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Native fungal strains from Yucatan, an option for treatment of biomethanated vinasse

Cepas fúngicas nativas de Yucatán, una opción para el tratamiento de vinazas biometanada

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Abstract

Vinasse is wastewater generated during ethanol production, with a high content of organic matter, and the presence of toxic and recalcitrant compounds that give it a dark brown color. Anaerobic digestion of vinasse is a low-cost method treatment that produces useful byproducts such as biogas. However, at anaerobic digestion of vinasse, a part of the organic matter, which includes melanoidins and phenolic compounds cannot be eliminated producing a wastewater named biomethanated vinasse (BV). It has been reported that fungi that produce ligninolytic enzymes can degrade recalcitrant compounds in raw vinasses but there is little information on the use of these microorganisms in the treatment of BV. In this work, seven fungi (*Trametes hirsuta* Bm-2 and AHB-6, *Phanerochaete chrysosporium* Bm-4, *Cochliobolus lunatus* AHB-1, and *Athelia rolfsii* AT5, AT13 and AT7) were evaluated for degrading BV. The pathogenic fungi *C. lunatus* and *A. rolfsii* species were not able to support the high concentration of toxic compounds present on the BV plate. On the other hand, *Trametes hirsuta* (Bm-2 and AHB-6) and *Phanerochaete chrysosporium* (Bm-4) can degrade and remove phenolic compounds present in BV in liquid medium. However, the Bm-2 strain it was the only microorganism able to produce a visible change in the color of the medium with a concentration of 25% BV, achieving 68.8% decolorization and 65.58% phenolic compound removal rate, with a maximum laccase activity (3415.9 U/ml) at 144 h. The results show the potential of *T. hirsuta* Bm-2 for degrading persistent toxic compounds present in biomethanated vinasses.

Keywords: Biomethaned vinasse, laccases, phenolic compounds, biodegradation, color removal.

Resumen

La vinaza es un residuo generado durante la producción de etanol, posee un alto contenido de materia orgánica y de compuestos recalcitrantes que le dan una coloración café oscuro. La digestión anaerobia de la vinaza es un método de tratamiento de bajo costo que produce subproductos útiles como el biogás. Sin embargo, en la digestión anaerobia de la vinaza, una parte de la materia orgánica, la cual incluye las melanoidinas y los compuestos fenólicos, no puede ser eliminada, generando un agua residual nombrada como "vinaza biometanada" (VB). Se ha registrado que los hongos que producen enzimas ligninolíticas pueden degradar compuestos recalcitrantes en la vinaza cruda pero existe poca información sobre el uso de estos microorganismos en el tratamiento de VB. En este trabajo, siete hongos (*Trametes hirsuta* Bm-2 y AHB-6, *Phanerochaete chrysosporium* Bm-4, *Cochliobolus lunatus* AHB-1, y *Athelia rolfsii* AT5, AT13 y AT7) fueron evaluados para degradar vinazas "biometanada" (VB, previamente tratadas por DA). Los hongos patógenos *C. lunatus* y *A. rolfsii* no fueron capaces de soportar la alta concentración de compuestos tóxicos en placas de VB; mientras, los hongos nativos *Trametes hirsuta* (Bm-2 y AHB-6) y *Phanerochaete chrysosporium* (Bm-4) fueron capaces de degradar y remover los compuestos fenólicos presentes en VB en medio líquido. La cepa Bm-2 fue la única capaz de producir un cambio visible en el color del medio con una concentración de 25% VB, alcanzado una decoloración de 68.8% y una remoción de compuestos fenólicos de 65.58%, con una máxima actividad de lacasa (3415.9 U/ml) a las 144 h. Los resultados muestran el uso potencial de *T. hirsuta* Bm-2 para degradar compuestos tóxicos persistentes presentes en vinaza "biometanada".

Palabras clave: Vinaza "biometanada", lacasa, compuestos fenólicos, biodegradación, eliminación de color.

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1 Introduction

The Renewable Fuels Association (RFA) pointed out that global production of ethanol reached over 108,641 million liters in 2018 (RFA, 2019). In Mexico it has presented an increase of 16.5 times the quantity produced in 2004 compared to 2014 and the trend is that it will continue to increase in the coming years (Pérez-Fernández and Venegas-Venegas, 2017). Also, the Tequila production, a traditional Mexican spirit, has seen a continuing growth due mainly to the increasing worldwide demand of Tequila, whose production reached 351.7 million of liters of Tequila in 2019 (CRT, 2019; Méndez-Acosta et al., 2010).

During ethanol production (such as biofuel, for industrial applications or alcoholic beverages) a waste product known as vinasse is generated which vary as function of the feedstock used in this process of which (between 10 and 18 liters for each liter of ethanol produced). This vinasse has a high content of organic matter (50 - 150 g/L of COD), and acid pH (3 - 5) and it has been reported the presence of toxic and recalcitrant compounds that give it a dark brown color of this waste product (Chowdhary et al., 2017; Noa-Bolaño et al., 2020). With the objective to diminish these characteristics, several studies have been carried out to develop efficient techniques in vinasse treatment, like physical-chemical and biological treatments. One of them, the anaerobic digestion of vinasse, which is a low-cost and low-energy method that not only produces the lowest amount of sludge but a useful by-products such as biogas (Gómez-Guerrero et al., 2019). However, the anaerobic digestion of vinasse do not eliminate the melanoidins and phenolic compounds (Parsaee et al., 2019; Serrano-Meza et al., 2020). Moreover, depending of the organic loading rate used in the anaerobic digestion reactor and the type of vinasse, a COD removal between 41 and 95% can be registered, which indicates that there is still organic matter that anaerobic digestion cannot degrade (España-Gamboa et al., 2012; García-Depraect et al., 2020; Gómez-Guerrero et al., 2019). This effluent obtained after the anaerobic digestion treatment of vinasse is known as biomethanated vinasse (BV) and its characteristics turns it into a wastewater that requires a previous conditioning treatment prior to its disposal in the environment. An option for treatment is biological decolorization and degradation, which is an environmentally friendly and cost competitive alternative in comparison with chemical decomposition (España-Gamboa et al., 2011). Several biological processes such as bioadsorption and biodegradation have reported color removal from raw vinasse by using fungi such as Coriolus, Aspergillus, and Phanerochaete; and certain bacterial sp. such as *Bacillus, Alkaligenes* and *Lactobacillus* (Chowdhary et al., 2017). Pazarlioglu et al. (2005) reported that these microorganisms produce extracellular enzymes including manganese peroxidase, lignin peroxidases, and lacasses under appropriate conditions, to be able of breaking many different chemical bonds. Recently, Ferreira et al. (2020), mention that the fungus *Pleurotus sajor-caju* can be applied in vinasse treatment, because this wastewater acts as a stressful environment for *P. sajor-caju* and that this microorganism maintains its homeostasis by increasing the activity of ligninolytic enzymes under reactive oxygen species (ROS) induced oxidative stress during vinasse degradation. However, most of this information is based on the treatment of raw vinasse and there is truly little information on the use of these ligninolytic microorganisms in the treatment of biomethanated vinasse.

