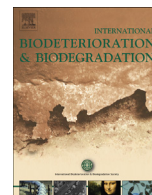
International Biodeterioration & Biodegradation 87 (2014) 18–25

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

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



Post-harvest sugarcane residue degradation by autochthonous fungi

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A R T I C L E I N F O

Article history:
Received 16 September 2013
Received in revised form
25 October 2013
Accepted 29 October 2013
Available online 21 November 2013

Keywords:
Post-harvest sugarcane residue
Lignocellulolytic enzymes
Fungus
Biodegradation

A B S T R A C T

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.

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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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http://dx.doi.org/10.1016/j.ibiod.2013.10.020

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

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