Journal of Environmental Chemical Engineering 6 (2018) 5177–5185

Contents lists available at ScienceDirect

Journal of Environmental Chemical Engineering

journal homepage: www.elsevier.com/locate/jece

Sustainable bioremediation of sugarcane vinasse using autochthonous macrofungi

Pablo M. Ahmed[a,*,1], Hipólito F. Pajot[b,1], Lucía I.C. de Figueroa[b,c], Carlos H. Gusils[a]

[a] Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Estación Experimental Agroindustrial Obispo Colombres (EEAOC)/Agroindustrial Technology Institute of Northwest Argentine, National Council of Scientific and Technical Research, Agroindustrial Experimental Station "Obispo Colombres", Av. William Cross 3150 (T4101XAC), Las Talitas, Tucumán, Argentina

[b] Planta Piloto de Procesos Industriales Microbiológicos (PROIMI-CONICET)/Pilot Plant for Microbiological Industrial Processes, Av. Belgrano y Caseros (T4000INI), S M de Tucumán, Tucumán, Argentina

[c] Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán/Chair in Superior Microbiology, National University of Tucuman, Faculty of Biochemestry, Chemestry and Pharmacy, Ayacucho 471 (T4000INI), S M de Tucumán, Tucumán, Argentina

ARTICLE INFO

Keywords:
Biodegradation
Sugarcane vinasse
Lignocellulolytic basidiomycetes
Laccases enzymes
Sustainable management

ABSTRACT

Vinasse is the main residue from bioethanol production. If discharged in water bodies, it can impair the photosynthetic process causing great damage to the environment. However, this wastewater could be considered a by-product and used as a substrate for obtaining value-added products. In the present work, two basidiomycetous fungi were selected based on their ability to decolorize vinasse and to synthesize lignocellulolytic enzymes in agar medium. Selected fungi were identified as *Pycnoporus* sp. P6 and *Trametes* sp. T3. In liquid cultures, both fungi demonstrated their capacity to decolorize and remove phenolic compounds from diluted vinasse, with the concomitant production of ligninolytic enzymes, mainly laccases, suggesting the participation of this enzyme in the bioremediation process. This study proposes that *Pycnoporus* sp. P6 and *Trametes* sp. T3 could be used in the design of cutting-edge sustainable biotechnological processes to remedy vinasse, simultaneously synthesizing oxidative enzymes, in an environmentally friendly way.

1. Introduction

The increase in the bioethanol consumption as biofuel opens up new opportunities for sugarcane crop, the main source of bioethanol in Latin America. Generally, in autonomous distilleries, ethanol comes from the sugarcane juice fermentation, but in annexed plants, all the juice (or a large volume of it) is usually derived from sugar production, and the resulting molasses are used in the bioethanol generation [1].

Argentina has 23 bioethanol-producing sugar mills; 20 of them in the Northwest region and 15 in the province of Tucumán with an alcohol production estimated in 250,000,000 L during the harvest period 2017 and beginning of 2018 (http://www.ipaat.gov.ar/wp-content/uploads/2017/07/TOTALES-HASTA-1RA-QUINCENA-MAYO-2018.pdf).

The wastewater from the bioethanol production is called vinasse, and it is typically generated in a ratio of 10 to 14 L of vinasse per L of ethanol [2]. Vinasse is a dark-brown effluent with a strong smell and acidic pH (3.5–5.0). It contains high concentrations of dissolved solids including non-volatile compounds from the fermentation broth, non-fermentable organic, phenolic and polyphenolic compounds, and remaining fermentescibles sugars. Sugarcane vinasse has high biological oxygen demand (BOD), with values between 45,000 and 60,000 mg L^{-1} and higher chemical oxygen demand (COD), ranging between 80,000–120,000 mg L^{-1} [3–5]. High amount of BOD and COD generally cause various issues especially in advanced wastewater treatment method such as membrane bioreactor [6]. Sugarcane vinasse also has a high content of mineral salts, high electrical conductivity and ash percentage [7].

The unrestricted discharge of vinasse causes severe damage to the environment. While in aquatic environments it can lead to a reduction of the photosynthetic activity and to a decrease of the dissolved oxygen concentration, in soils it incurs in alkalinity losses and reduces germination capacity. In addition, the vinasse dumping may disrupt the biogeochemical cycles of essential elements, such as carbon and nitrogen cycling [8]. There are several and effective physicochemical processes for the vinasse treatment [9], but they usually produce large

* Corresponding author.
E-mail addresses: pabloma@live.com.ar (P.M. Ahmed), hipolito_pajot@yahoo.com (H.F. Pajot), proimiunt@gmail.com (L.I.C. de Figueroa), cgusils@eeaoc.org.ar (C.H. Gusils).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.jece.2018.08.007
Received 6 June 2018; Received in revised form 11 July 2018; Accepted 1 August 2018
Available online 06 August 2018

quantities of secondary sludge or are expensive and not adequate enough for effluents with considerable seasonal and compositional variations, as vinasse [1]. Thus, bioremediation has become an attractive alternative since it is a non-polluting, eco-friendly and socially accepted procedure [10,11].

Basidiomycetes and Ascomycetes fungi have been used in bioremediation of vinasse because of their abilities to discolor and degrade natural and synthetic colorants present in this type of wastewater e.g. polyphenols, caramels, alkaline degradation products of hexoses, melanins and melanoidins, trough their ligninolytic enzymes [12–14]. In addition, several studies have shown that vinasse can be used as a raw material for the production of microbial biomass or secondary metabolites of industrial importance, including biopolymers, lipids, organic acids, pigments and enzymes [15–17]. The production of laccases enzymes by ligninolytic fungi through the use of vinasse as a nutritional source is presented as a green, economical and sustainable option for the management and disposal of this wastewater.

For all the foregoing, the objectives of the present work were to characterize physicochemically three vinasses from the sugar-alcohol activity of Tucumán, to evaluate its potential as a substrate for the production of oxidative enzymes at the same time that the vinasse's decolorizing capacity of autochthonous macrofungi was studied.

This work highlights the use of dual-purpose biotechnological processes to vinasses as a cutting-edge technology which could be employed for the treatment of wastewaters in an economic and environmentally friendly maintainable manner.

2. Materials and methods

2.1. Distilleries vinasses

As other agro-industrial wastewaters, vinasse composition is extremely variable. Thus, vinasses were collected from three different distilleries of the province of Tucumán, Argentina. All them were obtained from sugarcane molasses fermentation. The effluents pH and temperature values were registered *in situ* and controlled periodically. Vinasse samples were stored at 4 °C until processed.

2.2. Vinasses physicochemical characterization

Analytical determinations of vinasses were performed in compliance with established standards as recommended by the APHA [18].

The BOD_5/COD ratio of each sample was calculated and the biodegradability was determined based on the modified classification of Aziz and Tebbutt [19].

Glucose, fructose, and saccharose were determinate by HPLC (Waters e2695, USA), employing a Waters Sugar-Pak™ 1 column (300 mm × 6.5 mm, USA) filled with a microparticulate cation exchange gel in calcium form, according to ICUMSA Methods Book (Method GS7/4/8-23, 2007) [20].

2.3. Microorganisms

Six basidiomycetes from the culture collections of EEAOC and PROIMI, previously selected by their ability to degrade post-harvest sugarcane residues [21], were employed in this work. The microorganisms were grown in PDA medium (Merck) at 30 ± 2 °C and stored at 4 °C.