Tapia-Tussell et al. (2011), isolated *Trametes hirsuta* Bm-2 from wood decay in the Yucatan Peninsula, Mexico. This fungus produced high laccase activity without the addition of mediators and increased extracellular laccase activity eight-fold when it was grown on a medium induced with agro-industrial substrates, compared to the basal medium. The laccases are enzymes that catalyze the oxidation of various aromatic compounds, specifically phenolic compounds (*ortho-* and *para*-diphenols, aminophenols and polyphenols), anilines, polyamines and aryl diamines, as well as some inorganic ions, while concomitantly reducing molecular oxygen to water (España-Gamboa et al., 2017). Three laccases of *T. hirsuta* Bm-2 were purified and characterized; these enzymes showed high resistance to organic solvents, thermostability, and an ability to decolorize synthetic dyes and textile effluents (Pereira-Patrón et al., 2019). Therefore, this study is focused on the degradation of phenolic compounds and decolorization of biomethanated vinasse, using *Trametes hirsuta* Bm-2, as well as a comparison with other white rot fungi, analyzing its potential use for the treatment of highly polluting wastewater in Mexico.

Table 1. Biomethanated vinasse characterization.

Parameter	Value*
pH	7.6 ± 0.08
Chemical oxygen demand	39788 ± 5610
Phenolic compounds	1110 ± 63
Nitrogen total	1115 ± 7
Ammoniacal nitrogen	315 ± 7
Sulfate	ND
Sulfide	8 ± 0.01
Total phosphorus	26 ± 6

*All values except pH are in mg/L.; ND: not detected.

2 Materials and methods

2.1 Biomethanated vinasse (BV)

BV was obtained from a UASB reactor with an operational volume of 4.2 L operated under mesophilic conditions (30 ± 5 °C) and fed daily with 300 ml of ethanol hydrous vinasse with an Chemical Oxygen Demand (COD) of 110,065 mg/L (España-Gamboa et al., 2017). The physicochemical VB characteristics is showed in Table 1.

2.2 Fungal Strains

Trametes hirsuta species [Bm-2 (GQ280373.1) and AHB-6 (GQ280372.1)], *Phanerochaete chrysosporium* [Bm-4 (GQ280374.1)] and *Cochliobolus lunatus* [AHB-1 (GQ280375.1)], were isolated from wood decay and henequen by-products in the Yucatan Peninsula, Mexico. Soil-borne plant pathogenic fungus strains (*Athelia rolfsii*), AT5, AT13 and AT7 were provided by the GeMBio laboratory of the Yucatan Scientific Research Center. These fungi were selected for their high enzymatic activity in previous studies and their capacity for growth on vinasse.

2.3 Determination of enzymatic activity on plate

Laccase production was detected with an ABTS (2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) oxidation-based method. An 0.8 cm-diameter disk of mycelium from each strain was inoculated on ME plates (Malt Extract 2% and Agar 2%) containing 5 nM ABTS, and then incubated for four days at 35 °C. The formation of a dark-green halo on the ME plates indicated a positive extracellular laccase secretion. The diameter and intensity of the halo were used as an indicator of the level of enzyme production (Tapia-Tussell et al., 2011).

2.4 Growth on BV plate

The growth of selected strains was evaluated on a BV plate, and for these two concentrations of biomethanated vinasse were employed (5 and 25% v/v) in a Yeast Malt Peptone Glucose Agar (YMPGA) culture medium (Dextrose 1%, Malt extract 1%, Peptone 0.2%, Yeast extract 0.2%, KH2PO4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1%, Thiamine 0.01% and Agar 2%). The YMPGA medium was prepared and added to the concentration of biomethanated vinasse, then the medium was autoclaved at 121 °C and 0.15 MPa for 20 min.

On the center of each plate, a 1-cm diameter disk of mycelium from each strain was inoculated and then incubated for seven days in darkness at 35 °C. This procedure was carried out in triplicate for each strain to be evaluated.

2.5 Inoculum preparation

Each strain was grown in ME medium with 2% (w/v) wheat bran added. This medium was placed in darkness at 35 °C for seven days. From these pure cultures, 10 disks of the 1cm diameter were transferred, under aseptic conditions, to 250 ml Erlenmeyer flasks containing 100 ml of sterile YMPG medium supplemented with 2% (w/v) of wheat bran. The flasks were incubated for 3 days at 35 °C and 150 rpm. Finally, the pellets obtained were ground with an HG-300D homogenizer (Hsiang Tai Machinery Industry Co.,). The resulting mycelial suspension was used for the subsequent experiments.

2.6 Decolorization and phenolic compounds degradation assays

250 ml Erlenmeyer flasks were filled with 100 ml of BV diluted by the addition of distilled water to obtain concentrations of 5%, 10%, 15%, 20% and 25% (v/v) in solution. The pH of these solutions was adjusted to 5.5 and they were autoclaved at 121 °C and 0.15 MPa for 20 min. Then, 2 ml of mycelial suspension was inoculated into each flask and subsequently incubated for 196 h with orbital rotation (130 rpm) in a New Brunswick shaker at 28 ± 2 °C. Three replicates were incubated for each concentration. Samples were

collected every 48 h for enzymatic activity. Total phenolic content and color were measured at 0 h and 196 h (see below for details). In order to know the amount of degraded organic matter, COD was measured at 0 h and 196 h, in the BV concentrations of 20% and 25% with the fungus that registered the greatest color and phenolic compounds removal.

