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Post-harvest sugarcane residue degradation by autochthonous fungi

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ABSTRACT

Several fungal species were isolated from different sources: post-harvest sugarcane residue, soil, decomposing forest litter and from mycelia obtained from the inner parts of fresh fungal fruiting bodies collected in Las Yungas region (Argentina). These isolates were first screened for their ability to produce carboxymethyl cellulose (CMC) degradation and guaiacol oxidation. After primary screening, seventeen isolates were further tested for their ligninolytic ability by assessing polyphenoloxidase, laccase, manganese peroxidase and endoxylanase activities. Based on their lignocellulolytic activities, five isolates (named *Bjerkandera* sp. Y-HHM2, *Phanerochaete* sp. Y-RN1, *Pleurotus* sp. Y-RN3, *Hypocrea nigricans* SCT-4.4 and *Myrothecium* sp. S-3.20) were selected for liquid and solid-state fermentation assays in culture media including sugarcane debris. Lignocellulolytic enzymes production, dry mass loss and phenol concentration in the water soluble fraction were then evaluated. Results suggest that native strains with lignocellulolytic activity are suitable to increase post-harvest sugarcane residue decomposition and support the use of these strains as an alternative to pre and post-harvest burning. Biological treatments using *Phanerochaete* sp. Y-RN1, *Pleurotus* sp. Y-RN3 and *Myrothecium* sp. S-3.20 could be used to degrade and increase the accessibility to lignocellulose components of sugarcane residue.

1. Introduction

Widely common practices in sugarcane production include pre and post-harvest burning. Pre-harvest burning facilitates cutting and piling of sugarcane stalks. Post-harvest burning removes remaining obstruction to operations in establishing the ratoon crop (Mendoza et al., 2001). However, these practices produce environmental damages and health hazards due to the exposure to smoke and particulate matter.

Post-harvest sugarcane residue is a lignocellulosic material removed from the cane stalks and deposited on field surface. Average structural polysaccharide percentages on dried sugarcane residue are cellulose (38.1%), hemicellulose (29.2%), lignin (24.7%), ashes (3.4%) and extractives (4.7%) (Miléo et al., 2011).

When sugarcane residue is retained on soil surface, the residue blanket improves soil organic carbon and nitrogen content (Hemwong et al., 2008). However, residue mineralization is a very slow process, mainly due to its high C/N relationship (Digonzelli et al., 2011) taking almost one year to total decomposition (Robertson and Thorburn, 2007).

Accelerated natural decomposition of such residues can avoid the detrimental effects of burnt and biomass accumulation on soil surface. Fungi are the predominant organisms responsible for lignocellulose degradation (Sánchez, 2009). It has been reported that fungi improve agricultural wastes degradation such as rice straw (Yu et al., 2009; Kausar et al., 2010; Chang et al., 2012), wheat straw (Vares et al., 1995; Dorado et al., 1999; Dinis et al., 2009) and corn stover (Panagiotou et al., 2003; Wan and Li, 2010). Sugarcane residue degradation by fungi is possible due to the existence of enzymatic hydrolytic systems (mainly cellulases and hemicellulases) and oxidative extracellular ligninolytic systems (composed by laccases, lignin peroxidases and/or manganese peroxidases) (Sánchez, 2009).

The isolation of autochthonous fungal strains for their application in sugarcane residue degradation is mandatory, in order to avoid the introduction of exotic fungi into sugarcane fields. The aim of this work was the selection of lignocellulolytic fungi from green-harvest sugarcane fields and subtropical mountain forests in northwest Argentina (Las Yungas) and the assessment of their

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sugarcane residue degradation potential under liquid and solid-state cultivation by the evaluation of the activity of lignocellulolytic enzymes.

2. Materials and methods

2.1. Collection of fungal samples and isolation of lignocellulolytic fungi

Samples were collected from several sources.

Soil and sugarcane residue samples were collected from fields cropped with CP 65-357, LCP 85-384 and RA 87-3 sugarcane varieties in Leales, Tucumán (Argentina) during 2009–2010. These are the most widespread commercial varieties in the zone. Samples were collected and stored in plastic bags at 4 °C until processed. In order to isolate and purify fungal strains, residues were cut in small sizes, disinfected and placed on 2% potato-dextrose-agar (PDA) according to standard methods (Rayner and Todd, 1977). Ten-fold serial dilutions of each soil sample were made in sterilized distilled water and 100 μl then spreaded on PDA surface (D'Annibale et al., 2006) and incubated at 30 ± 1.5 °C for 72 h. In order to obtain pure/axenic cultures, morphologically different fungi colonies were sub-cultured on PDA medium.

Fungi were also isolated from decomposing forest litter and mycelia obtained from the inner parts of fungal fresh fruiting bodies collected in Las Yungas region (Parque Sierra de San Javier) and transferred onto malt extract agar (MEA).

Stock cultures were maintained on MEA slants at 4 °C.

2.2. Reference strain

Coriolopsis rigida CLPS 232 (Spegazzini Institute Culture Collection) was used as a positive control for lignocellulolytic screening assays. It has been previously studied for its capacity to degrade agroindustrial solid by-products (Saparrat et al., 2010a). This strain was kindly provided by Dr. M.C.N. Saparrat from the Universidad Nacional de La Plata, La Plata, Argentina and is deposited in the Spanish Type Culture Collection (CECT 20449).