Grammothele subargentea (CLPS 436), *Coriolopsis rigida* (CLPS 232) (Culture collection of "Carlos Spegazzini" Institute) and *Irpex lacteus* (IJFM A792) (Culture collection of the Biological Research Center (CIB)) were also included as positive controls of lignocellulolytic activities in agar plates, in order to validate the screening procedure [22,23].

2.4. Molecular characterization of the fungal strains

Fungal strains were identified by sequencing of the ITS1-5.8S-ITS2 fragment of rDNA [24], using the its1 (5′-TCCGTAGGTGAACCTG CGG-3′) and its4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (Macrogen, Korea).

The obtained sequences were manually edited and compared with fungal strains sequences deposited in the GenBank and Mycobank databases using BlastN [25]. The sequences most related to those obtained in this work were aligned by the MUSCLE algorithm provided in MEGA 6 Software [26]. The phylogenetic tree was inferred by the Maximum Likelihood method using the Tamura and Nei [27].

2.5. Preparation of vinasse based media

All vinasse-amended media were prepared using the distillery's vinasse 1 (V1). Raw vinasse was previously centrifuged (10,000 *g*, 15 min), sterilized at 121 °C, 15 min and adjusted to pH 4.5 ± 0.5 (0.1 N HCl). Diluted vinasse (10 and 50%, v/v) was prepared by diluting 100% raw vinasse in distilled water.

2.6. Vinasse discoloration in solid media

The trials were performed on Petri dishes inoculated with 6 mm diameter agar plug from the edge of 8-days old fungal cultures growing on PDA medium.

The decolorizing ability of the fungal strains was evaluated according to the diameter of the clearance haloes (dch) formed and classified as: (i) no decolorizing ability (dch ≤ 6 mm), (ii) moderate decolorizing ability (dch = 6–75 mm), and (iii) high decolorizing capability (dch ≥ 75 mm). Uninoculated plates were used as abiotic controls. The assays were made in triplicate and dch was measured twice, perpendicularly. An average of the measurements taken was announced.

2.7. Screening for lignocellulolytic enzymes

Phenoloxidases, polyphenoloxidases, lignin peroxidase, laccase, endoxylanases, and endoglucanases were assayed in Ligninase Basal Medium (LBM) as recommended by Pointing [28]. The ability to degrade cellulose also was recorded through the Index of Relative Enzyme Activity (ICMC) [29].

Mn-dependent peroxidase was examined placing 50 µL of MBTH/DMAB/Mn/H_2O_2 solution over fully grown mycelia (adapted from Castillo et al. [30]).

Plates were incubated at 30 ± 2 °C in darkness and examined daily during 14 days.

Results were recorded as (–) no reaction, (+) week positive reaction, (++) strong positive reaction.

2.8. Vinasse treatment in liquid media

Vinasse discoloration and the secretion of ligninolytic enzymes in liquid cultures were performed in 10% (v/v) vinasse final concentration (V10 medium) and V10 medium plus LBM components (V10 + LBM). The assays were evaluated in triplicate, using 250 ml Erlenmeyer flasks containing 50 ml of media inoculated with five agar plugs from the edge of 8-days old fungal cultures growing on PDA medium.

Ligninase Base Medium broth without vinasse was used as positive control for ligninolytic enzymes production. Inoculated and uninoculated flasks (abiotic controls) were incubated at 30 ± 2 °C and 250 rpm for 15 days. Samples from the different treatments were taken and centrifuged at 10.000 *g* for 15 min and 4 °C. Supernatants were collected for further analysis.

Treatments were statistically compared using Minitab 17.0 Software (2014) [31].

2.8.1. Discoloration and phenolic compounds analysis

Total phenolic compounds were measured according to Clarke et al. [32] with Folin-Ciocalteu reagent and caffeic acid as standard.

Discoloration (D) was calculated from absorbance measurements at 475 nm using a Multiskan™ GO Thermo Scientific (USA) microplates spectrophotometer, according to the formula of Sirianuntapiboon et al. [33]:

$$D(\%) = [(A_0 - A_t)/A_0]x\ 100$$

where, A_0 and A_t were the absorbances of the vinasse-amended medium at the start point (0) and at a cultivation time (t), respectively. Additionally, culture supernatants were subjected to spectral scanning between 300 nm and 800 nm in order to analyze color disappearance at the initial (day 0) and final (day 15) of the respective treatments.

2.8.2. Enzymatic assays

Laccase (EC 1.10.3.2, Lacc) activity was measured by monitoring the oxidation at 420 nm of 1.8 mM ABTS (= 36,000 $M^{-1}\,cm^{-1}$) in 50 mM acetate buffer (pH 4.0). One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 mmol of ABTS in 1 min [34].

Manganese peroxidase (EC 1.11.1.13, MnP) activity was determined based on Castillo et al. [30]. The reaction of 0.07 mM MBTH (3-Methyl-2-benzothiazolinone hydrazone) and 1 mM DMAB ((3-Dimethylamino) benzoic acid) was measured at 610 nm (ε = 53,000 $M^{-1}\,cm^{-1}$) in presence of 0.3 mM $MnSO_4.H_2O$ and 0.05 mM H_2O_2 in 100 mM succinate-lactate buffer (pH 4.5). Mn-independent peroxidase (MIP) activity was measured using the same assay but without adding $MnSO_4.H_2O$. One unit of MnP and MIP enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 mmol of substrate in 1 min.

Lignin peroxidase (EC 1.11.1.14, LiP) activity was determined by the oxidation of veratric alcohol at 310 nm [35]. The reaction mixture was composed of 0.1 mM H_2O_2 and 0.2 mM veratric alcohol in 50 mM citrate-phosphate buffer (pH 4.5). One unit of LiP activity (U) was defined as the amount of enzyme required for causing a change in absorbance unit per minute.

3. Results and discussion

3.1. Physical-chemical properties of distilleries effluents

An exhaustive characterization of three vinasses coming from the sugar-alcohol industry of Tucumán is presented in Table 1.

The herein studied sugarcane vinasses show values of pH, EC, TNU, TS, FS, VS, COD, BOD, metals and RS reported for these wastewater worldwide.

Despite the collected vinasses presented a high fraction of organic load (COD values between 74,290–137,610 $mgO_2\,L^{-1}$, Table 1), more than 50% of the same can be degraded by biological action in five days (BOD_5 values between 38,504–76,904 $mgO_2\,L^{-1}$). Biodegradability rates (BOD_5/COD) of 0.56, 0.52 and 0.58 were calculated for the distilleries vinasses V1, V2, and V3 respectively, demonstrating in all cases their nature biodegradable (Table 1).

Potassium and calcium where the preponderant metals detected in the assayed vinasses (Table 1), whilst Mg, Na, Fe, Cu, and Mn were found in lower concentrations (data not shown). The metals values found in the assayed samples demonstrate that raw vinasses contain a salts concentration which makes them little advisable for their use in agriculture, incurring overfertilization and others several adverse impacts on the soil structure, as well as to the crop productivity and water resources [36–38]. However, recent studies reported that the macro and micronutrients present in vinasses, at suitable concentrations, contributes to its uses as a source of nutrients for growth of microorganisms or the production of compounds with industrial importance [15–17].