2.7 Analytical procedures

The concentration of total phenolics was determined by the Folin-Ciocalteu method (Waterman and Mole, 1994), which measures the formation of a blue complex spectrophotometrically at 740 nm following the reduction of a phosphomolybdic-phosphotungstic reagent by phenolics. Gallic acid was used as the standard for plotting the calibration curve.

The decolorization was calculated according to the formula used by Sirianuntapiboon et al. (1995), according to the absorbance measurements at 475 nm. The reported color removal was calculated using the following equation:

$$Discoloration(\%) = \frac{(OD_i - OD_f}{OD_i} \times 100 \qquad (1)$$

where OD_i = initial absorbance, OD_f = final absorbance

Laccase activity was determined using the substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and was measured at 420 nm (ε_{max}= 36,000 L/mol·cm) (Wolfenden and Willson, 1982). One enzyme unit (U) is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute under assay conditions. The amount of enzyme production was expressed as U/ml.

COD content was determined via colorimetric methods (Hach Company DR-890).

2.8 Statistical analysis

Sampling was done in triplicate and the statistical differences were determined using a one-way analysis of variance (ANOVA) with 95% confidence limits. The statistical significance of the results was tested at $p < 0.05$ level using Tukey's test. All statistical analyses were performed using SAS 9.1 (SAS Institute, SAS Campus Drive, Cary, NC, USA).

3 Results and discussion

Determination of enzymatic activity on plate

The seven strains evaluated presented laccase activity. As can be seen in Figure 1, at 96 h, *T. hirsuta* (Bm-2 and AHB-6) strains showed dark green halos around the mycelial growth with diameters of 6.5 and 4.6 cm, respectively, *C. lunatus* AHB-1 also showed a 3.8-cm diameter halo. These results indicate that laccase activity in these strains is extracellular. Meanwhile, the *A. rolfsii* strains AT5, AT13 and AT17 had a smaller halo diameter. On the other hand, *Phanerochaete chrysosporium* Bm-4 did not show a halo indicating the excretion of laccase to the medium, but it could be noted that after 48 h, a color change due to oxidation of ABTS had occurred.

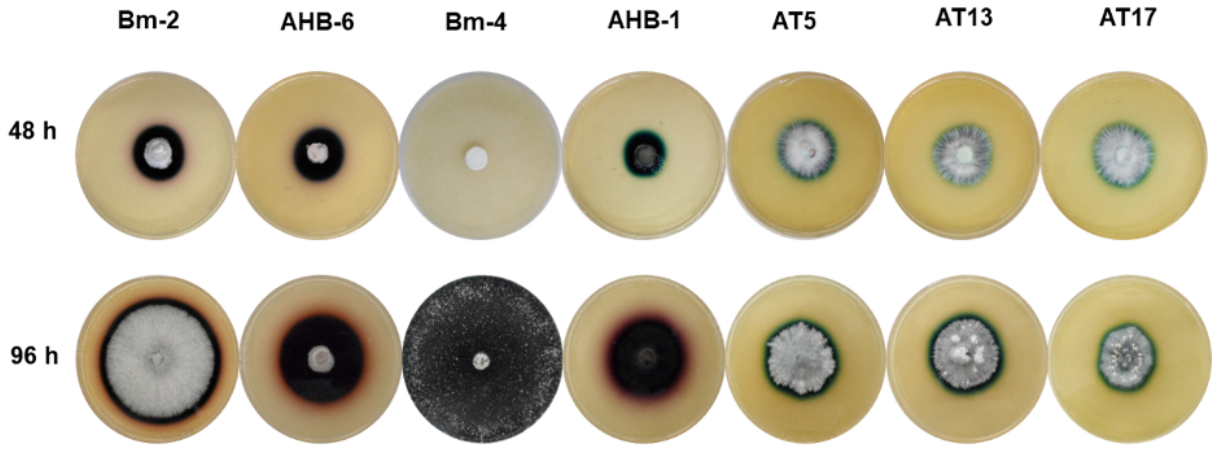

Fig. 1. Laccase activity in plate for fungus *Trametes hirsuta* (Bm-2 and AHB-6), *Phanerochaete chrysosporium* (Bm-4), *C. lunatus* (AHB-1), *Athelia rolfsii* (AT5, AT13 and AT7) at 48 h and 96 h.

These results agree with those reported by Tapia-Tussell et al. (2011), who detected the highest oxidative activity in *Trametes hirsuta* Bm-2 and AHB-6. In the case of *Phanerochaete chrysosporium* Bm-4, no extracellular laccase activity was produced since no halo formation was observed, except at the end of mycelia growth; ABTS oxidation was observed below the fungal colony, which might suggest that laccase activity in this fungus could be associated to the cell wall.

Baldrian (2006), mentions that localization of laccase is probably connected with its physiological function and determines the range of substrates available to the enzyme. Due to the properties of lignin, the enzymes that can breakdown it should be exclusively extracellular; however, this situation does not hold with laccases because it was reported that laccases of wood-rotting fungi are usually also found intracellularly and also associated with the cell wall and in spores. The cell wall and spore-associated laccases have been linked to the possible formation of melanin and other protective cell wall compounds.