2.3. Culture media and inocula

Potato-Dextrose-Agar (PDA) (l^{-1}): potato infusion (infusion from 200 g potatoes), 20.0 g dextrose and 17.0 g agar. The pH was adjusted to 5.0 with lactic acid 25% (Chand et al., 2005). Malt extract (ME) broth (l^{-1}): 20.0 g malt extract and 1.0 g yeast extract (pH 6.0) was prepared for liquid state fermentation assays. For agar plates assays 20.0 g agar was added. Sodium carboxymethyl cellulose (CMC) agar (l^{-1}): 2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 2.0 g CMC sodium salt, 0.2 g peptone, and 17.0 g agar (Kasana et al., 2008). For endoxylanase assay 20.0 g l^{-1} beechwood xylan was incorporated to a basal medium containing (l^{-1}): 0.5 g NaH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.3 g yeast extract, 0.3 g malt extract and 10.0 ml traze solution (0.01 g $CaCl_2$, 0.001 g $CuSO_4 \cdot 5H_2O$, 0.001 g $Fe_2(SO_4)_3$ and 0.001 g $MnSO_4$). Yeast extract–Malt extract (YM) broth (l^{-1}): 10.0 g glucose, 5.0 g peptone, 3.0 g malt extract and 3.0 g yeast extract (pH 6.4) (Pajot et al., 2011). SCR (sugarcane residue) broth (l^{-1}) containing 2.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g KCl, 0.5 $Mg_2SO_4.7H_2O$, 0.01 g $FeSO_4$, 4.0 g malt extract, 3.0 g glucose and 5.0 g sugarcane residue (pH 5.0) (Chang et al., 2012) was prepared. First, sugarcane residue was milled and sieved through a 1 mm mesh. For solid-state fermentation assays sugarcane residue moisture content was adjusted to 80% after sterilization by the addition of 2.0 g l^{-1} $NaNO_3$, 1.0 g l^{-1} KH_2PO_4, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $FeSO_4$, 4.0 g l^{-1} malt extract and 3.0 g l^{-1} glucose. Stock cultures were kept at 4 °C on 2% MEA slants supplemented with yeast extract (0.1%). In all screening test, inoculum consisted of one agar plug (6 mm diameter) obtained from the hyphal edge of 8 day old cultures grown on MEA. Agar plate screenings were performed in 90 mm diameter Petri dish containing 15 ml of culture medium. In liquid and solid state fermentation assays, 3 agar plugs of each fungal strain were used as inoculum.

2.4. Screening for lignocellulolytic activity

A two-step screening strategy was adopted to test enzymatic activities possibly involved in lignocellulosic material degradation. First, a primary screening was designed to evaluate strains with cellulolytic activity on CMC agar and oxidative enzymatic activity on MEA supplemented with guaiacol. Second, the positive cellulolytic and oxidative isolates were assayed for their ligninolytic activities.

2.4.1. Primary screening for lignocellulolytic activity

2.4.1.1. Extracellular cellulolytic activity on agar plates. The extracellular cellulase activity was measured after 48 h of incubation at 30 ± 1.5 °C in CMC agar. The plates were flooded with KI/I_2 for 5 min as described by Kasana et al. (2008). Diameter of the zone of clearance around the colonies was recorded. The clear zone diameter/colony diameter ratio was calculated and expressed as Index of Relative Enzyme Activity (I_{CMC}) (Pečiulytė, 2007).

2.4.1.2. Extracellular oxidative enzymatic activity on agar plates. Ligninolytic activity was estimated on MEA medium supplemented with 1 mM guaiacol. Formation of a reddish-colored halo around colonies was taken as positive reaction (Saparrat and Hammer, 2006). Plates were incubated at 30 ± 1.5 °C, in darkness, and were examined daily for 10 days.

2.4.2. Secondary screening for lignocellulolytic activity

Polyphenoloxidase, laccase, manganese peroxidase and endoxylanase enzyme production were evaluated through agar-plate diffusion assays. Uninoculated plates were included as abiotic controls and growth control plates (without enzymatic substrate) were also included. Plates were incubated at 30 ± 1.5 °C in darkness and were examined daily for 10 days.

2.4.2.1. Polyphenoloxidase (PPO) assay. PPO was detected through the use of MEA medium supplemented with 1% (w/v) tannic acid. The formation of a brown color surrounding the growth area was taken as the indication of PPO production (Pointing, 1999).

2.4.2.2. ABTS assay. MEA plates supplemented with 2 μM ABTS (2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid)) diammonium salt were evaluated for the formation of a green color around the fungal mycelia due to the oxidation of ABTS in the presence of laccase and peroxidase (Pointing, 1999).

2.4.2.3. Manganese peroxidase (MnP) assay. The presence of MnP was examined in MEA plates containing 100 ppm of $MnCl_2.4H_2O$. The formation of dark-brown flecks was considered as positive MnP production (Steffen et al., 2000). The presence of MnP was also assessed in agar plates with phenol red (0.03%). In this case, formation of a clearance halo around colonies was taken as a positive result (Boominathan et al., 1990).

2.4.2.4. Endoxylanase assay. Endoxylanase was detected on beechwood xylan agar plates. At 48 h the plates were flooded with iodine stain (0.25% w/v aqueous I_2/KI) for 5 min. Xylan degradation around the colonies appeared as a yellow-opaque area in contrast

with a blue/reddish purple color produced by undegraded xylan (Pointing, 1999).