Because the distillery's vinasse 1 showed the highest concentrations of COD, DBO, and RS, representing the potentially more polluting vinasse, it was selected to prepare the amended media to perform the discoloration tests in both solid and liquid conditions, as well as evaluate its use as a substrate for the production of ligninolytic enzymes.

3.2. Vinasse decoloration in solid media

Diluted sugarcane vinasse supported the growth of macrofungi. Similar observations were performed by other authors for other fungi, including *Pleurotus ostreatus, Phanerochaete chrysosporium*, *Ganoderma* sp. and *Mucor pusillus* [39,40].

All the tested fungi were inhibited by 50 and 100% (v/v) vinasse media, even in the presence of LBM components. However, when fungi were evaluated in solid V10 medium, isolates #1, #3, #7 and #8 showed moderate decolorizing ability (discoloration haloes ranged between 57 and 65 mm), while fungus #6 decolorized the entire plate (Figs. 1, 2) and isolate #5 did not produce discoloration haloes under the tested conditions.

Fungal strains #1, #3, #6, #7 and #8 able to decolorizing vinasse on agar plates, were selected for further studies.

Table 1
Physical-chemical characteristics of vinasses resulting from bioethanol production in three distilleries of Tucumán.

Parameters	Distillery vinasse 1 (V1)	Distillery vinasse 2 (V2)	Distillery vinasse 3 (V3)
pH	4.88 ± 0.01	4.93 ± 0.5	5.15 ± 0.11
EC [$mS\,cm^{-1}$]	30.60 ± 1.71	29.80 ± 2.68	32.20 ± 1.86
Turbidity [TNU]*	2588 ± 126.22	1,368 ± 148.66	2,575 ± 111.33
Humidity [%]	91.80 ± 2.42	95.80 ± 1.34	89.50 ± 3.15
TS [$g\,L^{-1}$]	81.24 ± 7.08	41.95 ± 0.30	104.89 ± 0.45
FS [$g\,L^{-1}$]	19.70 ± 3.68	13.87 ± 0.21	36.59 ± 0.62
VS [$g\,L^{-1}$]	61.54 ± 10.76	28.08 ± 0.30	68.30 ± 0.68
COD [$mg\,O_2\,L^{-1}$]	137,610 ± 2638.90	74,290 ± 583.33	109,852 ± 36.50
BOD_5 [$mg\,O_2\,L^{-1}$]	76,904 ± 4222.20	38,504 ± 1483.50	64,360 ± 2941.90
BOD_5/COD	0.56	0.52	0.58
K [$g\,L^{-1}$]	8.20	5.30	11.00
Ca [$g\,L^{-1}$]	2.20	1.40	2.40
RS [g %]	2.98	0.16	0.91

The average temperatures *in situ* of the vinasses were 98 ± 2 °C.
*TNU: turbidity nephelometric units.
EC, electric conductivity, TS, total solids, FS, fixed solids, VS, volatile solids, COD, chemical oxygen demand, BOD_5, biochemical oxygen demand, K, potassium, Ca, calcium.
BOD_5/COD, biodegradability rate.
RS, residual sugars. *RS* = (*Saccharose value* * 1.05) + *glucose value* + *fructose value*

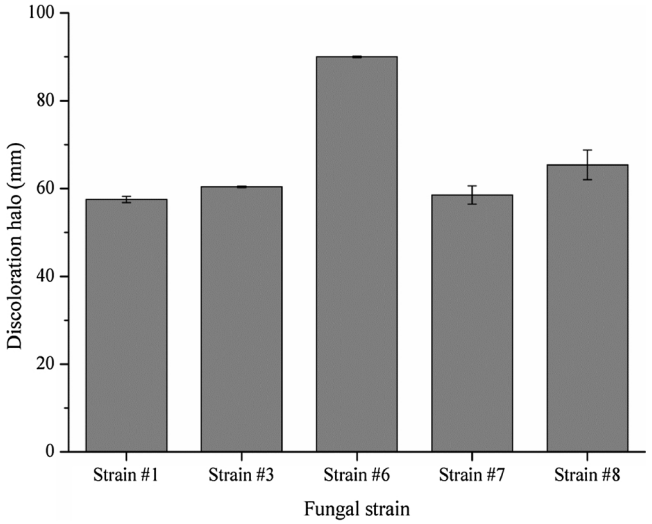

Fig. 1. Sugarcane vinasse discoloration in solid medium plus 10% vinasse (V10), after 10 days of incubation.

3.3. Lignocellulolytic enzymes in solid media

All the strains produced extracellular oxidases (reddish haloes in guaiacol amended plates), laccases (green haloes in ABTS and pink haloes in syringaldazine amended plates), polyphenoloxidases (dark brown haloes in tannic acid amended plates) and Mn peroxidases (blue haloes around wells in DMAB/MBTH plates). Only one strain (#7) did not synthesize lignin peroxidase (absence of clearance zones in modified plates with Azure B). Likewise, all the strains were able to degrade xylan and carboxymethylcellulose (CMC), implying potential to degrade hemicelluloses and celluloses (endoglucanase activity). Strain #6 presented the highest I_{CMC} (Index of Relative Enzyme Activity = 1.28 ± 0.05 mm; Table 2).

As expected, the control strain *Grammothele subargentea* synthesized enzymes with oxidative activity against guaiacol, ABTS, syringaldazine (Syr), Mn^{2+} and tannic acid. However, it showed no ability to decolorize Azure B, evidencing the absence of the lignin peroxidase (LiP) enzyme (Table 2). *Irpex lacteus* A792 produced guaiacol intense oxidation and to a lesser degree of ABTS, Syr, and Mn^{2+}. It was the only one of the reference strains with the potential to oxidize lignin (positive LiP activity), whereas *Coriolopsis rigida* showed only oxidative activity against ABTS. All reference strains degraded CMC with intense haloes formation and with $I_{CMC} \geq 1.00$, evidencing potential to hydrolyze celluloses (Table 2).

3.4. Molecular identification

The analysis of the ITS1-5.8S-ITS2 sequences showed that the fungal strains #1, #3, #7 and #8 belong to subclade 3 of the *Trametes* genus according to Welti et al. [41], and then were named *Trametes* sp. T1, T3, T7, and T8, whereas the fungus #6 is closely related to the *Pycnoporus* genus and was named *Pycnoporus* sp. P6 ([41]; Fig. 3).

The analysis included 38 sequences and 473 positions. The phylogenetic history was inferred using the Maximum Likelihood method, based on the model of Tamura and Nei [27]. The presented tree is 1000 replicates consensus obtained by bootstrap. The percentage of replicates in which each taxon appears is displayed.