Previous investigations have shown that *Trametes hirsuta* is a representative of basidiomycetes displaying a high lignolytic potential and producing high-redox-potential laccases (Rebrikov et al., 2006). Moreover, laccase from *Trametes hirsuta* can efficiently degrade a wide variety of compounds like synthetic dyes and antibiotics. This makes this biocatalyst very suitable for the treatment of wastewater from different industries (Navada and Kulal, 2019; Rosales et al., 2002). Zapata-Castillo et al. (2015), reported that *Trametes hirsuta* Bm-2 produces three laccase isoenzymes which have some properties in common, but differ in others. Their tolerance to temperature and organic solvents highlights the importance of these laccases for use in organic synthesis processes and/or for the treatment of textile industry effluents. This study showed that individual laccase fractions lacking mediators were not able to decolorize indigo carmine dye, but color removal was complete when syringaldehyde (a phenolic compound) was added to the reaction system. This suggest that laccases present in *Trametes hirsuta* Bm-2 are suitable for the treatment of biomethanated vinasse, since this wastewater has a high phenolic compounds content, which will active the laccases, helping to remove the phenolic compounds and color at the same time.

Phanerochaete chrysosporium is a white rot fungus that has been extensively studied for its exceptional ligninolytic properties, fast growth and easy handling under field conditions. This organism has the ability to mineralize a range of recalcitrant organic pollutants such as chlorophenols, nitrotoluenes and polycyclic aromatic hydrocarbons. Many of the useful properties of the fungus are due to its extracellular enzyme package (Gnanamani et al., 2006). In contrast to several other white rot fungi, it is generally accepted that *P. chrysosporium* produces lignin peroxidase (LiP) and manganese peroxidase (MnP), but no laccase (Kersten and Cullen, 2007). However, there are a few reports, such as Srinivasan et al. (1995), where it is shown that *P. chrysosporium* produces extracellular laccase, although laccase production appears to be relatively low even with ABTS, which is considered one of the more sensitive substrates for laccase assay. Thus, in this study it can be observed that *Phanerochaete chrysosporium* (Bm-4) is able to produce laccase, although it is not extracellular but probably associated with the cell wall, making it one of the first research papers to propose this kind of laccase production in *P. chrysosporium*.

In the case of *C. lunatus* (AHB-1) and *Athelia rolfsii* (AT5, AT13 and AT7), these microorganisms are pathogens for plants and they may have demonstrated laccase production because this enzyme has been shown to be an important virulence factor in many diseases caused by fungi. Among other roles, laccase can protect the fungal pathogen from the phytoalexins (toxic compounds synthesized by plants in response to microbial infection) and tannins in the host environment (Mayer, 2002).

Growth on BV plate

As can be seen in Figure 2, *T. hirsuta* (Bm-2 and AHB-6), *P. chrysosporium* (Bm-4) and *C. lunatus* (AHB-1) were the only fungi capable of growing on the BV plate at a concentration of 5% in EMA medium. Nevertheless, on the BV plate at a concentration of 25% in the YMPGA medium, the strain *C. lunatus* (AHB-1) was no longer able to grow and a remarkable inhibition on *T. hirsuta* (AHB-6) growth was observed. These results provide evidence that white rot fungi *T. hirsuta* and *P. chrysosporium* are able to tolerate high concentrations of phenolic compounds and melanoidins, the main components present in biomethanated vinasse.

Ferreira et al. (2010), evaluated the growth of select fungi in media containing different concentrations of vinasse (25, 50 and 100%).

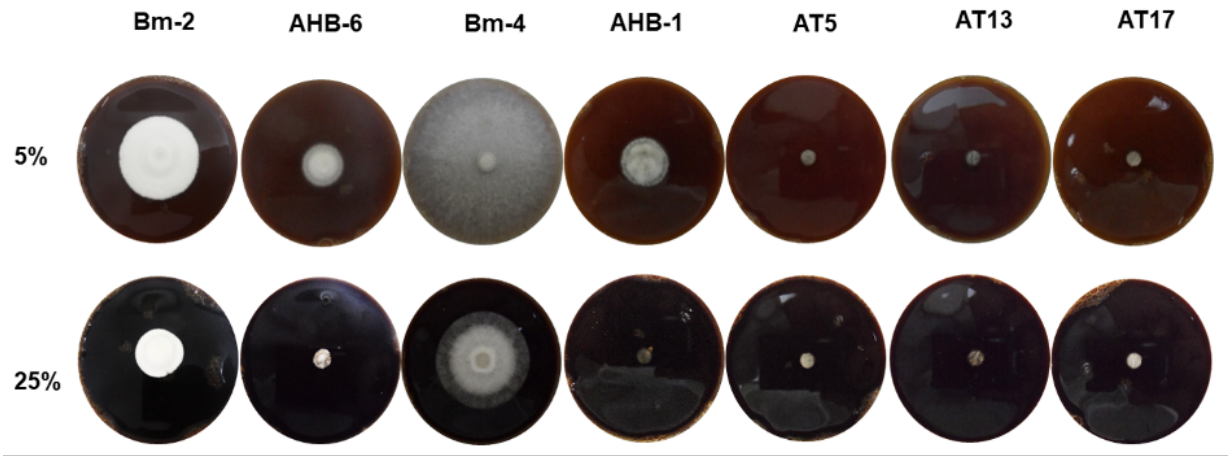

Fig. 2. Growth on seventh incubation day of the fungi *Trametes hirsuta* (Bm-2 and AHB-6), *Phanerochaete chrysosporium* (Bm-4), *C. lunatus* (AHB-1), *Athelia rolfsii* (AT5, AT13 and AT7) on BV plate at concentration of 5 and 25% (v/v) in YMPGA medium.

They found that the *P. chrysosporium* fungus, when cultivated in 25 and 50% concentrations, did not show any significant differences at 1% probability level when compared to the control sample. However, at 100% a reduction in growth occurred. A similar result was reported by Ahmed et al. (2018), *P. chrysosporium* fungus, was able to growth on plate with 10% of vinasse but when the concentration was increased (50 and 100%), the fungus was inhibited. These results are in agreement with those obtained in this work, since *P. chrysosporium* and *T. hirsuta* were able to grow at a high concentration of BV. However, it is evident that when there is an increase in BV in the medium, this has an inhibiting effect on growth.