2.5. DNA isolation

Fungi cultures of each isolate were grown in 50 ml of YM broth at 30 °C for 72 h on an orbital shaker (250 rpm). Mycelia were then collected by centrifugation at 10,000 rpm (10 min at 4 °C), resuspended in 2 M NaCl and finally washed twice with sterile water (Fang et al., 1992). Washed mycelial mats were frozen with liquid nitrogen and ground to powder by using a sterile pestle and mortar. Grounded mycelia were extracted once with phenol:-chloroform:isoamyl alcohol (25:24:1) and washed twice with chloroform:isoamyl alcohol (24:1). Two volumes of absolute ethanol and 0.1 volume of 3 M potassium acetate were added to the final aqueous phase in order to achieve DNA precipitation, mixed by inversion and then centrifuged (10 min at 4 °C, 8000 rpm). The pellet was then washed twice with 70% ethanol, dried and finally resuspended in distilled water.

2.6. rDNA amplification, sequencing and analysis

Fungal ITS1-5, 8S-ITS2 regions and D1/D2 domains of the LSU rDNA gene were amplified by using Internal Transcribed Spacer 1 (ITS-1) (5′-TCC GTA GGT GAA CCT GCG G-3′) and NL-4 (GGT CCG TGT TTC AAG ACG G-3′) primers (White et al., 1990). Nucleotide sequencing of the genes was performed by Macrogen (Korea). Sequences were analyzed, and edited if necessary, using the Invitrogen Vector NTI Advance 10.3.0 software (Invitrogen, San Diego, CA, USA). DNA sequences were submitted to GenBank under the accession numbers listed in Table 3. Strain identification was performed by comparison with the AFToL and GenBank databases, using BLAST software provided. Arbitrarily, a ≥99% identity criterion was employed to identify strains at the species level. Sequences showing 99%–96% identity, were tentatively identified to the genus level. Sequences showing 96% identity were considered unidentified.

For each strain, an illustrative phylogenetic trees was constructed including available sequences from representative related strains. The evolutionary history was inferred using the Maximum Parsimony method, as included in MEGA5 (Tamura et al., 2011). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated.

2.7. Liquid-state fermentation (LF)

LF assays were performed with fungal strains selected in the secondary screening. Secretion of lignocellulolytic enzymes in liquid cultures was evaluated in duplicate using 250 ml Erlenmeyer flasks containing 50 ml of the medium (ME broth and SCR broth). Inoculated and uninoculated flasks (used as abiotic controls) were incubated at 30 °C and 250 rpm for 5 d. Liquid cultures were then centrifuged at 9000 rpm during 15 min and 4 °C. Supernatant was collected for further enzyme analysis.

2.8. Solid-state fermentation (SSF)

SSF assays were conducted to study the effect of fungal treatments on sugarcane residue degradation. SSF fungal strains were grown in 50 ml Erlenmeyer flasks containing 2 g of dried and sterilized sugarcane residue (80% initial moisture content). Treatments and abiotic controls were incubated at 30 ± 1.5 °C up to 14 d. Samples were sacrificed after 7 and 14 d incubation. Total solid substrates were suspended in 30 ml sodium acetate buffer (50 mM, pH 4.5) and extracted in an orbital shaker (3 h at 30 °C, 150 rpm). Samples were then filtered through Whatman N°1 filter paper and centrifugated (15 min at 4 °C, 9000 rpm). Water soluble fractions were finally collected for further assays (Wan and Li, 2010). After enzyme extraction, solid substrates were dried for 24 h at 105 °C and weighed. Assays were performed by duplicate. Sugarcane residue degradation was expressed as the loss of dry mass (%), calculated as the difference between the dry mass on substrates with and without inoculation (Saparrat et al., 2008).

2.9. Analysis and enzyme assays

Total phenolic compounds were measured in the water soluble fraction according to Singleton et al. (1999) with Folin-Ciocalteu reagent using tannic acid as standard. Soluble proteins were measured by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation at 420 nm of 1.8 mM ABTS (ε = 36,000 M^{-1} cm^{-1}) in 50 mM acetate buffer (pH 4.0). Mn-independent peroxidase activity was measured using the same assay but adding 5 mM H_2O_2 and subtracting the previously determined laccase activity. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS in 1 min (Bourbonnais and Paice, 1990).

Manganese peroxidase (MnP) (EC 1.11.1.13) was determined according to Castillo et al. (1994). The reaction of 0.21 mM MBTH (3-methyl-2-benzothiazolinone hydrazone) and 3 mM DMAB (3-dimethylaminobenzoic acid) was measured at 610 nm (ε = 53,000 M^{-1} cm^{-1}) in presence of 2.7 mM $MnSO_4.4H_2O$ and 0.45 mM H_2O_2 in succinate-lactate buffer (100 mM, pH 4.5). Results were corrected by activities in test samples without manganese. One unit of manganese peroxidase activity (U) was defined as the amount of enzyme required to oxidize 1 μmol of substrate in 1 min.

Endo-β-1,4-glucanase (EC 3.2.1.4) and endo-β-1,4-xylanase (EC 3.2.1.8) were measured with CMC and beech wood xylan as the substrates, respectively (Ghose, 1987; Ghose and Bisaria, 1987). Reducing sugars were determined by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugars per min under the assay conditions.

β-Glucosidase (EC 3.2.1.21) activity was estimated using 1.5 mM of p-nitro phenyl β-D-glucopyranoside (pNPG) in sodium acetate buffer (50 mM) and incubated at 40 °C for 60 min. Reaction was terminated by the addition of 0.2 M sodium carbonate. The p-nitrophenol liberated was measured at 420 nm (ε = 16,000 M^{-1} cm^{-1}) (Miake et al., 2000).