Pycnoporus and *Trametes* are usually isolated from rotting wood and can degrade lignocellulose, textile colorants, recalcitrant compounds and other industrial materials [42–44]. *Pycnoporus* and *Trametes* strains were able to decolorize V10 medium, an unsurprising fact since both genera have been extensively studied for their ability for wastewater decolorization. Thus, Eugenio et al. [45] investigated the degradation of lignosulfonates by strains of the *Pycnoporus* genus and determined its complete mineralization. Pointing and Vrijmoed [46] demonstrated that a strain of *Pycnoporus sanguineus* was able to biodegrade azo dyes (Orange G, Amaranth) and triphenylmethanes (Phenol Bromo Blue, Malachite Green, Violet Crystal, etc.). Ottoni et al. [43] performed discoloration assays in solid medium and evaluated the potential of three *Trametes versicolor* strains against different concentrations of two textile dyes, Black Reactive 5 and Poly R-478. Minussi et al. [35] demonstrated the decolorizing ability of four white rot fungi including *T. versicolor* and *Trametes villosa* on synthetic dyes and a real textile effluent, in solid medium added with 0.05% Reactive Blue 19, 0.025% Reactive Red 195, 0.05% Reactive Yellow 145, 0.05% Black Reactive 5 and effluent from a textile industry, respectively.

Taking into account the high ability to decolorize vinasse and synthesize lignocellulolytic enzymes on solid media both fungi, *Pycnoporus* sp. P6 and *Trametes* sp. T3 was selected as the best candidates for subsequent vinasse remediation trials.

3.5. Vinasse decolorization in liquid media

Pycnoporus sp. P6 reduced 51% of the initial color and 78% of total phenolics of V10 after 12 days. In V10 + LBM, results were similar with 40% decolorization and a reduction of total phenolic compounds of 86% after 8 days (Figs. 4, 5). *Trametes* sp. T3 showed 43% decolorization, and 80% decrease of total phenolic compounds after 10 days incubation, while in V10 + LBM maximum decolorization reached 57% and the maximum reduction of total phenolic compounds achieved was 80% after 10 days (Fig. 4 and Fig. 66).

The initial pH in both 10% (v/v) vinasse-amended media was 4.7 ± 0.2. Vinasse color, pH and total phenolic compounds in uninoculated V10 and V10 + LBM media remained unchanged for the duration of the trials (99 ± 1%) (Figs. 5, 6), suggesting that vinasse discoloration could be attributed to the degradation or transformation of one or more of its components by *Pycnoporus* sp. P6 or *Trametes* sp. T3 fungi.

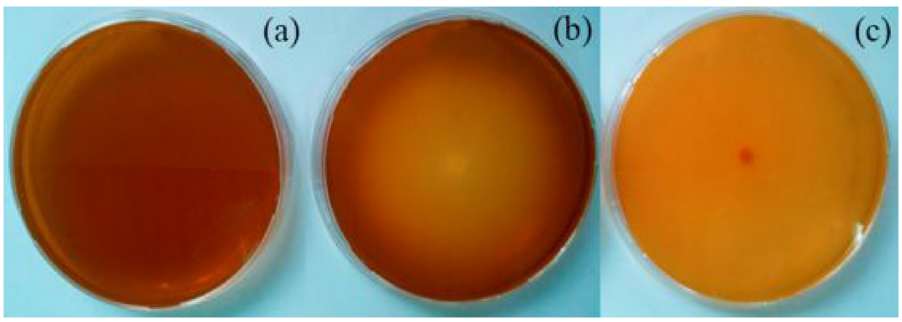

Fig. 2. Biodecoloration of 10% (v/v) vinasse-amended solid medium (V10) by the fungal strains #3 (b) and #6 (c), after 10 days of incubation. In (a) an abiotic control of the V10 medium is shown.

Table 2
In vitro synthesis of lignocellulolytic enzymes by the autochthones fungal strains #1, #3, #6, #7 and #8 in LBM agar plus specific substrates according to enzyme activity.

Macrofungi	Guaicol	ABTS	Syr	Azure B	$MnSO_4$	Tannic acid	Xylan	Cellulolytic activity	
								I_{CMC}	Clearing zone
Coriolopsis rigida	–	+	–	–	–	–	++	1.00 ± 0.00	+
Grammothele subargentea	++	++	++	–	++	++	++	1.00 ± 0.00	+
Irpex lacteus A-792	++	+	+	++	+	–	++	1.08 ± 0.06	+
Fungal strain #1	++	++	+	+	++	++	++	1.08 ± 0.05	+
Fungal strain #3	++	++	+	++	++	++	++	1.23 ± 0.14	++
Fungal strain #6	++	++	++	++	++	++	++	1.28 ± 0.05	++
Fungal strain #7	++	++	+	–	+	++	++	1.01 ± 0.13	+
Fungal strain #8	++	++	+	++	+	++	++	0.86 ± 0.02	+

(−): No halo production; (+): Low-intensity halo; (++): High-intensity halo.

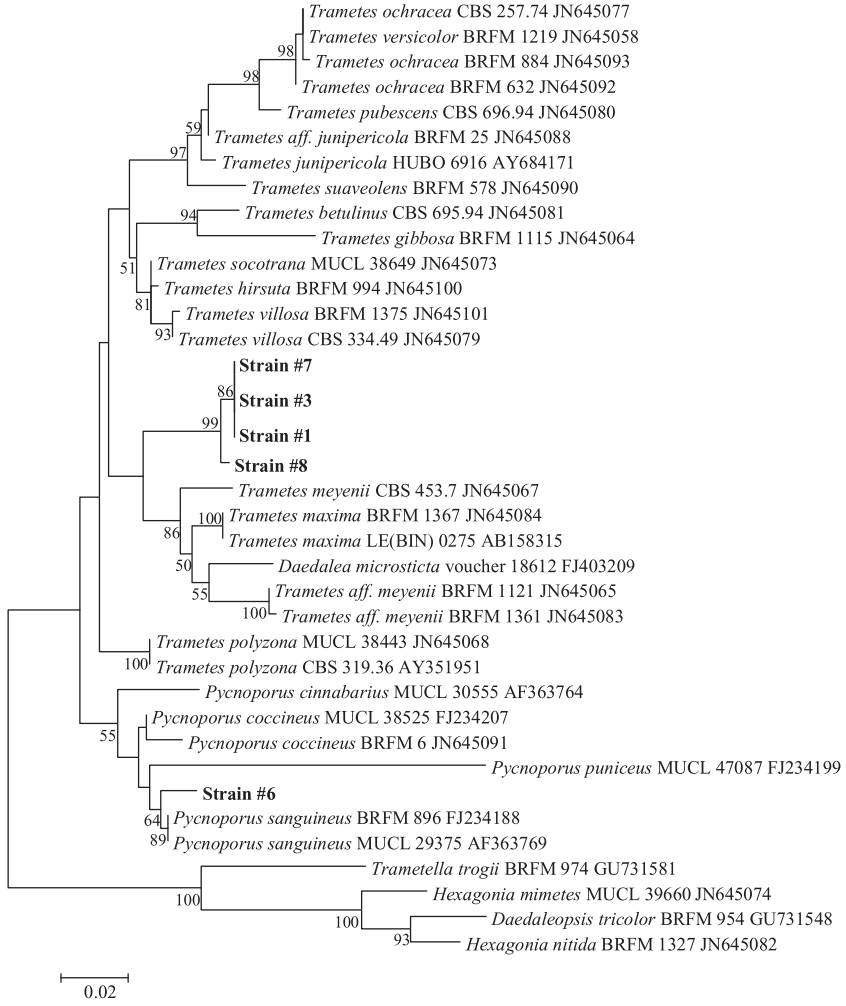

Fig. 3. Phylogenetic analysis of basidiomycetous strains based on sequences from the ITS1-5.8S-ITS2 region of rDNA.

