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Utilization of the Fungus *Pycnoporus* sp. for Remediation of a Sugarcane Industry Effluent

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Abstract: Lignocellulosic fungi are highly versatile organisms with valuable applications in bioremediation processes, including the biodegradation of agro-industrial effluents. In this work, the use of a native strain of the white-rot fungus, *Pycnoporus* aff. *sanguineus*, was studied for its use in the bioremediation of the sugar industry waste called vinasse, originating from the San Martín del Tabacal Sugar Mill, located in the north of the Salta province, Argentina. We studied under controlled laboratory conditions the bioremediation process of 3 concentrations of vinasse (5, 10, and 25% in distilled water) with a native isolated strain. The results showed biomass growth at all three tested concentrations, with a maximum at the highest vinasse concentration (25%), while the percentages of color and Chemical Oxygen Demand (COD) removal indicated that the most efficient treatment was with 10% vinasse. The results obtained are promising for the treatment of effluents from the sugar industry using white-rot fungi considering the valuable subproducts of *Pycnoporus* spp. biomass.

Keywords: mycoremediation; industrial waste; vinasse

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1. Introduction

The sugar agroindustry has historically been one of the most significant economic activities in the Northwestern region of Argentina. In recent years, sugarcane production in this country has steadily increased; while the 2015 harvest totaled 17.7 million tons, by 2021 this figure rose to nearly 23.5 million tons. All this production is concentrated in the Northwestern region, with 11.80% corresponding to the Province of Salta [1]. The San Martín del Tabacal Sugar Mill, located in the north of the province, is the most important, accounting for 80% of the provincial production [2].

Among the by-products of sugar production, bioethanol production from molasses fermentation stands out [3]. In addition to bioethanol, this process generates a very dark liquid residue called "vinasse", produced in a ratio of 1:12-15 bioethanol:vinasse [4–6]. The properties of vinasse can vary, primarily depending on the raw material used, the distillation process, and the treatment carried out to separate the alcohol from the already fermented substrate [7,8]. However, in general, this effluent is characterized by being a dark brown liquid with a honey-like smell and malt-like taste [9]. It is acidic (pH≈4) and has a high content of suspended solids and dissolved salts (including ions such as NH_4^+, NO_3^-, NO^-, PO_4^{3-}, SO_4^{2-}, K^+, Ca_2^+, Mg_2^+) that are reflected in high electrical conductivity and ash percentage values [10]. Additionally, it contains a very high organic load, making it hundreds of times more polluting than domestic wastewater [11–14]. Vinasse chemical composition is remarkably diverse, ranging from relatively simple, low molecular weight compounds like glucose, fructose, and sucrose to a series of complex organic molecules such as melanoidins, humic acids, lignins, metal sulfides, and phenolic compounds like flavonoids, which are responsible for its dark color and recalcitrant nature. Various heavy metals such as cadmium, manganese, iron, zinc, nickel, and lead have also been detected [15]. The dark pigmentation of vinasse is due to several factors. Firstly, the presence of phenolic components such as tannic and humic acids, which often act as toxic substances. Secondly, products generated during the thermal decomposition of sucrose and reducing sugars like glucose or fructose, and most notably, the presence of melanoidins, high molecular weight compounds that are difficult to degrade, resulting from the Maillard reaction [6,16–18].

Of all the waste generated by alcohol production industries, distillery vinasse stands out as the most harmful to the natural environment. It is produced in large quantities and has high pollution potential, not only due to its high organic load but also because of its dark color [19]. Contamination of water bodies with vinasse leads to decreased light penetration, thus reducing photosynthetic activity and dissolved oxygen. Additionally, the high nutrient load causes eutrophication of the system, contributing to the increase of insect populations and disease vectors [12,20]. Furthermore, the phenolic compounds and melanoidins in vinasse have antioxidant properties that can reduce or inhibit microbial activity, affecting natural biogeochemical cycles such as carbon and nitrogen cycles, which are essential for ecosystems [21].