2.10. Statistical analysis

Statistical analysis were performed using InfoStat (2011). Correlations between enzymatic abilities and fungal origin were analyzed using chi-squared test for categorical variables. All comparisons were done using right unilateral tests and $p < 0.05$ was considered significant. A one-way ANOVA and Tukey's test ($p \leq 0.05$) were used to evaluate differences in phenol concentration and dry mass loss between fungal treatments in LF and SSF.

3. Results and discussion

3.1. Collection of fungal samples and isolation of lignocellulolytic fungi

Forty-nine fungal strains were isolated from different sources and were screened for their lignocellulolytic potential. Nine of

them were taken from sugarcane crop residues, 16 from soil and 24 from Las Yungas forest. Among these 24, four strains were isolated from decomposing forest litter and 19 from fresh fruiting bodies founded in dead trees, decomposing wood and one from burnt wood.

3.2. Primary screening for lignocellulolytic activity

All the isolates were evaluated for their ability to produce colorless zones in CMC agar plates (Table 1) due to the presence of endoglucanase enzymes. Eight fungal strains presented strong extracellular colorless halos around colonies and $I_{CMC} > 1$. The I_{CMC} of SCT-4.4 and SCT-5.1.1 were near to one. No statistically significant correlation was found between endoglucanase activity and fungal origin ($\chi^2 = 0.57$, $p = 0.4505$).

As shown in Table 1, 14 fungal strains presented positive reaction on guaiacol supplemented agar. Seven strains isolated from Las Yungas showed intense positive reaction in guaiacol agar plates and three showed weak positive reactions. Four strains from sugarcane fields only exhibited a weak positive reaction. A significant association between guaiacol oxidation and fungal strains presence was detected ($\chi^2 = 8.53$, $p = 0.0141$).

From these screenings, 17 strains which produced a clear halo on CMC agar plates around their colonies ($I_{CMC} > 1$) and positive reaction on guaiacol plates, were selected for further research. Four strains from sugarcane residue (SCT-2.7, SCT-2.14, SCT-3.8, SCT-4.4), three originating from soil (S-3.8, S-3.20, S-3.22) and ten strains isolated from Las Yungas (Y-H6, Y-H7, Y-H11, Y-H13, Y-H14, Y-HHM2, Y-HHM10, Y-RN1, Y-RN3, Y-RM).

Table 1
Screening of lignocellulolytic activity on solid medium with carboxymethyl cellulose and ME agar supplemented with guaiacol. SCT: fungal strains isolated from sugarcane residue; S: fungal strains isolated from soil and Y: fungal strains isolated from Las Yungas forest.

Fungal strains	Extracellular cellulolytic activity		Extracellular oxidative enzyme activity
	I_{CMC}	Clearing zone	Reddish halo
SCT-2.3	1.12 ± 0.09	++	−
SCT-2.4	0.78 ± 0.08	+	−
SCT-2.7	1.12 ± 0.03	++	+
SCT-4.1	0.84 ± 0.04	+	−
SCT-2.12	1.80 ± 0.12	++	−
SCT-2.14	1.03 ± 0.04	+	+
SCT-3.4	0.87 ± 0.02	+	−
SCT-4.4	0.85 ± 0.02	+	−
SCT-5.1.1	1.00 ± 0.01	+	−
S-1.4	0.67 ± 0.04	+	−
S-2.4	0.92 ± 0.01	+	−
S-2.7	0.88 ± 0.03	+	−
S-2.9	1.26 ± 0.11	++	−
S-2.10	0.97 ± 0.12	+	−
S-2.13	0.47 ± 0.04	+	−
S-3.5	0.61 ± 0.05	+	−
S-3.8	0.70 ± 0.08	+	−
S-3.9	0.34 ± 0.06	+	−
S-3.12	0.64 ± 0.03	+	−
S-3.18	0.82 ± 0.03	+	−
S-3.19	0.39 ± 0.08	+	−
S-3.20	0.77 ± 0.20	+	+
S-3.22	ND	ND	+
S-3.24	ND	ND	−
S-3.25	1.27 ± 0.09	++	−
Y-H1	0.27 ± 0.02	+	−
Y-H2	1.34 ± 0.38	++	−
Y-H4	0.89 ± 0.09	+	−
Y-H6	0.79 ± 0.06	+	+
Y-H7	0,97 ± 0.10	+	++
Y-H8	0.75 ± 0.06	+	−
Y-H11	0.63 ± 0.09	+	++
Y-H13	0.94 ± 0.14	+	++
Y-H14	0.87 ± 0.04	+	++
Y-H19	0.88 ± 0.03	+	−
Y-H20	0.98 ± 0.01	+	−
Y-HHM1	0.87 ± 0.02	+	−
Y-HHM2	0.81 ± 0.04	+	+
Y-HHM6	0.95 ± 0.03	+	−
Y-HHM7	0.92 ± 0.02	+	−
Y-HHM10	1.47 ± 0.03	++	++
Y-HHM12	0.97 ± 0.02	+	−
Y-HFM1	1.06 ± 0.02	+	−
Y-HFM2	0.99 ± 0.01	+	−
Y-RN1	0.33 ± 0.06	+	+
Y-RN2	0.29 ± 0.07	+	−
Y-RN3	1.49 ± 0.20	++	++
Y-RM	0.65 ± 0.03	+	++
Y-HL	1.03 ± 0.06	+	−
C. rigida 232	1.15 ± 0.11	++	++

(−): Negative reaction; (+): positive reaction with weak halo under the colony; (++): positive reaction with strong extracellular halo. ND: not detected.