Fig. 4. Sugarcane vinasse before and after treatment by ligninolytic macrofungi: (a) Untreated 10% (v/v) raw vinasse (abiotic control), (b) 10% vinasse medium (V10) treated by *Pycnoporus* sp. P6 after 12 days, (c) 10% (v/v) vinasse medium plus LBM nutrients (V10 + LBM) treated by *Trametes* sp. T3 at 10 days.

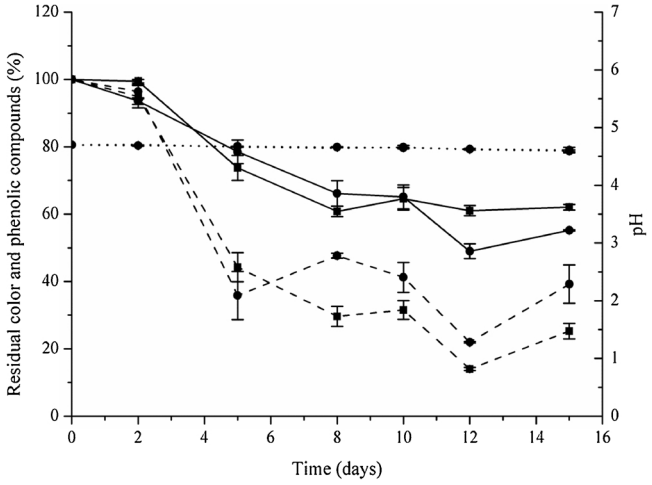

Fig. 5. Profiles of residual color (—), residual phenols (—) and pH (…) obtained during the treatment by *Pycnoporus* sp. P6: (■) V10 + LBM medium, (●) V10 medium.

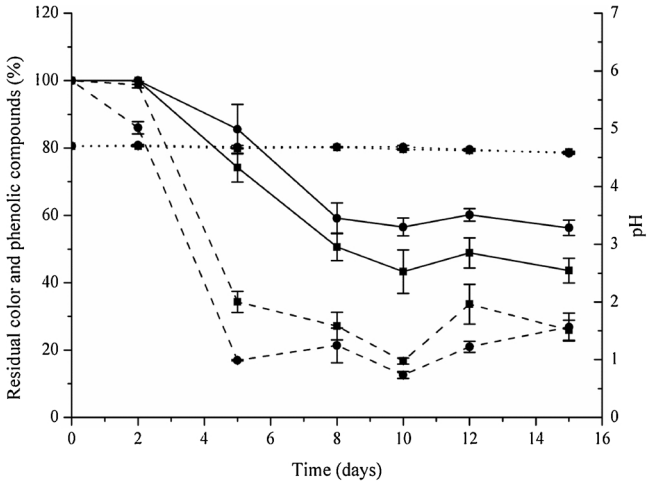

Fig. 6. Profiles of residual color (—), residual phenols (—) and pH (…) obtained by *Trametes* sp. T3: (■) V10 + LBM medium, (●) V10 medium.

3.6. Analysis of absorbance spectra

The absorption spectra of V10 + LBM and V10 media formulated with 10% (v/v) vinasse did not show peaks in the visible region. However, peaks were recorded in the UV region (220–340 nm), indicating the presence of polyphenols [47], one of the main compounds in distillery vinasses. After 15 days of treatment, a decrease in the absorbance in the UV region was verified for all the tested conditions. When *Pycnoporus* sp. P6 was used, an average decrease in absorbance of 47% in V10 + LBM medium and 35% in the V10 medium (Fig. 7) was reached, whereas the decrease obtained by *Trametes* sp. T3 was on average 55% in V10 + LBM and 57% in V10 (Fig. 8). The changes observed in the UV region spectra can be attributed to a transformation of the vinasse compounds by both fungal strains. However, the absence of individual absorption peaks makes it impossible to attribute the observed decrease to the degradation of specific substances [48,49].

The maximum enzymatic activities from *Pycnoporus* sp. P6 and *Trametes* sp. T3 cultures are shown in Table 3.

The enzyme activities recorded by both macrofungi in the vinasse-amended media were higher than those found in the control LBM broth, being obtained among 250% (MIP for *Pycnoporus* sp. P6 in V10 medium) and 1300% (MnP for *Trametes* sp. T3 in V10 medium) more activity in presence of the effluent depending on the enzyme, medium

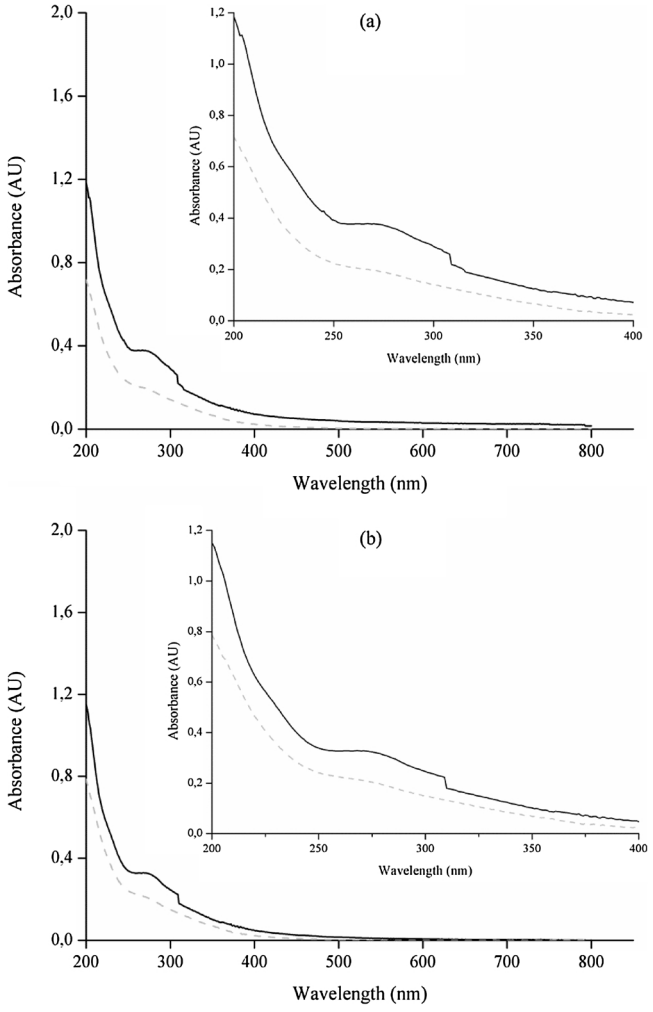

Fig. 7. Absorption spectra of (a) V10 + LBM medium and (b) V10 medium: (—) Untreated media before to start the treatment and (—) treated media after 15 days by *Pycnoporus* sp. P6.

and fungal strain. The titers of Lacc activity were the highest among all the determined, being around tenfold more when vinasse was present in culture media (Table 3). These results showed that sugarcane vinasse acts as an inducer of the production of ligninolytic enzymes as its bioremediation take place.