Due to these inhibitory characteristics on the growth of microorganisms, the possibility of using vinasses and BV as biocides for phytopathogens has been studied. Santos et al. (2008), showed that the capacity for inhibition depends on the raw material from which the vinasse is produced (beet, sugar cane or wine), the type of pathogenic fungi on which it acts, and the concentration of vinasse in the culture medium, reaching inhibition values from 10 to 100%. It has been reported that the inhibitory capacity of vinasses is mainly due to the compounds that provide the vinasses with color (polyphenols and melanoidins), where polyphenols have greater antimicrobial effects than melanoidin at the same concentration. However, the reported concentration of polyphenol in vinasses is significantly lower than that of melanoidins. Therefore, it can be concluded that melanoidins are the main contributors to the antimicrobial effects, while polyphenol hexose degradation products only contribute a minor part (Arimi et al., 2014). In figure 2, we can see that *C. lunatus* (AHB-1) and *Athelia rolfsii* (AT5, AT13 and AT7) were not able to support the high concentration of toxic compounds present on BV plate, thus BV wastewater can act as a fungicide for these microorganisms. On the other hand, *T. hirsuta* (Bm-2 and AHB-6) and *P. chrysosporium* Bm-4 are potential strains able to diminish toxic compounds in this wastewater; therefore, they were selected for the treatment of aqueous dilutions of effluents and to evaluate this ability.

3.1 Decolorization and phenolic compounds degradation assays

As can be seen in Figure 3, the three selected fungi presented a percentage of color removal in each of the BV concentrations evaluated. However, *T. hirsuta* (Bm-2) was able to diminish color in biomethanated vinasse more efficiently, registering the highest percentage of decolorization (87.69%) at a concentration of 5% v/v of BV and the lowest percentage of decolorization (68.8%) in the highest concentration of BV evaluated (25% v/v). The second fungus with a good performance in decolorization was *P. chrysosporium* (Bm-4), achieving a value of 83.7% at a concentration of 5% v/v of BV, while the lowest percentage of color removal was at a concentration of 25% v/v of BV, registering a value of 35%.

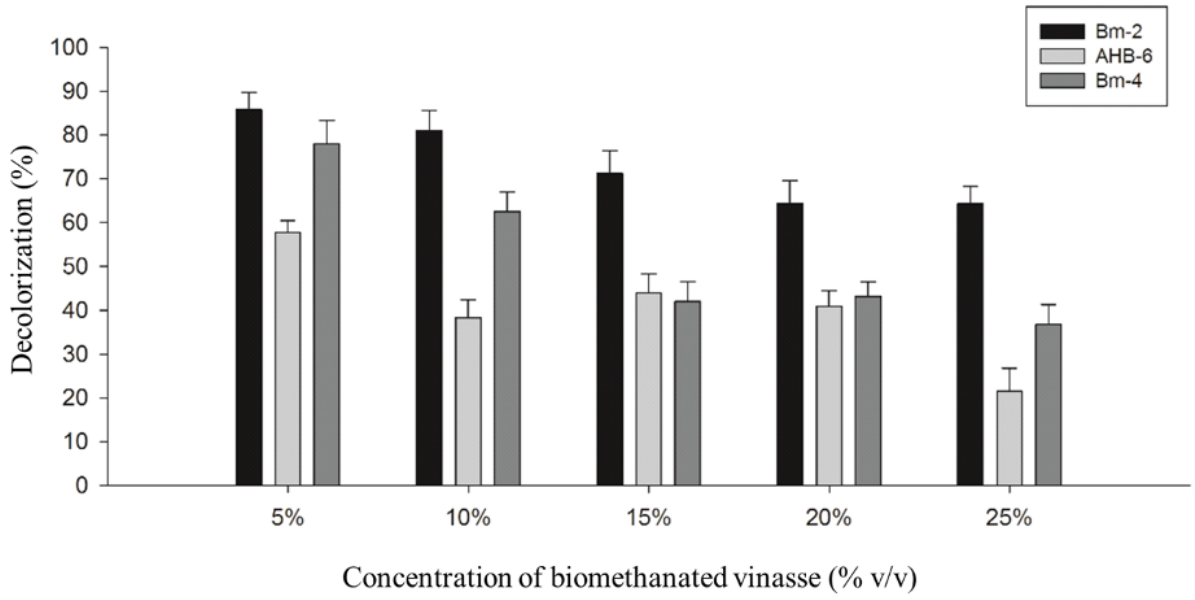

Fig. 3. Decolorization of biomethanated vinasse with T. hirsuta (Bm-4 and AHB-6) and *P. chrysosporium* (Bm-4) using different concentrations of BV in the liquid medium.

This last percentage is almost half of that obtained with Bm-2 at that same concentration. Finally, the *T. hirsuta* fungus AHB-6 was the microorganism with the lowest decolorization yield, obtaining its highest yield at a concentration of 5% v/v of BV (58%) and the lowest decolorization (20%) at a concentration of 25% v/v of BV.

It can also be observed in Figure 3, that the decolorization performance of fungi decreases when the BV concentration is higher in the liquid medium. Regarding the removal of phenolic compounds, as can be seen in Figure 4, the three fungi presented different removal percentages at the various concentrations evaluated. As in color removal, the Bm-2 strain showed the highest efficiency in the removal of phenolic compounds, registering removal percentages of 76.22%, 75.69%, 69.9%, 66.78% and 65.58% at a BV concentration (v/v %) of 5, 10, 15, 20 and 25%, respectively. These results show that the increase in the concentration of BV in the liquid medium apparently decreases the removal percentage of phenolic compounds. However, ANOVA statistical analysis performed on the removal percentages obtained with Bm-2 at the different concentrations showed that there are no significant differences ($p < 0.05$). Therefore, it is suggested that the inhibition presented by Bm-2 with respect to the removal of phenolic compounds is lower compared to the other microorganisms evaluated. Thus Bm-2 is a good option for the treatment of wastewater such as biomethanated vinasse.