3.3. Secondary screening for lignocellulolytic activity

Two strains from soil from sugarcane fields (S-3.20 and S-3.22) were positive for ABTS oxidation, polyphenoloxidase (tannic acid oxidation) and both manganese peroxidase activities (Table 2).

Y-HHM2, Y-HHM10, Y-RN1 and Y-RN3 showed positive results in all enzymatic assays. All evaluated fungi presented xylanase activity, implicating hemicellulose degradation.

Considering the results of these screening assays, five fungal isolates (Y-HHM2, Y-RN1, Y-RN3, SCT-4.4 and S-3.20) were selected for sugarcane residue fermentation assays.

As shown in Table 2, strain Y-HHM10 was capable of positive reaction in all enzymatic substrates. Unfortunately, this strain had the lowest radial growth rate (data not shown) making its production for degradation assays difficult. Due to this growth dynamics, Y-HHM10 was not selected for further research.

3.4. Molecular identification

Table 3 shows the results of the molecular identification of the selected fungal strains. According to the adopted criteria, strain

Table 2
In-vitro production of polyphenoloxidase, laccase, manganese peroxidase and endoxylanase on solid media supplemented with enzymatic substrates.

Fungal strains	Tannic acid oxidation[a]	ABTS oxidation[b]	$MnCl_2$ oxidation[c]	Phenol red decoloration[d]	Xylan degradation[e]
SCT-2.7	−	+	−	−	+
SCT-2.14	−	+	−	−	+
SCT-4.4	−	−	−	−	+
SCT-5.1.1	−	−	−	+	+
S-3.8	−	−	−	−	+
S-3.20	+	+	+	+	++
S-3.22	+	+	+	+	++
Y-H6	−	−	−	−	+
Y-H7	+	+	−	−	+
Y-H11	+	+	+	−	++
Y-H13	+	++	−	−	+
Y-H14	+	+	−	−	++
Y-HHM2	+	+	++	+	+
Y-HHM10	+	+	+	+	+
Y-RN1	+	+	++	+	+
Y-RN3	+	++	+	+	++
Y-RM	+	++	−	−	++
C. rigida 232	+	+	+	−	+

(−): Negative reaction; (+): positive reaction; (++): intense positive reaction.
[a] Brown zone around colonies.
[b] Dark green zone around colonies.
[c] Dark brown precipitated.
[d] Phenol red agar medium-colorless.
[e] Clear zone around colonies.

Table 3
Molecular identification of fungal isolates.

#	Accession number	AFToL/Wasabi					NCBI				Identification tentative
		AFTOL-ID	Closest match	Collection number	Accession number	Identity (%)	Closest match	Collection number	Accession number	Identity (%)	
Y-HHM2	KF578081	484	*Phlebia radiata*	FLP 6140	AY854087	93	*Bjerkandera* sp.	ZLY2010 isolate M127	HM595615	100	*Bjerkandera* sp.
Y-RN1	KF578080	1802	*Trichosporon lignicola*	FLP 6140	DQ836002	92	*Phanerochaete sordida*	KHL12054	EU118653	96	*Phanerochaete* sp.
Y-RN3	KF578085	564	*Pleurotus ostreatus*	6689 (T)	AY645052	99	*Pleurotus ostreatus*	6689 (T)	AY450345	99	*Pleurotus* sp.
			Agaricus ostreatus	CBS 102510 (T)			*Pleurotus populinus*	9936	AY450346		
SCT-4.4	KF578083	52	*Hypocrea americana*	OSC 100005	AY544649	96	*Hypocrea nigricans*	NBRC 31289	JN943370	100	*H. nigricans*
S-3.20	KF578084	186	*Hydropisphaera erubescens*	ATCC 36093	AY545726	93	*Myrothecium verrucaria*	BBA 71017 (CBS 328.52(T) = ATCC 9095(T))	AJ302003	99	*Myrothecium* sp.
		155	*Roumegueriella rufula*	CBS 346.85	DQ518776	93					

SCT-4.4 was identified to the species level and renamed *Hypocrea nigricans* SCT-4.4, meanwhile, strain S-3.20 was identified and renamed *Myrothecium* sp. S-3.20. Despite of showing 100% identity with GenBank sequences, Y-HHM2 could only be identified to the genus level since no valid description could be found for *Bjerkandera* sp. ZLY2010 isolate M127 and the strain was consequently renamed *Bjerkandera* sp. Y-HHM2. Similarly, Y-RN3 was renamed *Pleurotus* sp. Y-RN3 since identities between different *Pleurotus* species over the analyzed sequence are usually over 99%. Further studies are required for the full identification of this strain. Strain Y-RN1, showing 96% identity with *Phanerochaete sordida* KHL12054, could barely be identified at the genus level and was then renamed *Phanerochaete* sp. Y-RN1.

Phylogenetic trees (Maximum Parsimony) based on ITS1-5,8S-ITS2 regions and D1/D2 domains of the LSU rDNA gene sequence, showing relationship of the isolated strains with closely related species and genera are presented as supplementary content.