Results showed a significant correlation between the reduction of color and phenolic compounds in both media treated either by *Pycnoporus* sp. P6 ($r > 0.89$, $p = 0.000$) and by *Trametes* sp. T3 ($r > 0.83$, $p = 0.000$). Maximum color and total phenolics removal occurred simultaneously with higher titers of laccase (Figs. 9, 10). Laccases act oxidizes phenolic groups and has been related to the detoxification of vinasses in previous works. For example, España-Gamboa et al. [5] evaluated the treatment of 10% (v/v) sugarcane vinasse by *Trametes versicolor* in an air–pulsed bioreactor and demonstrated that most color and total phenols were removed when Lacc activity achieved a maximum of 428 U L^{-1}. Verma et al. [50] reported the bioremediation of sugarcane vinasses using a hybrid technology with a *Cerrena unicolor*. As results of their assays, the authors informed that Lacc synthesis was directly correlated with vinasses discoloration. Ferreira et al. [40] treated raw vinasse, vinasse plus glucose and vinasse amended with synthetic fungi media with *P. sajor-caju*, and determined Lacc as the highest enzymatic activity produced during the treatments (400–450 U L^{-1}). Aguiar et al. [15] showed the production of several ligninolytic enzymes by *Pleurotus ostreatus* in a medium with vinasse and sugarcane bagasse, being Lacc activity the one that registered the

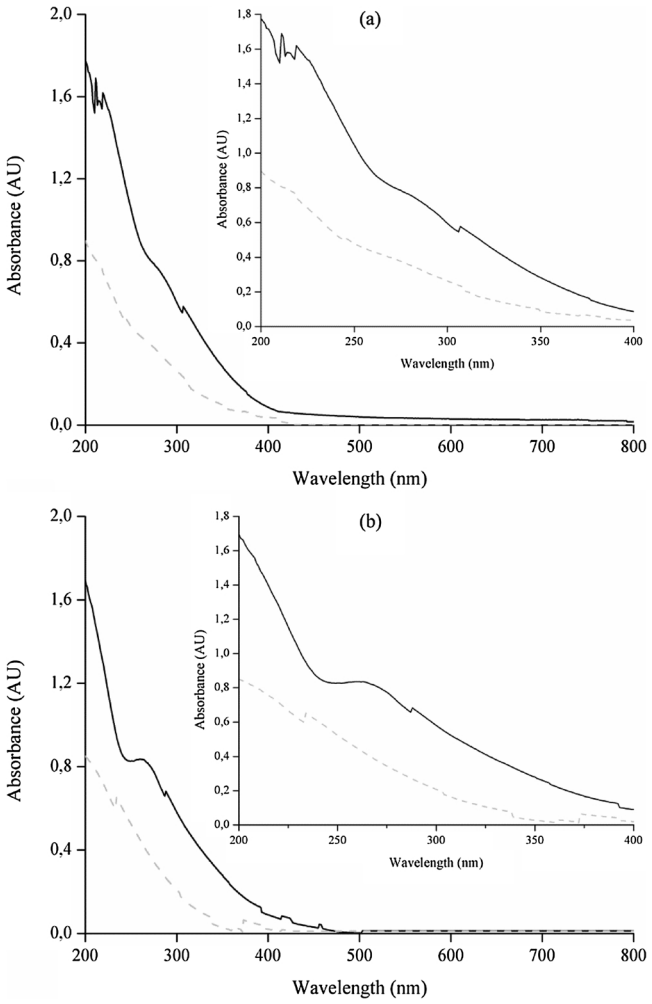

Fig. 8. Absorption spectra of (a) V10 + LBM medium and (b) V10 medium: (—) Untreated media before to start the treatment and (—) treated media after 15 days by *Trametes* sp. T3.

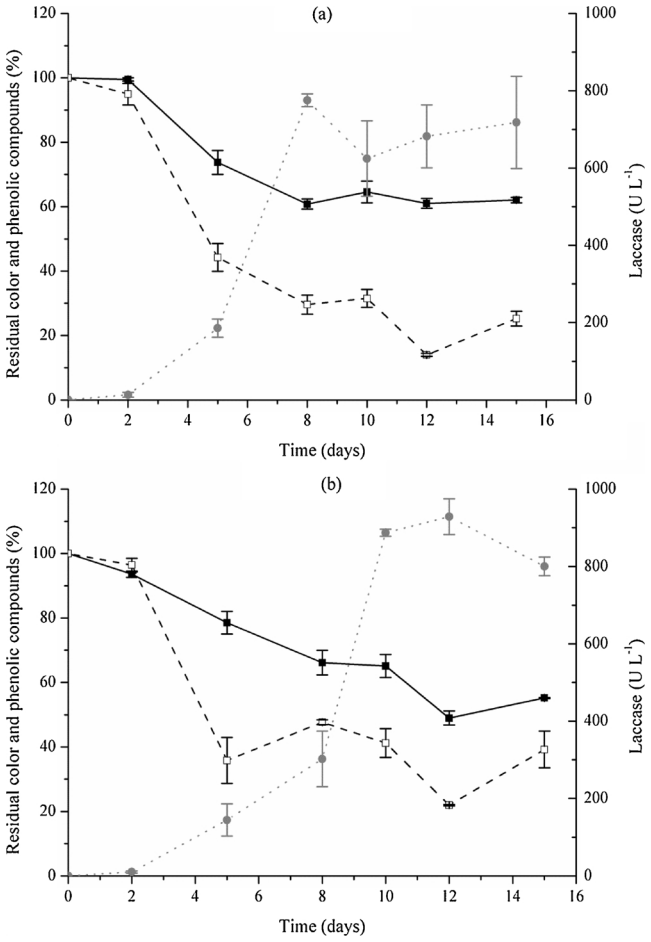

Fig. 9. Profiles of residual color (–■–), residual phenolic compounds (–□–) and laccase activity (…●…) obtained during sugarcane vinasse treatment by *Pycnoporus* sp. P6 in (a) V10 + LBM and (b) V10.

highest titles (325.23 U L^{-1}). Even though recent studies demonstrate Lacc synthesis from the vinasse bioremediation employing ligninolytic macrofungi, the candidates analyzed and presented in this work were able to produce highlighted values of activity, especially if it considers that they are native strains which were not modified.

4. Conclusion

The biological treatment of native sugarcane vinasses can be achieved with the concomitant production of valuable metabolites, leading to the development of innovative and environmental-friendly technologies for the treatments of agro-industrial wastewaters. Autochthonous macrofungi as *Pycnoporus* sp. P6 and *Trametes* sp. T3 demonstrated great ability to decolorize and remove phenolic compounds from sugarcane vinasse while producing laccases and other ligninolytic enzymes. It was demonstrated that vinasses can be used as sole culture medium component, acting as an excellent inducer of lignocellulolytic enzymes, mainly with laccase activity.

The important results obtained in the present work, based on experiences of discoloration and decrease of phenolic compounds, expose the potential possibility of using the autochthonous basidiomycetes

Table 3
Enzymatic activities of the autochthonous macrofungi *Pycnoporus* sp. P6 and *Trametes* sp. T3 cultured in LBM + V10 and V10 media. The number in parentheses shows the maximum enzymatic activity measured in control medium LBM broth.

Fungal strain	*Pycnoporus* sp. P6		*Trametes* sp. T3	
Medium/ Enzyme activity	LVM + V10	V10	LVM + V10	V10
MnP [U L^{-1}]	19.83 ± 3.85 (2.64)	20.20 ± 1.04 (2.64)	8.93 ± 0.25 (0.87)	11.74 ± 0.27 (0.87)
MIP [U L^{-1}]	11.30 ± 1.47 (1.73)	4.37 ± 0.23 (1.73)	8.71 ± 0.35 (0.74)	9.45 ± 0.09 (0.74)
Lacc [U L^{-1}]	775.30 ± 16.5 (78.32)	928.62 ± 46.45 (78.32)	213.44 ± 16.83 (21.36)	198.75 ± 16.00 (21.36)

MnP, manganese-dependent peroxidase, MIP, manganese-independent peroxidase, Lacc, laccase.
Lignin peroxidase was not detected under assay conditions.

