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Development of xylophagous fungi native to Veracruz in sugarcane (*Saccharum* spp.) distillery vinasses on inert materials of zeolite and agricultural lime

Schettino-Salomón, Blas[1]; Llarena-Hernández, R.C.[2], Hernández-Martínez, R.[3]; Hernández-Rosas, F.[1]

[1] Colegio de Postgraduados Campus Córdoba, Postgrado en Innovación Agroalimentaria Sustentable, Km 348, carretera federal Córdoba-Veracruz, Congregación Manuel León, Amatlán de los Reyes, Veracruz, C.P. 94953.

[2] Facultad de Ciencias Biológicas y Agropecuarias. Universidad Veracruzana. Carretera Peñuela Amatlán de los Reyes, Veracruz. México. C.P. 94945.

[3] IxMx-SECIHTI-Colegio de Postgraduados Campus Córdoba, Km 348, carretera federal Córdoba-Veracruz, Congregación Manuel León, Amatlán de los Reyes, Veracruz, C.P. 94953.

* Correspondence: fhrosas@colpos.mx

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ABSTRACT

Objective: To establish the development conditions of white-rot fungi with the use of vinasse as substrate and zeolite and commercial agricultural lime as support.

Design/methodology/approach: Pure vinasse from a sugarcane distillery was used as the main nutrient on a support of vinnase-zeolite and vinasse-lime in 1:1 proportion. The variable of study was the daily radial growth (mm-day^{-1}) of each of the four isolates on vinasse-inert culture medium. The experimental unit was glass Petri dishes of 90×15 mm by triplicate. A Mann-Whitney U test was conducted (using R Studio).

Results: Four fungal isolates (G=*Ganoderma* sp. K., P=*Pleurotus* sp. B., Py=*Pycnoporus* sp. K., and Tr=*Trametes* sp. C.) were cultured on zeolite-vinasse and agricultural lime-vinasse and their daily radial growth was recorded. Two fungal isolates were able to develop tissue covering the Petri dish in 9 days. Zeolite-vinasse was the best treatment, showing a median of 2.1 mm-day^{-1} on isolate G and 1.6 mm-day^{-1} on isolate P with significant differences ($p<0.05$).

Limitations on study/implications: All the white-rot fungi strains test were cultured at 28 °C and minimal development conditions with the use of a byproduct of the sugarcane industry and the use of non-conventional inerts.

Findings/conclusions: The development of mycelium by the isolated fungi G and P on a substrate with zeolite-vinasse recorded the highest growth compared to the agricultural lime.

Keywords: Zeolite, agricultural lime, vinasse, white-rot-fungi.

INTRODUCTION

Sugarcane (*Saccharum* spp.) is one of the crops that generates an important agroindustrial activity in Mexico. It is grown in 15 states with a surface of 800 thousand hectares, operated

by 49 sugar mills (SADER, 2021). Within this agroindustry, the production of sugarcane ethanol is considered a solid industry (López-Ortega *et al.*, 2021). Vinasse is a black liquid of unpleasant odor generated as a final byproduct of the distillation process of sugarcane juice to obtain ethanol. This vinasse contains a high content of organic matter that could trigger a negative environmental impact if it is not treated adequately (Montiel-Rosales *et al.*, 2022). It is estimated that for every liter of ethanol produced, 10 to 15 L of vinasse is obtained (Cortes-Rodríguez *et al.*, 2018). The excessive incorporation of vinasses into soils modifies the pH and conductivity and can cause toxicity where it is dumped (Nakashima and de Oliveira Junior, 2020). Among the conventional treatments to treat contaminant effluents of agroindustrial activities, anaerobic degradation can be achieved through activated muds, rotative biological contactors, shallow ponds, or percolating filters. However, the processes can be inhibited with the presence of phenol compounds, salt and sulfur ions (Carpanez *et al.*, 2022; Fernandes *et al.*, 2020). Among the treatments of vinasses, the use of fungal biodegradation is considered an alternative potential. Xylophagous fungi called white-rot fungi have demonstrated the ability to degrade different synthetic and natural contaminants, through their lignocellulosic enzymes (Grelska and Noszczyńska, 2020; Saglam *et al.*, 2018). These enzymes are not specific, although they allow the degradation of recalcitrant compounds (Del Gobbo *et al.*, 2022). There are precedents in the bioremediation of sugarcane vinasses performed in a fixed-bed bioreactor with *Pleurotus ostreatus* (FUNGICOL S.A.S) for the removal of substances that cause coloring in the vinasse, decoloring 83% after 36 days (Tapie *et al.*, 2016). "Airlift" bioreactors with concentric tube have also been evaluated, managing to discolor the vinasse between 58 and 78% using a strain of *Pleurotus sajor-caju* CCB020 (Aragão *et al.*, 2020). Other species such as *Trametes* sp. T3 showed 75% of discoloring when it is immobilized on polyurethane, and a production of laccase enzymes of 112 U L^{-1} after 21 days (Ahmed *et al.*, 2022). Usually, all these practices are conducted in liquid media on bioreactors. Among the techniques used in the removal of contaminants from effluents, the use of zeolites has been helpful in the treatment of residual water, mainly because of their crystalline structure, capable of storing water within its pores that can be liberated through heat (de Magalhães *et al.*, 2022). As materials for remediation, they are more efficient in the removal of heavy metals in residual waters than in the soil (Otunola and Ololade, 2020). Given the accomplishments developed, the following research had the single objective of establishing the conditions of development on zeolite and commercial agricultural lime as inert bases for the development of biomass from local isolates of xylophagous fungi using vinasse as the main substrate.