3.5. Liquid-state fermentation (LF)

Phenolic compounds could be released by the enzymatic activities related to lignocellulolytic systems. Saparrat et al. (2010b) reported phenol reduction in water-soluble fraction of *Scutia buxifolia* leaves treated with the ascomycete fungus *Ciliochorella* sp. which only exhibits β-1,4 endoglucanase activity (no laccase or peroxidase).

In liquid cultures, the basidiomycetes *Bjerkandera* sp. Y-HHM2 and *Phanerochaete* sp. Y-RN1 and the deuteromycetes *Myrothecium* sp. S-3.20, significantly reduced phenol concentration, possibly implying its use as carbon/energy source for fungal growth. In the case of *Pleurotus* sp. Y-RN3, a slight decrease in phenol concentration, was also observed but this difference was not statistically significant. On the other hand, *H. nigricans* SCT-4.4 presented an increase over the abiotic control, even though it proved to be not significant (Fig. 1).

Lignocellulolytic enzymes production were evaluated in selected strain, since they are usually related to agricultural waste degradation. Lacasse and Mn-independent peroxidase activities were detected in low levels in ME and SCR broth, while MnP could not be detected on liquid media (Table 4).

Measured hydrolytic enzymes activities are shown in Table 5. Endo-β-1,4-glucanase activity in *Myrothecium* sp. S-3.20 in SCR broth increased up to 2-fold compared to ME broth. *Pleurotus* sp. Y-RN3 was only able to produce endoglucanase when incubated in SCR-broth. Endoxylanase had considerable levels of activity on liquid cultures of *H. nigricans* SCT-4.4 and *Phanerochaete* sp. Y-RN1.

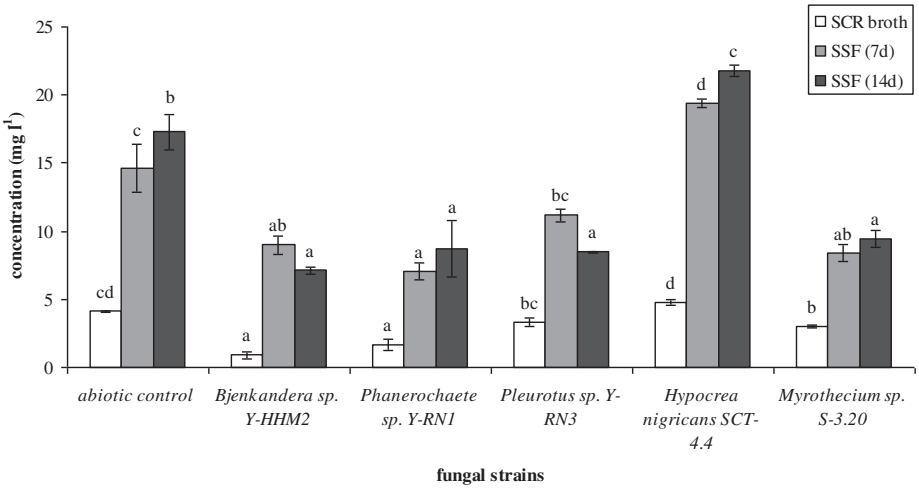

Fig. 1. Phenol concentration (mg l^{-1}) during LF and SSF of sugarcane residue uninoculated and inoculated with the different strains. Means ± SD (bars) followed by the same letter are not significantly different according Tukey test, $p \leq 0.05$.

Table 4
Laccase, Mn-independent peroxidase and manganese peroxidase enzyme production by five fungal strains grown on LF on day 5, SSF on day 7 and 14 of incubation. Enzyme activity is expressed as U ml^{-1} ± SD and U g^{-1} ± SD.

Fungal strains	Ligninolytic activities			
	ME broth (U ml^{-1})	SCR broth (U ml^{-1})	SSF 7d (U g^{-1})	SSF 14d (U g^{-1})
	Laccase			
Bjerkandera sp. Y-HHM2	ND	ND	ND	ND
Phanerochaete sp. Y-RN1	ND	0.0233 ± 0.0015	ND	ND
Pleurotus sp. Y-RN3	0.0511 ± 0.0005	ND	10.8434 ± 1.8895	8.2800 ± 0.3526
Hypocrea nigricans SCT-4.4	ND	ND	ND	ND
Myrothecium sp. S-3.20	0.0159 ± 0.0013	0.0470 ± 0.0052	8.3911 ± 1.8527	11.9050 ± 1.6433
	Mn-independent peroxidase			
Bjerkandera sp. Y-HHM2	0.0450 ± 0.0015	0.0332 ± 0.0061	6.6389 ± 0.7309	1.7209 ± 0.2037
Phanerochaete sp. Y-RN1	ND	0.0186	0.9067 ± 0.1197	1.5465 ± 0.2225
Pleurotus sp. Y-RN3	ND	ND	ND	32.887
Hypocrea nigricans SCT-4.4	ND	ND	ND	ND
Myrothecium sp. S-3.20	0.0132	0.0945	0.2523	0.3986
	Manganese peroxidase			
Bjerkandera sp. Y-HHM2	0.0551 ± 0.0050	ND	6.3972 ± 1.7940	1.1548 ± 0.2644
Phanerochaete sp. Y-RN1	ND	ND	6.3839 ± 0.5917	6.3250 ± 1.7400
Pleurotus sp. Y-RN3	ND	ND	1.6251 ± 0.2385	3.0137 ± 0.8868
Hypocrea nigricans SCT-4.4	ND	ND	ND	ND
Myrothecium sp. S-3.20	ND	ND	ND	ND

ND: not detected under these assay conditions.