On the other hand, *P. chrysosporium* Bm-4 presented its highest percentage of phenolic compounds removal (50.76%) at a concentration of 5% of BV. However, at a higher concentration of BV the efficiency decreased dramatically, reaching a figure of 2.58% when 25% BV was used. The behavior of the *T. hirsuta* strain AHB-6 was different compared to the other microorganisms, because it registered its best phenolic compound removal performance at 20% of BV, achieving a figure of 29.72%, while at a concentration of 25% of BV, AHB-6 removed 16.77% of the phenolic compounds. This yield was greater than that recorded by Bm-4, and therefore it is suggested that *T. hirsuta* is better for the elimination of phenols at high concentrations of biomethanated vinasse than the *P. chrysosporium* species.

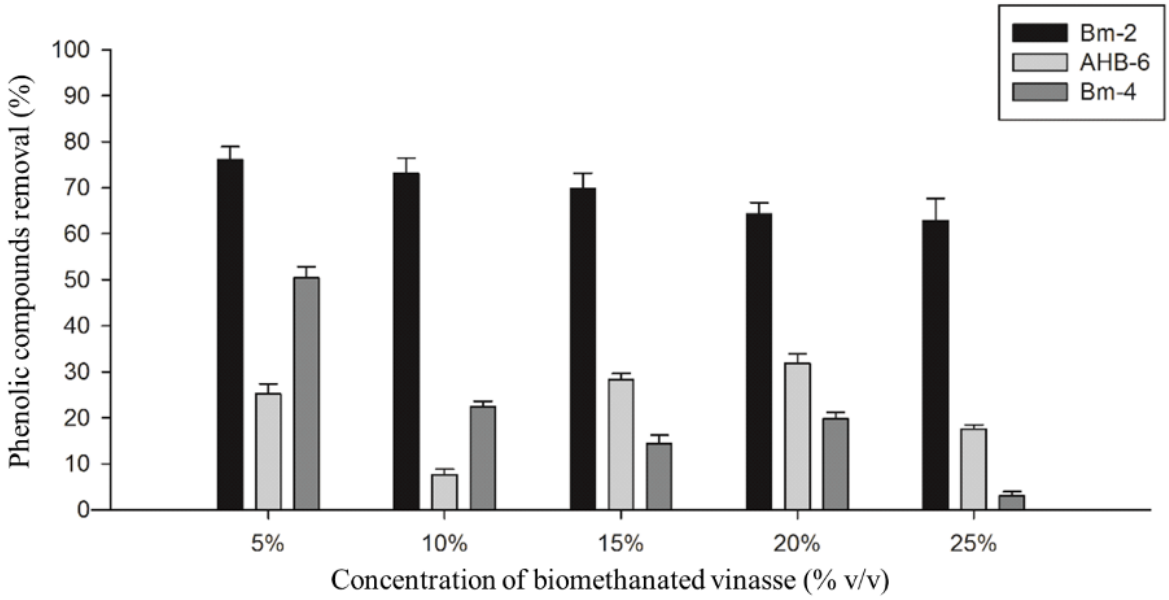

Fig. 4. Phenolic compounds removal in biomethanated vinasse with *T. hirsuta* (Bm-4 and AHB-6) and *P. chrysosporium* (Bm-4) using different concentrations of BV in the liquid medium.

Figure 5 shows the values of laccase activity in each fungus evaluated at the different concentrations of BV, and also the change in color with respect to the control for each dilution employed in the liquid medium.

In Figure 5, it can be seen that the microorganism with the highest production of laccase activity in the different concentrations of BV evaluated is *T. hirsuta* Bm-2. This fungus registered a maximum laccase activity of 1391.6 U/ml, 3475.0 U/ml, 3663.8 U/ml, 3411.1 U/ml and 3415.9 U/ml at BV concentrations of 5, 10, 15, 20 and 25%, respectively. On the other hand, the microorganisms *T. hirsuta* AHB-6 and *P. chrysosporium* Bm-4 had low laccase activity, registering a maximum value throughout the experiment of 214.1 U/ml and 122.0 U/ml respectively, at a concentration of 5% of BV for both AHB-6 and Bm-4.

In Figure 5a, corresponding to the concentration of 5% v/v of BV, it can be seen that Bm-2 was the microorganism that produced the most enzymatic activity, reaching its maximum value (1391.6 U/ml) after 96 hours. Under these conditions, Bm-2 achieved 87.69% decolorization of the medium with BV and removed 76.22% of the phenolic compounds present. On the other hand, Bm-4 in medium with 5% v/v BV registered 83.7% decolorization and 50.76% removal of phenolic compounds. These values are very close to those reached by Bm-2. However, as can be seen in Figure 5a, the laccase activity in Bm-4 was much lower than that recorded by Bm-2, reaching its maximum value (122 U/ml) after 192 hours. It is supposed that Bm-4 can remove color and phenols due to the presence of laccase associated with the cell wall, which is consistent with the results obtained in the determination of enzymatic activity on the plate. There is little information about microorganisms that have laccase associated with the wall. Kaur et al. (2019) found that laccase synthesizing Acinetobacter sp. was firmly cell-attached, and they mentioned that there is no report available on bacterial laccases, where enzymes attached to the prokaryotic cells and showed "whole cell" bonded biocatalytic potential. Enzymes bonded to cells offers great mileage from the industrial point of view, because there is no need for purification of laccase before its use. Thus, Bm-4 could be a good option for the treatment of BV at low concentrations (5 and 10%). However, further study is needed on the properties of these laccases bonded to the cell wall. Similarly, it is necessary to evaluate the presence of other ligninolytic enzymes such as manganese and lignin peroxidase, which have been reported in the degradation of phenolic compounds and melanoidins in vinasse (Srinivasan et al., 1995).

