in SSF represents a sustainable approach for producing protein-rich biomass and bioactive compounds, aligning with circular economy principles and offering opportunities for biotechnological innovation in food and waste valorization.

CRediT authorship contribution statement

Elisa Dufou-Hurtado: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Marcela Gaytán-Martínez:** Writing – review & editing, Supervision. **Angel H. Cabrera-Ramírez:** Writing – review & editing, Formal analysis, Data curation. **Emiro A. Leal-Urbina:** Writing – review & editing. **Mario E. Rodríguez-García:** Writing – review & editing, Formal analysis. **Aurea K. Ramírez-Jiménez:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fuco.2025.100742.

Data availability

Data will be made available on request.

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