Phanerochaete sp. Y-RN1 was able of producing endoxylanases only in liquid cultures. Little β-glucosidase activity could be detected (<0.1 U ml^{-1}) in liquid cultures of assayed fungi.

3.6. Solid-state fermentation (SSF)

The effect of fungal treatments over phenolic compounds concentration is shown in Fig. 1. *Phanerochaete* sp. Y-RN1 reached 52% phenolic compounds degradation during the first 7 d, without further reduction until 14 d. *Myrothecium* sp. S-3.20 phenolic compounds degradation increased over time, reaching 42.81% and 45.28% at 7 and 14 d, respectively. So did *Pleurotus* sp. Y-RN3 showed a similar behavior, with 23.79% and 50.96% phenol compounds degradation after 7 and 14 d, respectively. The highest phenol consumption was registered in *Bjerkandera* sp. Y-HHM2 cultures (phenol concentration dropped by 58.71% in 14 d). Only *H. nigricans* SCT-4.4 was able to increase phenol concentrations significantly ($p \leq 0.05$). This could be due to the action of hydroxyl radicals, produced in turn by enzymatic mechanisms. Such radicals could be able to transform non phenolic compounds into phenolics, thereby increasing the phenolic compounds titres.

According to Jurado et al. (2009) laccase treatment performed on steam-exploded wheat straw reduced phenolic compounds by polymerization of free phenols. However, Tsioulpas et al. (2002) suggested that other mechanisms beside laccase production could be involved in phenolic compounds removal. Our data confirm this statement since no laccase activity was detected in *Bjerkandera* sp. Y-HHM2 cultures (Table 4) but a reduction of phenolic compounds was observed.

The ability of selected fungal strains to reduce dry sugarcane residue biomass during 7 and 14 d of incubation was evaluated (Fig. 2). *Bjerkandera* sp. Y-HHM2 and *Myrothecium* sp. S-3.20 produced more than 10% of dry mass loss at 14 d. No dry mass loss was detected in *Pleurotus* sp. Y-RN3 cultures, under the assayed conditions. However, it should be noted that according to Dorado et al. (1999), optimal incubation times for *Pleurotus eryngii*, are usually

Table 5
Endo-β-1,4-glucanase, endoxylanase and β-glucosidase enzyme production by five fungal strains grown on LF on day 5, SSF on day 7 and 14 of incubation. Enzyme activity is expressed as U ml^{-1} ± SD and U g^{-1} ± SD.

Fungal strains	Cellulolytic and hemicellulolytic activities			
	ME broth (U ml^{-1})	SCR broth (U ml^{-1})	SSF 7d (U g^{-1})	SSF 14d (U g^{-1})
	Endo-β-1,4-glucanase			
Bjerkandera sp. Y-HHM2	ND	ND	371.50 ± 197.02	435.09 ± 61.53
Phanerochaete sp. Y-RN1	ND	ND	278.63 ± 131.35	478.60 ± 184.59
Pleurotus sp. Y-RN3	ND	16.22 ± 2.08	ND	ND
Hypocrea nigricans SCT-4.4	ND	ND	ND	ND
Myrothecium sp. S-3.20	2.76 ± 0.01	5.90 ± 0.01	139.31 ± 65.67	522.11 ± 61.53
	Endoxylanase			
Bjerkandera sp. Y-HHM2	ND	ND	ND	ND
Phanerochaete sp. Y-RN1	38.53 ± 7.78	22.55 ± 0.01	ND	ND
Pleurotus sp. Y-RN3	ND	ND	ND	ND
Hypocrea nigricans SCT-4.4	20.71 ± 18.95	259.28 ± 63.77	1864.25 ± 376.63	2687.22 ± 2329.22
Myrothecium sp. S-3.20	ND	ND	768.96 ± 125.54	606.79 ± 367.77
	β-glucosidase			
Bjerkandera sp. Y-HHM2	0.0029 ± 0.0006	ND	1.0886 ± 0.0317	0.6078 ± 0.6386
Phanerochaete sp. Y-RN1	0.0020 ± 0.0008	ND	0.7244 ± 0.1575	20.2614 ± 3.8544
Pleurotus sp. Y-RN3	ND	ND	0.0214 ± 0.0515	ND
Hypocrea nigricans SCT-4.4	0.0009 ± 0.0001	0.0654 ± 0.0019	0.6017 ± 0.0533	1.3649 ± 0.3895
Myrothecium sp. S-3.20	0.0057 ± 0.0008	0.0823 ± 0.0070	0.8514 ± 0.0354	17.5148 ± 3.1971

ND: not detected under these assay conditions.