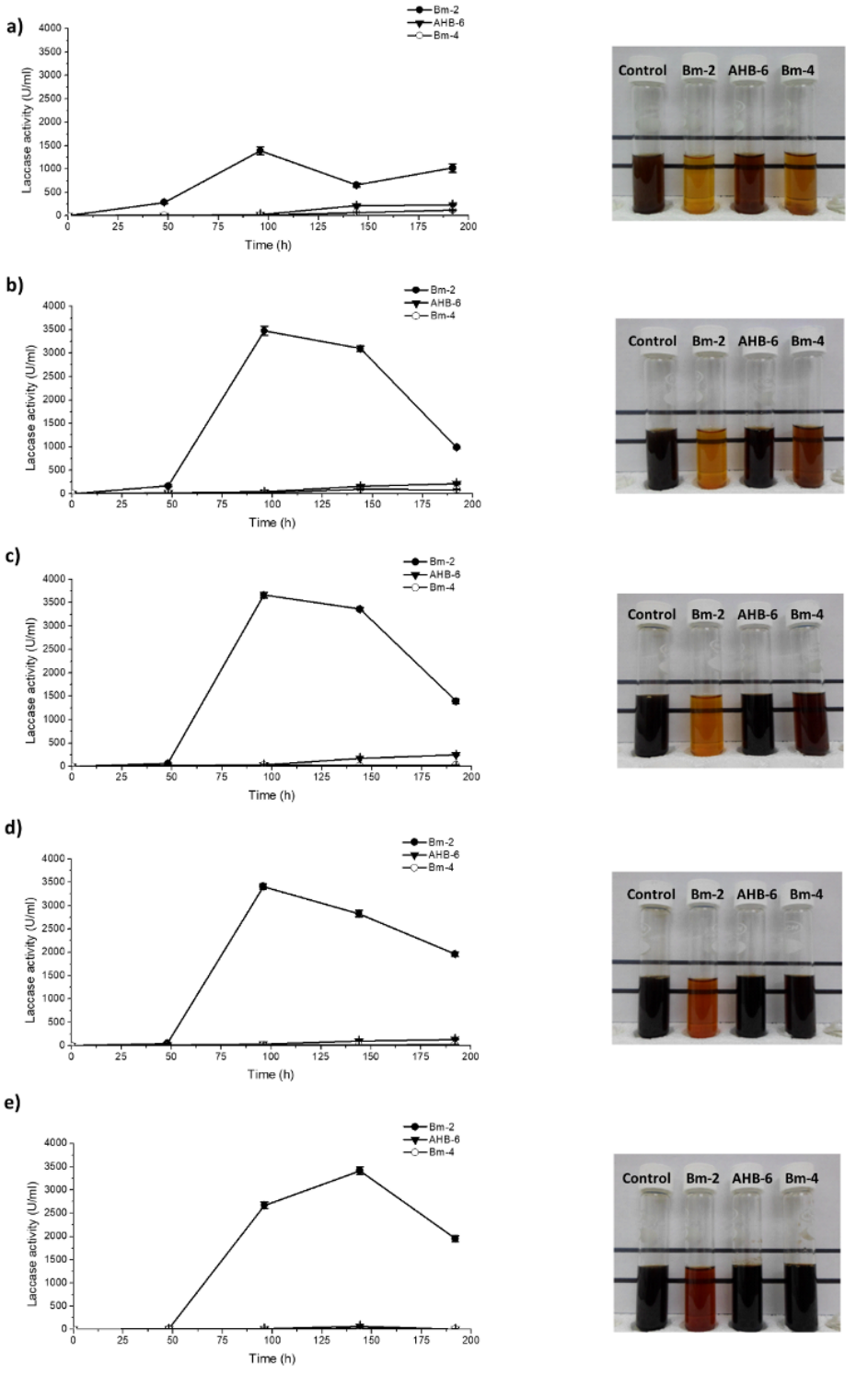

Fig. 5. Laccase activity and color change in biomethanated vinasse treated with *T. hirsuta* (Bm-4 and AHB-6) and *P. chrysosporium* (Bm-4): a) 5% v/v, b) 10% v/v, c) 15% v/v, d) 20% v/v, e) 25% v/v.

At 5% concentration of BV, *T. hirsuta* AHB-6, presented a behavior similar to Bm-4, since AHB-6 recorded 58% decolorization and 22.7% phenolic compounds removal. However, it showed a maximum enzymatic activity of 233.3 U/ml. Therefore, it would be necessary to assess whether there are any other enzymes in this microorganism capable of degrading phenolic compounds and melanoidins, as well as assessing the possible existence of laccase associated with the cell wall. However, it is evident from Figure 5, that AHB-6 is not able to tolerate high concentrations of BV, and therefore, for the treatment of this type of waste, AHB-6 is not the most suitable microorganism. It is important to highlight that although Bm-2 and AHB-6 are strains of the same species, they demonstrated very different behavior in the decolorization of these effluents. This may because they were isolated from different sources and as laccases are induced by multi-gen families, different types of laccases may be produced in each strain (Thurson, 1994). In a previous study, Pereira-Patrón et al. (2019) identified and characterized laccase genes produced by T. hirsute Bm-2 in a liquid medium, with (lac-T) and without (lac-B) induction. Both laccase-predicted proteins consisted of 521 amino acids, 4 copper-binding regions, a signal peptide, and 5 potential glycosylation sites. However, the in-silico analysis predicted that the 5'UTR of both genes is folded into structures with several hairpins, whose number and the location were different between the genes, radiating from a central loop region. Therefore, although the 5'UTRs of lac-T and lac-B are both long, the expression levels are drastically different, in that the medium with induction showed laccase activity 50 times higher than the basal medium.

As can be seen in Figure 5e, Bm-2 was the only microorganism able to produce a visible change in the color of the medium with a concentration of 25% BV, achieving 68.8% decolorization and 65.58% phenolic compounds removal, with a maximum laccase activity (3415.9 U/ml) after 144 hours. This laccase activity was 2.5 times greater than the maximum laccase activity achieved at a concentration of 5% BV. The increase is likely because at higher concentrations of BV there will be a greater quantity of phenolic compounds, which function as inducers and/or mediators of the enzyme, to unfold the more complex substrates (Zapata-Castillo et al. 2015). After 192 hours, the laccase activity dropped to a value of 1953.4 U/ml. The decrease in activity was due to the limitation of the substrate in the last hours of experimentation (Malmstrom et al., 1969). The foregoing results agree with Tapia-Tussell et al. (2015), who studied the expression of laccase genes (lcc1 and lcc2) in *T. hirsuta* Bm-2. The results indicated that phenolic compounds specifically regulate the laccase genes of this fungus and, in general, the compound with the highest influence on enzyme production was guaiacol, given that transcript levels in the presence of guaiacol were 40 times higher than in the control. Due to that *T. hirsuta* Bm-2 presented the best yields for phenolic compounds and color removal, the COD content was quantified in the tests with the highest concentration of BV (20 and 25%). These concentrations were selected, because at the industrial scale, the aim is to dilute the wastewater as little as possible by giving it any kind of treatment. At 20% BV concentration, the COD initial content was 25,600 mg/L and *T. hirsuta* Bm-2 was able to decrease to a final value of 5,900 mg/L, reaching a COD removal of 76.9%. On the other hand, at 25% BV concentration, *T. hirsuta* Bm-2 registered a COD removal of 84.7%, decreasing the initial COD value (27,400 mg/L) to a final COD value of 4,200 mg/L. Demonstrating that this microorganism is capable of consuming the organic matter that the microorganisms in the previous anaerobic treatment were not capable of degrading.