Various strategies have been suggested to address the treatment and management of vinasse. These include incineration, anaerobic and aerobic digestion using yeasts, and disposal in evaporation ponds. Among its most notable uses are composting and direct application to agricultural fields as fertilizer [22]. The fertigation method and the use of evaporation ponds are the most common practices in Northwestern of Argentina. Particularly at the San Martín del Tabacal Sugar Mill, the area of these vinasse evaporation ponds covers about 106 hectares. However, in recent decades, the use of white rot fungi from genera such as Phanerochaete, Trametes, Pleurotus, Pycnoporus, and Schizophyllum has been proposed as an attractive alternative for treating this waste. The growing acceptance of these organisms for the degradation of xenobiotic and recalcitrant compounds is due to the presence of a non-specific extracellular enzymatic battery capable of breaking down many bonds [23]. Among the exoenzymes that are part of the ligninolytic multi-enzyme complex, we can mention peroxidase systems, such as lignin peroxidases and manganese peroxidases, as well as laccase, cellulase, and hemicellulose systems. Those enzymes work synergistically in breaking down a wide variety of macromolecules [24]. Several of these projects not only aimed to reduce the contaminant load of the effluent but also sought alternatives for enzyme extraction or fungal biomass production, with the advantage of being edible, as in the case of *Pleurotus* sp. [25,26]. Other research focused on developing fungi with favorable characteristics in the medical field and obtaining natural pigments, as in the case of *Pycnoporus sanguineus* [27,28].

In this study, we evaluated the biomass production and bioremediation capacity of a local strain of *Pycnoporus* sp. on vinasse produced in the region.

2. Materials and Methods

The fruiting bodies of the fungi were collected from decaying trunks and branches located in a natural area in Vaqueros, La Caldera Department, Salta, within the Yungas province or the Tucumano-Oranense jungle 24°40'55.846"S 65°24'52.145"W). These samples were then transported to the laboratory for morphological description and strain isolation.

Sugarcane vinasse was obtained from the Sugar Mill San Martín de El Tabacal, located in the Orán Department, Salta. At the start of the bioassay, the effluent was characterized by measuring pH, electrical conductivity, true color, Chemical Oxygen Demand (COD), inorganic forms of nitrogen, soluble reactive phosphorus, and sulfates, according to APHA techniques [29].

A laboratory-scale bioassay was conducted, testing three dilutions of vinasse from El Tabacal with distilled water (V5%, V10%, and V25%). Each treatment had 4 replicates, using 250 mL bioreactors containing 100 mL of the respective medium, inoculated with a previously isolated strain of the fungus *Pycnoporus* sp. Additionally, control for each treatment were established using the same dilutions (C5%, C10%, and C25%), but without the fungus inoculation. These experimental units were randomly placed in an incubator at a temperature of 27 ± 1°C. After 60 days of incubation, biomass production was assessed by calculating its dry weight. The efficiency of *Pycnoporus* sp. as a bioremediation agent was also evaluated by measuring the removal of COD and True Color (TC).

To compare differences between treatments, ANOVA was performed previous assuring the data met the assumptions of normality and homoscedasticity. When normality was not met, non-parametric Kruskal-Wallis test was used. Contrasts between groups were calculated using Tukey test. Additionally, linear regressions were conducted with fungal biomass production and NH_4^+ consumption as dependent variables, and vinasse concentration (5%, 10%, and 25%) as the regressor variable using Infostat v. 2018.

3. Results

3.1. Characterization of the Effluent

The effluent was characterized by its dark brown color, high electrical conductivity values, acidic pH, and high levels of organic load and inorganic nutrients (Table 1).

Table 1. Characterization of vinasse produced by the El Tabacal sugar mill.