MATERIALS AND METHODS

The experiment was carried out in the facilities of the Applied Microbial Biotechnology laboratory of Colegio de Postgraduados, Campus Córdoba (N 18° 51' 22" W; 96° 51' 34"), in the municipality of Amatlán de los Reyes, Veracruz.

Samples of fungi collected

Fructiferous bodies of white-rot fungi were collected from trees fallen in the experimental fields of Colegio de Postgraduados, Campus Córdoba, located on N

18° 51' 22.967" W; 96° 51' 36.434". The collection was conducted according to the methodology by Guzmán (1990).

Procurement of vinasse

The vinasse was obtained from a storage tank in Alcoholera de Zapopan S.A. de C.V., Atoyac, Veracruz. The vinasse sample was taken directly from the storage tanks. After a few hours of the bioethanol production process, 20 L were collected. The vinasse was transported to the laboratory and stored in a cold room at 4 °C until its use (Del Gobbo *et al.*, 2022). The nutritional composition of vinasse was shared by the technical staff, and it is presented in the following Table 1.

Procurement of inerts

The zeolite used in this study was acquired from the company Grupo COYPUS SA. De C.V. Based on an X-ray study and EDS chemical analysis, it was found that zeolite is constituted by 10% Clinoptilolite and 90% Heulandite (Not published). Commercial lime from Agrical Grupo Calidra S.A de C.V. was used, whose constitution is $CaCO_3$, 90-98.5% and SiO_2, 0.1-2%.

Isolation and conservation of white-rot fungi

The sporocarps of xylophagous fungi collected were washed three times with purified water, the soil was removed with sterile gauze, submerged in ethanol at 70% and the excess water was eliminated on previously sterilized filter paper. Then, with the use of

Table 1. Nutritional and physicochemical composition of the sugarcane juice vinasse.

Test	Result	Contribution kg/L
N %	0.07	0.71
P %	0.013	0.31 (P_2O_5)
K %	0.223	2.73 (K_2O)
S %	0.057	0.58
Mg %	0.028	0.29
Ca %	0.033	0.34
Na ppm	< 250	< 250
Al ppm	< 50	< 0.05
Mn ppm	19.2	0.019
Cu ppm	< 2.5	< 0.003
Zn ppm	< 12.5	< 0.013
B ppm	< 25	< 0.025
Moisture %	97.7	-
Dry matter %	2.3	-
pH	3.71	-
CE mmhos/cm	8.81	-

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the blade of a #11 Braun® scalpel, the fungus pileus was dissected and a fragment of the tissue from the inside was taken. The tissue dissected was placed on potato dextrose agar (PDA) BD Bioxon® (39 g/L^{-1}). They were incubated at 28 °C in a Binder® incubator for 10 days (Liu *et al.*, 2016). After 10 days of growth in PDA, the fungi mycelium was dissected from the Petri dish, preserving the fungi tissue inside cryovials with distilled water and others with glycerol at 6% under refrigeration at 4 °C (Abatenh, 2018; Linde *et al.*, 2018). Then, it was resown in PDA medium to maintain fresh cultures for the experiments.

Experimental design

Two treatments were evaluated: 1) Mixture of 20 ml raw vinasse with 20 grams of zeolite, and 2) 20 ml vinasse with 20 g of agricultural lime. Each treatment was mixed homogeneously and placed in crystal Petri dishes of 90×15 mm and sterilized for 15 min at 15 Lb pressure.