Regarding to raw vinasse, Junior et al. (2020) evaluated the color removal on sugarcane vinasse using *Pleurotus sajor-caju*, these authors reaching values of decolorization between 82 and 92% and the vinasse decolorization was better in the media containing inducers such as ethanol and CuSO4. *T. hirsuta* (Bm-2) registered a range of decolorization of 68.8 - 87.9%, these values are lower than those reported by *P. sajor-caju*, nevertheless, no inducers for laccase were added to the BV medium and the chemical composition of BV is different to raw vinasse; for example, it is possible that raw vinasse contains small amounts of ethanol that could not be distilled, while BV, as it comes from an anaerobic digestion process, is not present (España-Gamboa et al., 2011). Ahmed et al. (2018), evaluated the capacity of native strains in the treatment of raw vinasse diluted (10% v/v), a Trametes sp. registered a color removal of 43%, and 80% decrease of total phenolic compounds after 10 days incubation. *T. hirsuta* (Bm-2) at the highest concentration of BV evaluated (25%) was able to remove the 65.58% of total phenolic compounds and registered a decolorization of 68.8%, these results are similar to Ahmed et al. (2018), but *T. hirsuta* (Bm-2) reached these values in a period of 8 days incubation.

There is little information on the fungal treatment of biomethanated vinasses since investigations are mainly focused on the treatment of raw vinasses, and therefore the results obtained in this study are relevant, in special in a country like Mexico where ethanol production is increasing over the years. Ravikumar et al. (2007) developed a bioremediation method for biomethanated distillery spent wash. They incubated several fungi in flasks containing 20% BV. However, although the fungi were able to remove chemical oxygen demand (63%), there was no change in the color of the effluent. Ligninolytic enzymes (LiP, MnP and laccase) present in some white rot fungal species are capable of degrading xenobiotics and organo-pollutants. *Phanerochaete chrysosporium* and Trametes versicolor are the most widely studied among these. *P. chrysosporium* JAG 40 resulted in 80% decolorization of the liquid medium of 6.25% anaerobically digested spent wash. Moreover, T. versicolor produces a 47 kDa extracellular enzyme identified as peroxidase, which is involved in the mineralization of melanoidins. In other words, the fungus produced 82% decolorization of 12.5% anaerobically-aerobically treated effluent (Chowdhary et al., 2017). These results reported in the literature are similar to those recorded by Bm-2 at the different concentrations evaluated. However, it is important to note that *T. hirsuta* Bm-2 was able to remove color (68.8%) and phenolic compounds (65.58%) at a higher concentration of dilution of BV (25%). This is reflected in a lower use of fresh water to achieve this dilution. thus, resulting in a benefit to the environment, both by reducing the consumption of fresh water and by reducing recalcitrant compounds that are normally disposed to the environment without a prior treatment. This makes this strain (Bm-2) a technological choice for the treatment of BV. It is proposed that for future research it would be important to evaluate the use of this new effluent for the fertilization and irrigation of crops and thus bring an economic benefit to farmers.

Conclusions

The pathogenic fungi *C. lunatus* (AHB-1) and *Athelia rolfsii* (AT5, AT13 and AT7) were not able to support the high concentration of toxic compounds present on the BV plate. On the other hand, *T. hirsuta* (Bm-2 and AHB-6) and *P. chrysosporium* Bm-4 are strains potentially able to diminish toxic compounds in this wastewater; therefore, they were selected for the treatment of aqueous dilutions of effluents. All strains evaluated: *T. hirsuta* (Bm-2 and AHB-6), and *P. chrysosporium* (Bm-4) have the ability to degrade and remove phenolic compounds present in BV. It is suggested that Bm-4 has the ability to remove color and phenols due to the presence of laccase associated with the cell wall, which is consistent with the results obtained in the determination of enzymatic activity on the plate.

The Bm-2 strain was the most efficient, since it was the only microorganism able to produce a visible change in the color of the medium with a concentration of 25% BV, producing 68.8% decolorization and 65.58% phenolic compounds removal, with a maximum laccase activity (3415.9 U/ml) after 144 hours.

Due to scarcity or an exponentially increased use of water in the world, the need to replenish water to a water source or system (groundwater, surface water) has also been raised (NRC, 2008). Thus, the importance of having strategies and technologies that allow this replenishment in such a way that water quality is not affected, justify the importance to develop better methodologies to process by-products such as vinasses. In Mexico, the use of raw vinasse for fertigation has been recorded, being necessary to dilute it at 10% v/v to avoid the saturation of the soil and contaminate nearby water bodies. With this work it was possible to appreciate that if an anaerobic digestion treatment for vinasse is carried out and later the implementation of ligninolytic fungi such as *T. hirsuta* (Bm-2) for the elimination of recalcitrant compounds, the consumption of fresh water will be reduced and a wastewater of better quality will be available to the environment.

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Nomenclature

ABTS: 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ANOVA: Analysis of variance
BV: Biomethanated vinasse
COD: Chemical Oxygen Demand
RFA: Renewable Fuels Association

UASB: Upflow Anaerobic Sludge Blanket
YMPGA: Yeast Malt Peptone Glucose Agar

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