Parameter	Units	Value
pH		4.18
True Color	UPt-Co	64200
Conductivity	uS/cm	9242
NH3	mg/L	520
NH4	mg/L	310
NO3	mg/L	100
PO4	mg/L	39.5
SO4	mg/L	700
COD	mg/L	45320

3.2. Description of the Fungal Strain

The identification and characterization of the strain were conducted through the study of morphological characteristics; based on Robledo et al. (2003) [29] it is reported as *Pycnoporus* aff. *sanguineus*.

The sexual fruiting bodies, with a hard to leathery texture, exhibited a fan-shaped (flabelliform) appearance, an intense orange coloration, and were strongly attached to the substrate at the base. Additionally, concentric rings were observed on the upper surface and tiny pores on the lower surface, numbering 5 to 6 per mm (Figure 1).

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Figure 1. Morphological characterization of the isolated Pycnoporus aff. Sanguineus, (**A**) Top view of a sexual fruiting body; (**B**) Bottom view; (**C**) Bottom view with augmentation where pores are observed.

3.3. Fungal Biomass Production

Regarding the DW of the fungus in the experimental units after 60 days of incubation, values ranged from 35.7 to 366.3 mg. Statistically significant differences were found between each of the treatments ($p<0.001$): V5% = 38.93 ± 2.8 mg; V10% = 92.68 ± 4.79 mg; V25% = 249.48 ± 49.93 mg, with the highest fungal development observed in the V25% treatment (Figure 2 A). These results were reflected in the linear regression model, which was highly significant between vinasse concentration and biomass production of the native strain of *Pycnoporus* aff. *sanguineus* ($R^2 = 0.95$; $p<0.0001$) (Figure 3).

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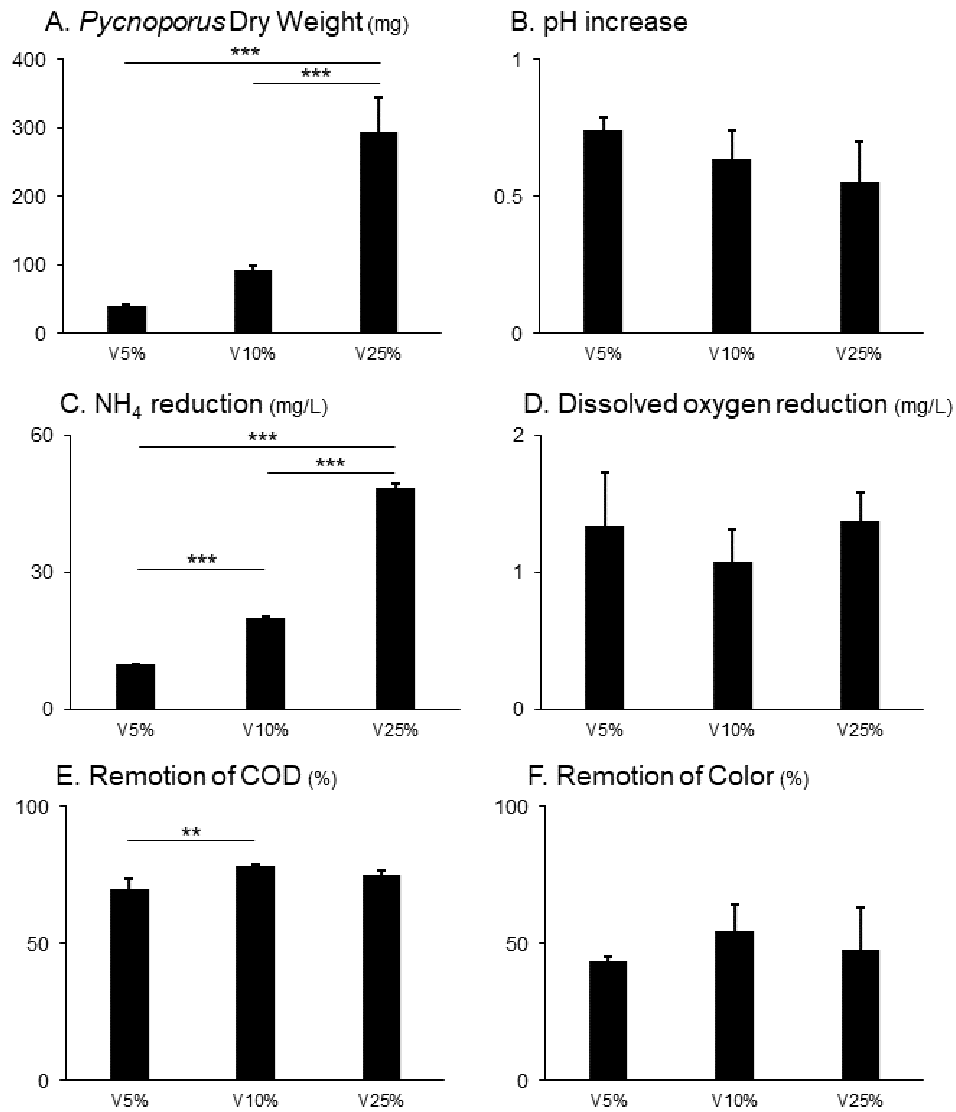