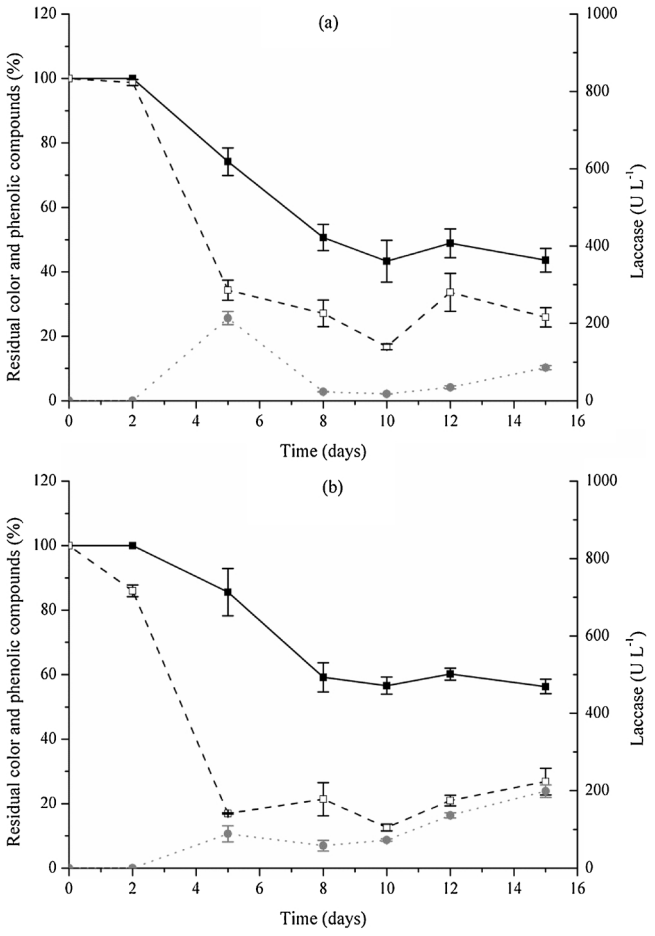

Fig. 10. Profiles of residual color (–■–), residual phenolic compounds (–□–) and laccase activity (…●…) obtained during sugarcane vinasse treatment by *Trametes* sp. T3 in (a) V10 + LBM and (b) V10.

Pycnoporus sp. P6 and *Trametes* sp. T3 in a sustainable biotechnology process. In direct relation, the simultaneous and increased synthesis of oxidases enzymes (Lacc, MnP, and MIP), for the treatment of sugarcane vinasse counteract the negative effects that their dumping and disposal originate on the environment.

Declarations of interest

None.

Acknowledgements

This work was supported by ANPCyT throughout the projects PICT 1154-2013 and PICT 2884-2014, Agroindustrial Experimental Station "Obispo Colombres" (EEAOC), National Scientific and Technical Research Council (CONICET), and Tucumán National University Research Council (CIUNT). The Authors gratefully acknowledge Dr. Silvia Zossi, Eng. Mónica Coronel and Pharma. Gabriela Juárez, for their contribution to assays realization and for useful technical advice, and Dr. M.C.N. Saparrat from the La Plata National University, who kindly provided the references fungal strains. Finally, the Authors also thank Dr. M.J. Martínez from CIB, Madrid, España, for her technical support, and the fuel ethanol companies that gave the effluents for this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jece.2018.08.007.

References

[1] B.S. Moraes, M. Zaiat, A. Bonomi, Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: challenges and perspectives, Renew. Sustain. Energy Rev. 44 (2015) 888–903.
[2] F.A.H. Oboody, H.B. Elamin, H.M. Ibrahim, Evaluation of biogas productivity from the mixtures of Vinasse, wastewater and filter mud, Int. J. Curr. Microbiol. Appl. Sci. 5 (4) (2016) 117–126.
[3] Y. Kharayat, Distillery wastewater: bioremediation approaches, J. Integr. Environ. Sci. 9 (2) (2012) 69–91.
[4] C.A. Christofoletti, J.P. Escher, J.E. Correia, J.F.U. Marinho, C.S. Fontanetti, Sugarcane vinasse: environmental implications of its use, Waste Manage. 33 (12) (2013) 2752–2761.
[5] E. España-Gamboa, T. Vicent, X. Font, J. Mijangos-Cortés, B. Canto-Canché, L. Alzate, Phenol and color removal in hydrous ethanol vinasse in an air-pulsed bioreactor using Trametes versicolor, J. Biochem. Technol. 6 (3) (2015) 982–986.
[6] A. Sepehri, M.H. Sarrafzadeh, Effect of nitrifiers community on fouling mitigation and nitrification efficiency in a membrane bioreactor, Chem. Eng. Process. 128 (2018) 10–18.
[7] O. Ahmed, A.M.E. Sulieman, S.B. Elhardallou, Physicochemical, chemical and microbiological characteristics of vinasse, a by-product from ethanol industry, Am. J. Biochem. 3 (3) (2013) 80–83.
[8] L.T. Fuess, M.L. Garcia, Implications of stillage land disposal: a critical review on the impacts of fertigation, J. Environ. Manage. 145 (2014) 210–229 (accessed 3 July 2018), http://www.ipaat.gov.ar/wp-content/uploads/2017/07/TOTALES-HASTA-1RA-QUINCENA-MAYO-2018.pdf.
[9] M.P. Wagh, P.D. Nemade, Treatment of distillery spent wash by using chemical coagulation (CC) and electro-coagulation [EC], Am. J. Environ Protect. 3 (5) (2015) 159–163.
[10] S. Kulshreshtha, Current trends in bioremediation and biodegradation, J. Bioremed. Biodegrad. 3 (2012) e114, https://doi.org/10.4172/2155-6199.1000e114.
[11] S. Singh, V.K. Gupta, Biodegradation and bioremediation of pollutants: perspectives strategies and applications, Int. J. Pharma Bio Sci. 10 (1) (2016) 53–65.
[12] R. Agarwal, S. Lata, M. Gupta, P. Singh, Removal of melanoidin present in distillery effluent as a major colorant: a review, J. Environ. Biol. 31 (4) (2010) 521–528.
[13] J. Dai, R.J. Mumper, Plant phenolics: extraction, analysis and their antioxidant and anticancer properties, Molecules 15 (10) (2010) 7313–7352.
[14] M.M. Arimi, Y. Zhang, G. Götz, K. Kiriamiti, S.-U. Geißen, Antimicrobial colorants in molasses distillery wastewater and their removal technologies, Int. Biodeterior. Biodegrad. 87 (2014) 34–43.
[15] M.M. Aguiar, L.F.R. Ferreira, R.T.R. Monteiro, Use of vinasse and sugarcane bagasse for the production of enzymes by lignocellulolytic fungi, Braz. Arch. Biol. Technol. 53 (5) (2010) 1245–1254.
[16] A. Bhattacharyya, A. Pramanik, S.K. Maji, S. Haldar, U.K. Mukhopadhyay, J. Mukherjee, Utilization of vinasse for production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) by Haloferax mediterranei, AMB Express 2 (1) (2012) 34.
[17] R. Sindhu, E. Gnansounou, P. Binod, A. Pandey, Bioconversion of sugarcane crop residue for value-added products–an overview, Renew. Energy 98 (2016) 203–215.
[18] American Public Health Association, American Water Works Association, and Water Environment Federation, Standard Methods for the Examination of Water and Wastewater, 21st ed., APHA, Washington, 2005.
[19] J.A. Aziz, T.H.Y. Tebbutt, Significance of COD, BOD and TOC correlations in kinetic models of biological oxidation, Water Res. 14 (4) (1980) 319–324.
[20] International Commission for Uniform Methods of Sugar Analysis (ICUMSA), Method Book, Bartens, Berlin, Germany, 2007.
[21] P.M. Ahmed, Biorremediación De Vinazas De Destilerías De Alcohol, Por Microorganismos Autóctonos Aislados De Ambientes Contaminados. Doctoral Thesis, Universidad Nacional de Tucumán, Argentina, 2016.
[22] D. Salvachúa, A. Prieto, M.E. Vaquero, Á.T. Martínez, M.J. Martínez, Sugar recoveries from wheat straw following treatments with the fungus Irpex lacteus, Bioresour. Technol. 131 (2013) 218–225.
[23] M.C.N. Saparrat, P.A. Balatti, A.M. Arambarri, M.J. Martínez, *Coriolopsis rigida*, a potential model of white-rot fungi that produce extracellular laccases, J. Ind. Microbiol. Biotechnol. 41 (4) (2014) 607–617.
[24] T.J. White, T. Bruns, S. Lee, J.W. Taylor, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (Eds.), PCR Protocols: A Guide to Methods and Applications, Academic Press, New York, USA, 1990, pp. 315–322.
[25] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, et al., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[26] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[27] K. Tamura, M. Nei, Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, Mol. Biol. Evol. 10 (3) (1993) 512–526.
[28] S.B. Pointing, Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi, Fungal Divers 2 (1999) 17–33.
[29] I. Khokhar, M.S. Haider, S. Mushtaq, I. Mukhtar, Isolation and screening of highly cellulolytic filamentous fungi, J. Appl. Sci. Environ. Manage. 16 (3) (2012) 223–226.
[30] M.D. Castillo, J. Stenstrom, P. Ander, Determination of manganese peroxidase activity with 3-methyl-2-benzothiazolinone hydrazone and 3-(dimethylamino)

