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Short communication

In-vitro degradation of Czapek and molasses amended post-harvest sugarcane residue by lignocellulolytic fungal strains

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ABSTRACT

Post-harvest sugarcane residue (SCR), deposited on sugarcane fields after green harvesting, could serve as a substrate for fungal biomass and lignocellulolytic enzymes production. In the present study, the mycelial growth of six strains (*Trametes* sp. Y–H11, *Bjerkandera* sp. Y-HHM2, *Phanerochaete* sp. Y-RN1, *Pleurotus* sp. Y-RN3, *Myrothecium* sp. S-3.20 and *Hypocrea nigricans* SCT-4.4) was measured *in-vitro* by applying a modified Gompertz equation. *In-vitro* assays showed shorter lag phases for fungi in modified Czapek, 0.3% and 1.0% molasses amended post-harvest SCR. Further increments in molasses concentrations produced a reduction on the specific growth rates for all tested fungi. Fungal degradation of post-harvest SCR and the concomitant enzyme production were tested under solid-state fermentation (SSF) of Czapek or molasses amended post-harvest SCR. Under SSF, *Pleurotus* sp. Y-RN3 produced the highest laccase titers but no hydrolytic activity could be detected. *Trametes* sp. Y–H11 and *Myrothecium* sp. S-3.20 showed high endoglucanase activities. Endoxylanase production was detected exclusively in Czapek amended media. These findings have implications for the fungal treatment of post-harvest SCR and its potential impact on the use of these residues in the production of biofuels and ligninolytic enzymes.

1. Introduction

The use of lignocellulolytic fungi for the degradation of different agro-industrial residues has become increasingly interesting. In recent years, such approach has been employed in the treatment of corn straw (Wan and Li, 2010; Guo et al., 2013), and sugarcane straw (Guerra et al., 2006) among others.

Such ability is related to the activities of hydrolytic (mainly cellulases and hemicellulases) and oxidative extracellular ligninolytic enzymes (laccases, lignin peroxidases and/or manganese peroxidase) (Sánchez, 2009). Fungal biopulping treatments greatly improve ethanol production from low cost agro-industrial residues by releasing easily fermentable sugars (Giles et al., 2015).

In sugarcane (*Saccharum* spp.) production systems, a portion of post-harvest sugarcane residues (SCR) is collected from fields and burned as energy source. The remaining portion, composed mainly of green and dry tops and leaves, is usually left on the field for agronomic reasons. Those residues present a relatively high hemicellulosic content. Their utilization for biofuel production, aside from being an eco-friendly process, could help to avoid reliance on corn starch and other edible biomass sources, making it a promising alternative to diminish environment and energy crises (Gupta and Verma, 2015).

Molasses, being rich in potash and nitrogen (Solomon, 2011) have shown to accelerate the decomposition rate of low nitrogen sugarcane residues (Sanclemente Reyes et al., 2011).

The present study is a continuation of a previous work, reporting degradation of post-harvest SCR by autochthonous fungi (Maza et al., 2014) and it is focused on the effects of different nutritional supplements (i.e. synthetic culture media vs. molasses) on the degradation of post-harvest SCR and enzymes production under solid-state fermentation (SSF). The *in-vitro* response of mycelial growth to different carbon and nitrogen sources was also analyzed applying modeling methods. The results attained could

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have implications in the management of post-harvest SCR for the production of biofuels.

2. Materials and methods

2.1. Fungal isolates, culture conditions and inocula

Basidiomycetes *Trametes* sp. Y–H11 (KF578082), *Bjerkandera* sp. Y-HHM2 (KF578081), *Phanerochaete* sp. Y-RN1 (KF578080) and *Pleurotus* sp. Y-RN3 (KF578085) were previously 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). Ascomycetes *Myrothecium* sp. S-3.20 (KF578084) and *Hypocrea nigricans* SCT-4.4 (KF578083) were isolated from soil and sugarcane residue samples, respectively (Maza et al., 2014). Stock cultures were kept on modified malt extract agar (MEA) (malt extract, 20.0 g L^{-1}; yeast extract, 1.0 g L^{-1}; agar, 17.0 g L^{-1}; pH 6.0) slants at 4 °C.

The modified Czapek broth containing in g L^{-1}: $NaNO_3$, 2.0; KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; malt extract, 4.0; glucose, 3.0; pH 5.5 was used for the solid-state fermentation (SSF) assay. All solid media employed in this study had 17.0 g L^{-1} of agar.