Figure 2. (**A**) Biomass production of Pycnoporus sp. on vinasse after 60 days of incubation on 5, 10 and 25% dilution of vinasse; (**B, C, D, E** and **F**) bioremediation results analysis of the vinasse. Statistically significant differences between treatments are shown as ** p<0.001, *** p<0.0001.

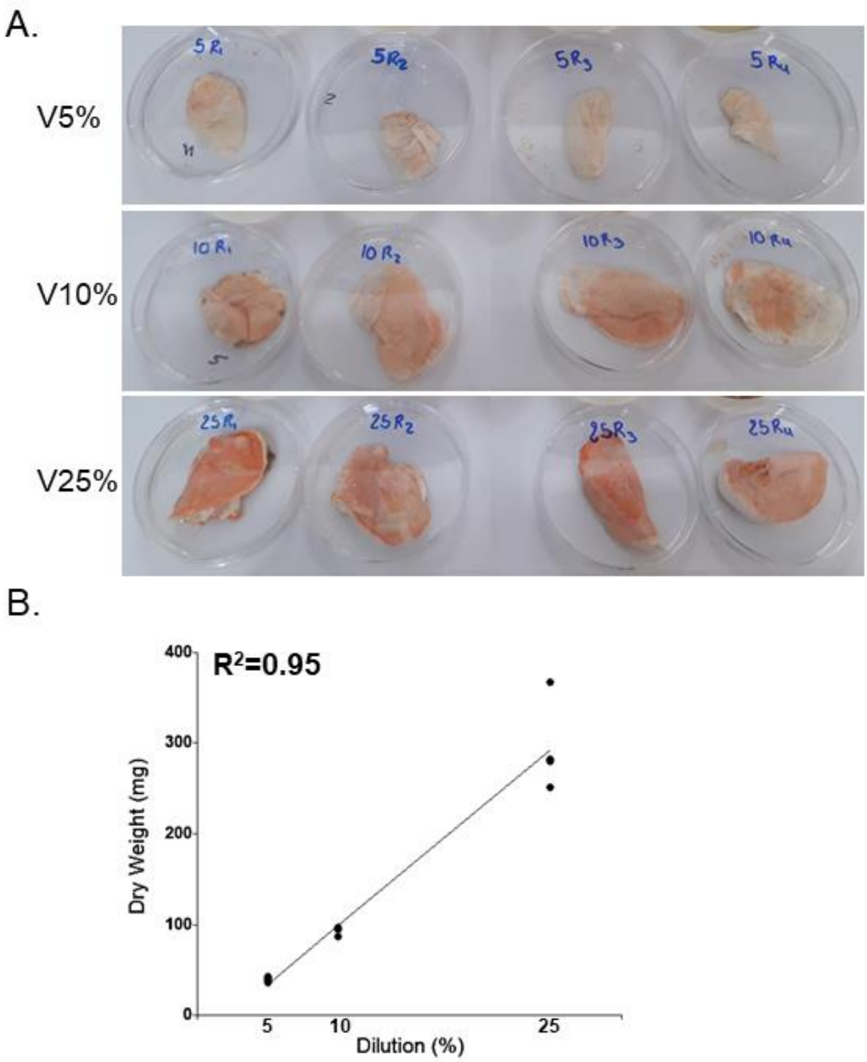

Figure 3. (A) Photos of *Pycnoporus* sp. growth after 60 days on 5, 10 and 25% of vinasse; and **(B)** Lineal Regression Model between dilution of vinasse (%) and dry weight (mg).

3.4. Efficiency of the Vinasse Remediation

In relation to the ammonium reduction the results ranged between 10 and 49 mg/L, and statistically significant differences were observed between each of the treatments, with average values of V5% = 10.05 ± 0.06 mg/L; V10% = 20.13 ± 0.38 mg/L; and V25% = 48.38 ± 0.95 mg/L (Figure 2 C). The linear regression model significantly fit the relationship between NH_4^+ reduction and the different vinasse concentrations ($R^2 = 1$; $p<0.0001$).

Regarding the removal of organic matter, measured through COD, the results varied between 65.25% and 78.79%. The treatments carried out at higher vinasse concentrations (V10% and V25%) proved to be the most efficient, with averages of 78.53% ± 0.38 and 75.13% ± 1.78 respectively, and both treatments were not statistically different from each other (Figure 2 E). Meanwhile, the treatment using the most diluted vinasse (V5%) showed a significantly lower average COD removal efficiency (69.95% ± 3.84). pH increase, dissolved oxygen reduction and remotion of the color were similar across all treatments (Figure 2 B, D and F).

4. Discussion

The study examined the vinasse from El Tabacal sugar mill, finding its dark brown color, acidic pH, and high values of electrical conductivity, organic load, and inorganic nutrients like those