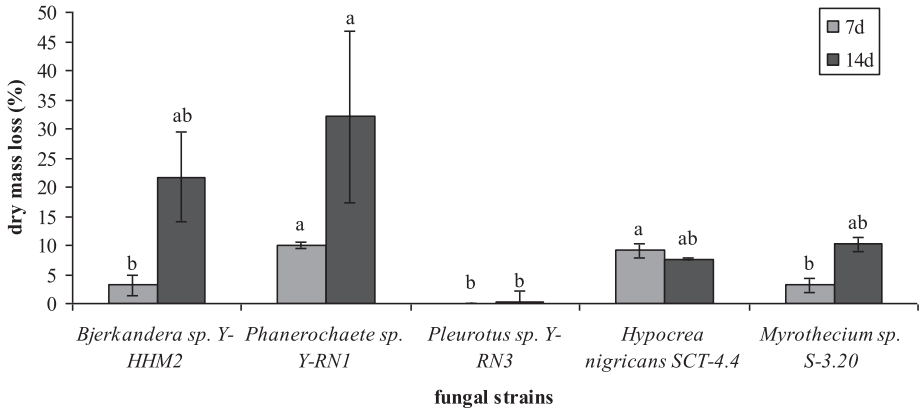

Fig. 2. Dry mass loss (%) of sugarcane residue by fungal strains on day 7 and 14. Means ± SD (bars) followed by the same letter are not significantly different according Tukey test, $p \leq 0.05$.

between 15 and 30 d. The highest dry mass loss was caused by *Phanerochaete* sp. Y-RN1 (32.07%) after 14 d cultivation. Similar results were obtained by Dorado et al. (1999) in wheat straw fermentation with *Phanerochaete chrysosporium* (45% of weight loss after 60 d).

Laccase activity showed maximum values for *Pleurotus* sp. Y-RN3 and *Myrothecium* sp. S-3.20 after 7 and 14 d, respectively (Table 4). *Pleurotus* sp. Y-RN3 produced laccase activity under all tested conditions but when growing on SCR-broth. On the other hand, *Myrothecium* sp. S-3.20 produced laccase in every tested condition, pointing to the constitutive production of this enzyme. However, no laccase activity was found for this species in an earlier study (Varnaitė and Raudonienė, 2005) after 60 days of cultivation on rye straw. *Phanerochaete* sp. Y-RN1 showed to be unable to produce laccase activity under SSF conditions, even when it does produce laccases on SCR-broth. Meanwhile *Bjerkandera* sp. Y-HHM2 and *H. nigricans* SCT-4.4 showed to be unable to produce laccase activities both, under LF and SSF assayed conditions (Table 4). *Pleurotus* sp. Y-RN3 showed maximum Mn-independent peroxidase production (32.89 U g^{-1}) after 14 d incubation. This oxidative activity was constitutively detected in *Bjerkandera* sp. Y-HHM2 and *Myrothecium verrucaria* S-3.20 cultures. A similar mechanism could be hypothesized in *Pleurotus* sp. Y-RN3 since similar results has been extensively reported for *Pleurotus* species (Kerem et al., 1992; Lang et al., 1998; Mansur et al., 2003; Souza et al., 2006). MnP activity was detected in *Bjerkandera* sp. Y-HHM2, *Phanerochaete* sp. Y-RN1 and *Pleurotus* sp. Y-RN3 SSF cultures (Table 4). Similar finding were previously reported for *Bjerkandera adusta* during incubation on wheat straw SSF (Dinis et al., 2009) and *Ceriporiopsis subvermispora* during sugarcane straw pretreatment in pulping process (Saad et al., 2008).

Endoglucanase activities were mainly detected on SSF (Table 5). *Myrothecium* sp. S-3.20 was able to produce endoglucanase activity in every assayed conditions. *Bjerkandera* sp. Y-HHM2, *Phanerochaete* sp. Y-RN1 and *Myrothecium* sp. S-3.20 showed maximum values of endoglucanase after 14 d cultivation, meanwhile the activity could not be detected in *H. nigricans* SCT-4.4 solid cultures. No endoxylanase activity could be detected in *Pleurotus* sp. Y-RN3 or *Bjerkandera* sp. Y-HHM2 cultures. *H. nigricans* SCT-4.4, on the other hand, showed endoxylanase positive activities under all assayed condition. *Myrothecium* sp. S-3.20 showed positive result only in SSF. *Phanerochaete* sp. Y-RN1 and *Myrothecium* sp. S-3.20 produced the highest values of β-glucosidase activity (20.26 and 17.51 U g^{-1}, respectively) on day 14 of incubation in SSF. No β-glucosidase activity was detected in *Pleurotus* sp. Y-RN3 cultures while *H. nigricans* SCT-4.4 showed it in all evaluated conditions.

4. Conclusions

Even when post-harvest sugarcane residue degradation is clearly a multi-enzymatic process, no simple correlation could be established between enzymatic activities and dry biomass weight loss. White-rot fungus *Phanerochaete* sp. Y-RN1 and *Bjerkandera* sp. Y-HHM2 produced the highest dry biomass weight loss on day 14 of incubation, showing endo-β-1,4-glucanase, β-glucosidase and peroxidase activities. However, *Myrothecium* sp. S-3.20 produced all tested enzymes but MnP, causing only a limited dry mass weight loss, possibly due to the presence of hydrolytic enzymes that rapidly consume substrate sugars resulting in fungal biomass growth. *Pleurotus* sp. Y-RN3, did not produce hydrolytic enzymes so initial growth in the substrate is slow. Further studies are required to confirm sugarcane residue degradation capabilities of this strain.

In general, maximum values of enzyme activities were measured under SSF growth conditions on day 14 of incubation, pointing to the existence of some induction mechanisms, possibly triggered by C/N ratio, filamentous growth and strict aerobic conditions.

Data shows that native strains, especially *Phanerochaete* sp. Y-RN1, *Pleurotus* sp. Y-RN3 and *Myrothecium* sp. S-3.20 are suitable to increase sugarcane residue degradation through lignocellulolytic enzymes production, supporting their use as an alternative to pre and post-harvest burning.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2013.10.020.

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