benzoic acid, Anal. Biochem. 18 (2) (1994) 399–404.
[31] I. Minitab, MINITAB Release 17: Statistical Software for Windows, Minitab Inc., USA, 2014.
[32] M.A. Clarke, R.S. Blanco, M.A. Godshall, T.B.T. To, Color components in sugar refinery processes, Proceedings of the Intl. Meeting of Sugar Industry Technologists 44 (1985) 53–87.
[33] S. Sirianuntapiboon, P. Phothilangka, S. Ohmomo, Decolorization of molasses wastewater by a strain No.BP103 of acetogenic bacteria, Bioresour. Technol. 92 (1) (2004) 31–39.
[34] R. Bourbonnais, M.G. Paice, Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation, FEBS Lett. 267 (1) (1990) 99–102.
[35] R.C. Minussi, S.G. De Moraes, G.M. Pastore, N. Duran, Biodecolorization screening of synthetic dyes by four white-rot fungi in a solid medium: possible role of siderophores, Lett. Appl. Microbiol. 33 (1) (2001) 21–25.
[36] M. Tejada, J.L. Gonzalez, Effects of two beet vinasse forms on soil physical properties and soil loss, Catena 68 (1) (2006) 41–50.
[37] D. Pant, A. Adholeya, Biological approaches for treatment of distillery wastewater: a review, Bioresour. Technol. 98 (12) (2007) 2321–2334.
[38] M. Arienzo, E.W. Christen, W. Quayle, A. Kumar, A review of the fate of potassium in the soil-plant system after land application of wastewaters, J. Hazard. Mater. 164 (2) (2009) 415–422.
[39] S. Rodríguez, M. Fernández, R.C. Bermúdez, H. Morris, Tratamiento de efluentes industriales coloreados con *Pleurotus* spp, Rev. Iberoam. Micol. 20 (2003) 164–168.
[40] L.F.R. Ferreira, M. Aguiar, G. Pompeu, T.G. Messias, R.R. Monteiro, Selection of vinasse degrading microorganisms, World J. Microbiol. Biotechnol. 26 (9) (2010) 1613–1621.
[41] S. Welti, P.A. Moreau, A. Favel, R. Courtecuisse, M. Haon, D. Navarro, et al., Molecular phylogeny of *Trametes* and related genera, and description of a new genus Leiotrametes, Fungal Divers 55 (1) (2012) 47–64.
[42] R.A.M. Watanabe, P.D.T.F. Sales, L.C. Campos, T.A. Garcia, M.C. Valadares, F. Schimidt, et al., Evaluation of the use of *Pycnoporus sanguineus* fungus for phenolics and genotoxicity decay of a pharmaceutical effluent treatment, Ambiente E Agua - Interdiscip. J. Appl. Sci. 7 (3) (2012) 41–50.
[43] C.A. Ottoni, C. Santos, Z. Kozakiewicz, N. Lima, White-rot fungi capable of decolourising textile dyes under alkaline conditions, Folia Microbiol. (Praha) 58 (3) (2013) 187–193.
[44] A. Levasseur, A. Lomascolo, O. Chabrol, F.J. Ruiz-Dueñas, E. Boukhris-Uzan, F. Piumi, et al., The genome of the white-rot fungus *Pycnoporus cinnabarinus*: a basidiomycete model with a versatile arsenal for lignocellulosic biomass breakdown, BMC Genomics 15 (1) (2014) 486.
[45] M.E. Eugenio, J.M. Carbajo, M.C. Terrón, A.E. González, J.C. Villar, Bioremediation of lignosulphonates by lignin-degrading basidiomycetous fungi, Bioresour. Technol. 99 (11) (2008) 4929–4934.
[46] S.B. Pointing, L.L.P. Vrijmoed, Decolorization of azo and triphenylmethane dyes by *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase, World J. Microbiol. Biotechnol. 16 (3) (2000) 317–318.
[47] J. Beltran-Heredia, J. Torregrosa, J.R. Dominguez, J. Garcia, Treatment of black-olive wastewaters by ozonation and aerobic biological degradation, Water Res. 34 (14) (2000) 3515–3522.
[48] M. Coca, M.T. García, G. González, M. Peña, J.A. García, Study of coloured components formed in sugar beet processing, Food Chem. 86 (3) (2004) 421–433.
[49] S.C. Shen, K.C. Tseng, J.S.B. Wu, An analysis of Maillard reaction products in ethanolic glucose-glycine solution, Food Chem. 102 (1) (2007) 281–287.
[50] A.K. Verma, C. Raghukumar, C.G. Naik, A novel hybrid technology for remediation of molasses-based raw effluents, Bioresour. Technol. 102 (3) (2011) 2411–2418.