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reported in numerous studies. The vinasse exhibited a pH of 4.18, matching reported values and attributed to organic acids produced during fermentation. The electrical conductivity was 9,242 µS/cm, lower than those in other regional studies. The initial COD was 45,320 mg O_2/L, comparable to other findings. Nitrate and phosphate levels aligned with previous research, while sulfate levels were lower. The vinasse's nutrient content enhanced fungal biomass production, suggesting potential for bioremediation. The study supports the use of white-rot fungi in bioremediation of sugarcane waste.

The characteristic dark brown color, acidic pH, and high values of electrical conductivity, organic load, and inorganic nutrients present in the vinasse from El Tabacal sugar mill were like those reported for vinasse in numerous studies [4,31,32]. The pH of the sample, 4.18, matched values reported previously since vinasses derived from the fermentation of molasses generally have pH values ranging between 4.2 and 5 [33]. This is related to the presence of organic acids generated during the fermentation process by yeasts and occasionally by bacteria [13]. The dissolved salt load, expressed in values of electrical conductivity (9,242 µS/cm), was lower than those obtained in studies conducted on vinasse from other countries in the region such as Mexico: 19,500 µS/cm, Colombia: 17,000 µS/cm [34]. Regarding COD, the initial value recorded in the vinasse prior to biotreatment was 45,320 mg O_2/L, comparable to those obtained for vinasses by other researchers [12]. Finally, in terms of nutrient content, we determined that nitrate and phosphate values were like those reported by Gil Rolón (2018) [35], who also worked with vinasse from the El Tabacal sugar mill, while the sulfate levels obtained in the measurements (700 mg/L) were lower than those reported in the same work (4000 mg/L).