In growth studies, the inoculum consisted of one agar plug (5 mm diameter) removed from the hyphal edges of 8 d old cultures grown on MEA. In SSF assays, three mycelium agar plugs (5 mm diameter) were cut along the edge of the actively growing colonies cultivated on MEA plates for 10 d at 30 °C.

2.2. Evaluation of radial growth on different culture media

Fungi growths were evaluated in Petri dishes (90 mm diameter) containing seven different media. A MEA medium was employed as control. The effect of synthetic medium supplementation was assessed by adding 5 g L^{-1} of post-harvest SCR to a Czapek modified culture medium (pH 6.0). The effect of molasses on the degradation of post-harvest SCR was measured in media containing 5 g L^{-1} of post-harvest SCR plus 0.3%, 1.0%, 2.0%, 5.0% or 10.0% molasses (pH 6.0). Agar plugs were aseptically transferred to the center of the different culture media. The plates were incubated for 10 d at 30 ± 1.5 °C in continuous darkness. Fungal growth was established by measuring two perpendicular diameters on a daily basis. The average value of the two diameters was used in modeling. Five replicates were analyzed for each treatment. A complete randomized design was used.

2.3. Solid-state fermentation (SSF)

Raw post-harvest SCR was sampled in a mechanically harvested sugarcane (*Saccharum officinarum* L. var. LCP 85–384) fields in Tucumán, Argentina. Samples were oven-dried at 60 °C to constant weight. The residue was mainly composed of leaves chopped 10–15 cm in length and small pieces of sugarcane stalks.

For SSF assays, fungal strains were grown in plastic bags containing 5 g of dried post-harvest SCR amended with either Czapek modified broth or 0.3% molasses solution, to obtain a moisture content of about 75% (w w^{-1}). Residues were then sterilized at 121 °C for 20 min. Bags were inoculated with three agar plugs, mixed and incubated at 30 ± 1.5 °C for 30 d. Substrates were manually turned once a week. An abiotic control, without fungal inoculation, was also incorporated. All experiments were performed in duplicate.

After incubation, bag contents 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 centrifuged (15 min at 4 °C, 9000 rpm). Water soluble fractions (WSF) were finally collected for further assays (Wan and Li, 2010). The remaining solid substrates were dried at 105 °C to constant weight. Post-harvest SCR degradation was expressed as the loss of dry mass (%), calculated with reference to an uninoculated substrate (Saparrat et al., 2008).

2.4. Determination of phenol contents

Total phenolic compounds were measured in the WSF according to Singleton et al. (1999) with Folin-Ciocalteu reagent, using tannic acid as standard. Concentration of phenolic compounds was expressed as tannic acid equivalents (mg tannic acid equivalents L^{-1} liquid phase).

2.5. Enzyme assays

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation at 420 nm of 1.8 mM ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) 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 μmol of ABTS in 1 min (Bourbonnais and Paice, 1990).

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 the dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of activity was defined as the amount of enzyme required to liberate 1 mmol of reducing sugars per min under the assay conditions.

2.6. Data analysis

Measurements of colony diameters were subjected to statistical analysis using InfoStat (Di Rienzo et al., 2011). Colony diameters were adjusted to the modified Gompertz model by Zwietering et al. (1990) and the growth parameters were estimated from the model: $D(t) = D_0 + D_{max} \times \exp(-\exp((\mu \times \exp(1)/D_{max})(\lambda - t) + 1))$ where $D(t)$ is the diameter of the fungal colony at time t (mm), D_0 is the agar plug diameter (mm) of inoculation, D_{max} is the maximum colony diameter (mm), μ is the specific growth rate (mm/d) and λ is the lag phase (d). Graphical validation was used to assess the performance of the obtained models. Plots of observed versus predicted values were examined visually to evaluate the overall reliability of the model (Garcia et al., 2009) (data not shown).

For SSF assays, all analyses were conducted in duplicate and data were subjected to analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$) to evaluate differences in phenolic compounds concentration and dry mass loss between fungal treatments.

3. Results and discussion

3.1. Evaluation of radial growth on different culture media

From the modified Gompertz equation (Zwietering et al., 1990), three parameters could be calculated: maximum specific growth rate (μ), asymptote or maximal growth reached (D_{max}) and lag phase (λ) (Table 1). No large growth variation was observed between fungal strains amended with Czapek modified culture medium and those amended with different molasses concentrations.

As general trends, shorter lag phases were observed in the fungal strains growing in Czapek or molasses (0.3%–1.0%) amended post-harvest SCR. Specific growth rates increased in media with 2.0 and 5.0% molasses and most fungi showed a slight reduction of