Four isolates from the fungi collected after 10 days cultured on PDA were used. From the fungus mycelium, 1 cm^2 was taken from each of the isolates and placed in the middle of the crystal Petri dishes with the mixture of vinasse-zeolite and vinasse-agricultural lime. The Petri dishes with each treatment were sealed with 45 mm plastic film. Triplicates of each isolate were made.

Radial growth

The radial growth of mycelium on the treatments of raw vinasse mixture on vinasse-zeolite and vinasse-agricultural lime was recorded, where the methodology of radius of the colony was used (Bilay *et al.*, 2000; Zubyk and Klechak, 2023). The growth speed (Vc) was estimated through the equation:

$$Vc = \frac{Ri - Rf}{\Delta t}$$

Where: Ri is the initial radius of the colony in mm and Rf is the final radius of the colony in mm and Δt is the linear duration in days. The experiment ended when the mycelium colonized the limits of the Petri dish.

Statistical analysis

The response variable was the radius of the colony. The data distribution was verified and subjected to a Mann-Whitney-U non-parametric analysis, to compare differences in the radial growth on the vinasse-zeolite and vinasse-agricultural lime mixture ($p<0.05$) according to McKnight and Najab, (2010). Statistical analyses were performed with the Rstudio software (R core team, 2022). The experimental unit was a Petri dish with treatments of 1) vinasse-zeolite and 2) vinasse-agricultural lime, and 3 repetitions of each of the treatments from each isolate were carried out.

RESULTS AND DISCUSSION

From the wood rot, 4 fungi were obtained. The determination of mycelium was made following the macroscopic traits (Guzmán, 1990; Stalpers, 1978; Bettucci *et al.*, 1971). Each isolate received a code (Figure 1).

Two isolates showed mycelial development on zeolite and agricultural lime. In both isolates, growth was observed on the treatments. The isolates codified as G and P began to develop on day 4 and 5; and until day 8 and 9, they covered the Petri dish, respectively. The Mann-Whitney-U analysis on the treatments of the isolate G on vinasse-zeolite and vinasse-agricultural lime showed that the radial growth in vinasse-zeolite (median=2.1 mm, Interquartile Range=1.2, 3.5 mm) differed significantly ($p<0.05$) from the growth on vinasse-agricultural lime (median=1 mm, Interquartile Range=0.4, 1.9 mm). Meanwhile, the isolate P on vinasse-zeolite (median=1.6, Interquartile Range 1.1, 3.2 mm) differed significantly ($p<0.05$) from vinasse-agricultural lime (median=0.7 mm, Interquartile Range=0.3,1.8).

During the growth of isolates on the vinasse-zeolite and vinasse-agricultural lime treatments, the mycelium showed different morphology; the isolate G on vinasse-zeolite, vinasse-agricultural lime presented vegetative mycelium, while the isolate P had aerial growth reaching the superior margins of the Petri dish on both treatments (Figure 2).

Because of the microporous structure of zeolite, it allows the transference of matter between the intracrystalline space and the medium surrounding it, in addition to its composition in AlO_4 which confers negative charges that can be neutralized by compensation cations (de Magalhães *et al.*, 2022; De Smedt *et al.*, 2015). These characteristics provide important properties in the capacity for adsorption and ionic exchange for the possible use of zeolite as support for immobilization (Borges-Rodríguez *et al.*, 2012). Meanwhile,