The increase in fungal biomass production observed in this work, as the concentration of vinasse increases, could be explained by the increase in the concentration of nutrients and carbonaceous materials in the medium, resulting in a more enriched environment that stimulates fungal growth. Similar results were reported in works with *Pycnoporus* sp. [36] and *Pleurotus* sp., where 25% vinasse showed a stimulating effect on strain growth [37]. The same was found for vinasse treated by white-rot fungi of other species such as *Phanerochaete chrysosporium*, *Ganoderma* sp., and *Trichoderma reesei* [38].

On the other hand, both the increase in ammonium (NH_4^+) consumption and the increase in pH, as vinasse concentration increases, can be explained by the occurrence of biodegradation processes of organic matter, because of the work of bond-breaking, performed by the fungal exoenzymatic battery [15]. As complex structures are transformed into simpler molecules through mineralization processes and more specifically in the case of NH_4^+, by ammonification processes, the release of ammonium molecules into the medium is triggered, generating an increase in the pH of the effluent. However, simultaneously, the fungus, when feeding, uses this nitrogen from the medium in the manufacture of proteins and other important molecules for its biomass growth. This is consistent with the results obtained in this work, where a higher ammonium consumption is reflected in the V25% treatment, where fungal biomass production was the highest.

The similarity in the difficult biodegradability of substrates such as celluloses, hemicelluloses, lignin, and compounds such as melanoidins and humic acids enables the use of white-rot fungi in bioremediation processes. Numerous studies demonstrate the participation of lignocellulolytic enzymatic systems in the decolorization process of vinasses from alcohol distilleries. Even though no significant differences were found in the percentages of color removal between the different treatments after 60 days of incubation in the present study, we can mention that the average removal values were like those reported by Ahmed (2016) [36], who working with 10% diluted vinasse and the same fungus species, *Pycnoporus* sp., achieved color removals of 51% after 12 days of treatment. These decolorization efficiencies were also like those achieved by other fungi, such as *Ganoderma* sp. (65%), *Aspergillus niger* (63%), and *Trichoderma reesei* (55%). While for other species like *Phanerochaete* sp. and *Pleurotus shimeji*, higher values (85%) have been reported [14].

Some of the compounds responsible for the color in vinasses also contribute to the high COD values as they are susceptible to oxidation for their breakdown into smaller molecules. In our study, we observed that treatments with higher vinasse concentrations were the most effective, with average removals exceeding 70%, with the V10% treatment showing the highest percentage (78.53%). These values were somewhat higher than those reported by Ahmed (2016) [36], who reported removals of 67.83% using 25% diluted vinasse and the fungus *Pycnoporus* sp. In other studies, researchers found similar values to ours regarding the reduction of COD in sugarcane vinasse treated by the fungus *Pleurotus* sp. [14,38]. Gil Rolón (2018) [35] working with vinasse of the same origin as ours and a strain of the fungus *Pleurotus ostreatus*, reported average removals of 76.35 and 70.96%, in vinasse dilutions of 10% and 25%, respectively. Comparatively, our native strain of *Pycnoporus* aff. *sanguineus* demonstrated greater efficiency in COD removal (78.53%).

Finally, *Pycnoporus* is a representative genus of saprophytic homobasidiomycetes that have lignocellulytic potential [39]. The red or orange pigments characteristic of the fruiting bodies (basidiocarps) of this fungus are compounds derived from cinnabarin [40]. which also have antibiotic, antiviral, and antitumor activities [41,42]. Thus, bioremediation with *Pycnoporus* sp. could be very interesting in relation of its derivates.

5. Conclusions

The need to implement clean and environmentally friendly technologies for the treatment of agro-industrial effluents highlights our work as a significant contribution to the study of bioremediation processes for these wastes. The results obtained show the potential of the native strain isolated from *Pycnoporus* aff. *sanguineus* to be used in vinasse bioremediation processes, which proved to be an excellent substrate for fungal biomass development and a promising percentage of color and COD removal were achieved.

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Conflicts of Interest: The authors declare no conflicts of interest

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