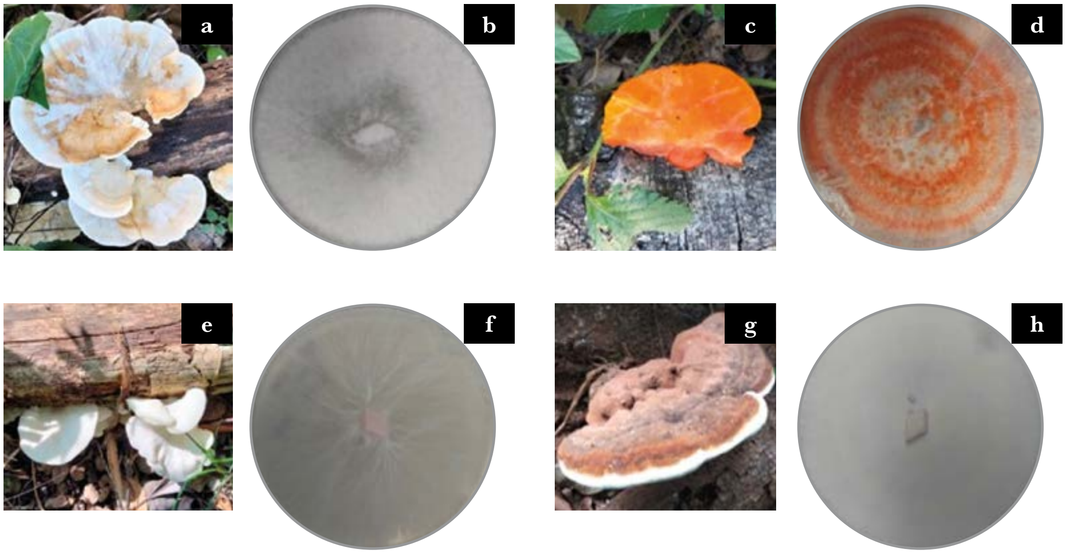

Figure 1. White-rot fungi in situ and on PDA after 10 days: a) *Trametes* sp. C (T), b) *Trametes* sp. C (T) on PDA, c) *Pycnoporus sanguineus* aff. K. (Py), d) *Pycnoporus sanguineus* aff. K (Py) on PDA, e) *Pleurotus djamor* aff, B, (P), d) *Pleurotus djamor* aff. B (P) on PDA, g) *Ganoderma* sp. K. (G), h) *Ganoderma* sp. K on PDA.

Table 2. Mycelial development of white-rot fungi on vinasse-zeolite and vinasse-agricultural lime. The mean of the radial growth (mm-day^{-1}) is presented, and the standard deviation, difference between medians of zeolite and agricultural lime (mm-day^{-1}), respectively.

Isolate code	Vinasse-Zeolite (mm-day^{-1}) ± Standard deviation	Vinasse-Agricultural lime (mm-day^{-1}) ± Standard deviation	Median (mm-day^{-1})
G	2.27±1.44	1.24±1.14	2.1 *vs.* 1.0*
P	1.97±1.27	1.04±1.01	1.6 *vs.* 0.7*

*=Significant difference ($p<0.05$).

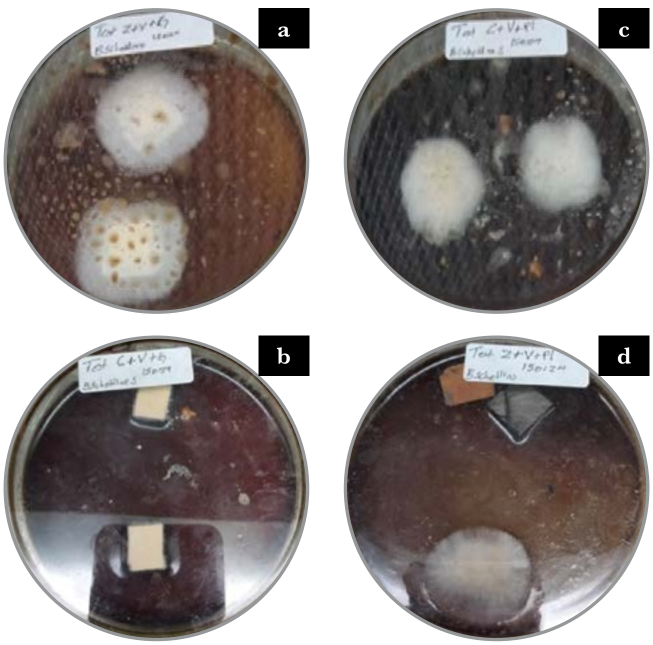

Figure 2. Demonstrateive assay of mycelium growth on treatments at day 7, where: a) Isolate G with vinasse-zeolite, b) Isolate G with vinasse-agricultural lime, c) Isolate P with vinasse-agricultural lime, and d) Isolate P with vinasse-zeolite.

the capacity for biodegradation of xylophagous fungi is linked to the enzymatic oxidative capacity of oxidases, peroxidases, oxidoreductases, especially laccases, which facilitate the degradation of complex contaminant structures to intermediate metabolites that are easier to degrade (Vaksmaa *et al.*, 2023).

CONCLUSIONS

The fastest growth on the treatments of vinasse-zeolite and vinasse-agricultural lime was obtained on vinasse-zeolite. Therefore, it is possible to use vinasse as a source of substrate to obtain fungal biomass of xylophagous fungi on inert materials such as zeolite and agricultural lime as support. It is the first study where an inert of mineral origin like zeolite is used, agricultural lime as support, and vinasse as substrate for the development of xylophagous or white-rot fungi. The colonization on treatments was achieved in 8 and 9 days. The development of the fungi allowed metabolizing the vinasse that served as substrate on the support of zeolite and agricultural lime. This study allows the bases to understand with future vision the level of traces retained in zeolite and agricultural lime, so that vinasses have a lower environmental impact when they are dumped in agricultural fields or runoffs that can affect living organisms in bodies of water.

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