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Promising cellulolytic fungi isolates for rice straw degradation

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The objective of this study was to evaluate the potential of eight fungal isolates obtained from soils in rice crops for straw degradation *in situ*. From the initial eight isolates, *Pleurotus ostreatus* T1.1 and *Penicillium* sp. HC1 were selected for further characterization based on qualitative cellulolytic enzyme production and capacity to use rice straw as a sole carbon source. Subsequently, cellulolytic, xylanolytic, and lignolytic (*Pleurotus ostreatus*) activity on carboxymethyl cellulose, oat xylan, and rice straw with different nitrogen sources was evaluated. From the results obtained it was concluded both isolates are capable to produce enzymes necessary for rice straw degradation. However, their production is dependent upon carbon and nitrogen source. Last, it was established that *Pleurotus ostreatus* T1.1 and *Penicillium* sp. HC1 capability to colonize and mineralize rice straw, in mono-and co-culture, without affecting nitrogen soil content.

Keywords: rice straw, biodegradation, *Pleurotus ostreatus*, *Penicillium* sp., ligninolytic enzymes, cellulolytic enzymes

Introduction

The increase in world population and its effect on food demand, in addition to reducing environmental impact derived from food production, has raised a challenge for the agricultural industry in order to improve resource utilization and diminish food industry impact on non-renewable resources (Godfray *et al.*, 2010). Within this context, crop residues play an important role in agricultural sustainability. Thus, it is necessary not to consider them as waste, but to acknowledge them as a renewable resource (Misselbrook *et al.*, 2012).

Rice straw is one of the most produced agricultural residues worldwide. For each kg of cropped grain 1 to 1.5 kg rice straw is produced. Therefore, a worldwide estimate of byproduct is about 650–975 million tons/year (Binod *et al.*, 2010). Furthermore, rice straw is an important source of soil nutrients. Approximately 40% of the nitrogen (N), 30 to 35% of the phosphorus (P), 80 to 85% of the potassium (K), and 40 to 50% of the sulphur (S) taken up by the rice plant remains in the vegetative parts after the rice grain is harvested (Dobermann and Fairhurst, 2002). None the less, given its chemical composition, cellulose (32–47%), hemicellulose (19–27%), and lignin (5–24%), its degradation in soil is very slow. Therefore, farmers prefer to remove it from the field as a faster alternative, taking away nutrients (Dobermann and Fairhurst, 2002), incurring in expenses and demanding space, time, and technology (Matsumura *et al.*, 2005). *In situ* burning is another option increasing air pollution and consequently affecting public health (Binod *et al.*, 2010).

To avoid burning and field removal, harvest residues can be incorporated into field soil. It has been reported this practice improves the soil's physico-chemical conditions and organic matter content, in the long term (Pathak *et al.*, 2006; Das *et al.*, 2008; Ortiz Escobar and Hue, 2008; Tejada *et al.*, 2008). However, it has also been described crop productivity can significantly decrease immediately after straw incorporation to soil, as a consequence of nitrogen immobilization by competing microbial population during the degradation process (Shindo and Nishio, 2005). In the same manner, CH_4 production can be tripled after a field is flooded in comparison with emissions generated during burning, when residue decomposition is performed in an anaerobic atmosphere (Kakua *et al.*, 2000; Glissmann *et al.*, 2001; He *et al.*, 2009).

Direct straw incorporation disadvantages can be lowered incorporating it partially or completely degraded. Bio-augmentation with lignocellulolytic fungi is an alternative. It has been demonstrated microorganism use and/or their enzymes increase recalcitrant compound degradation kinetics (lignin, cellulose, and hemicellulose) in these plant materials (Taniguchi *et al.*, 2005; Eun *et al.*, 2006; Harada *et al.*, 2008; Dinis *et al.*, 2009; Kausar *et al.*, 2010; Singh *et al.*, 2011). Additionally, it has been found fungi growth improves soil's physical structure (Yao *et al.*, 2011).

In Colombia, the National Rice Federation (Federación Nacional de Arroceros-FEDEARROZ) has evaluated the use of the fungus *Trichoderma viride*, a soil borne pathogens control agent, with excellent results as a straw decomposer; although this organism was not isolated or produced for this purpose. Performing crop residue handling and nutrient

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recycling using *T. viride*, these investigators achieved to increase rice yield between 1.0 to 1.4 t/ha, demonstrating this alternative is ecological and economically sustainable (Garcés and Ospina, 2009; Castilla, 2012). In the present study we evaluated the lignocellulolytic activity of eight fungi, previously isolated from rice crops, on defined cellulosic substrates and rice straw; thus establishing their potential for *in situ* straw degradation. Based on *Trichoderma viride* successful results, we hypothesized better results could be attained if microorganisms for this specific activity were selected.

Materials and Methods

Microorganisms

Evaluated microorganisms in this work were previously isolated from soils where rice-harvesting took place, and preselected for their cellulolytic and/or lignolytic activity (Gutiérrez-Rojas *et al.*, 2012). They had been previously identified as *Penicillium* sp. (codes HC1, HC7.4, HC7.5, and HC7.5'), *Trichoderma* sp. (HC8), *Paecilomyces* sp. (S7.1), *Streptomyces* sp. (S7.1), and *Pleurotus ostreatus* (T1.1) and are conserved on agar plates at 4°C at the Fermentation Laboratory, Biotechnology Institute, Universidad Nacional de Colombia. Isolates reactivation was performed in potato dextrose agar (PDA) for 8 days incubation period at 25°C.

Microorganism selection

To select microorganisms for subsequent phases of the study, cellulolytic enzyme production was qualitatively evaluated, as well as the capacity to utilize rice straw as the only carbon source. For qualitative evaluation Congo red hydrolysis halos in carboxymethyl cellulose (CMC) were employed (Kasana, *et al.*, 2008). As negative and positive controls *Escherichia coli* ATCC 25922 and *Trichoderma reesei* ATCC 26921 were used, respectively. All assays were performed in triplicates. Microbial respiration activity on rice straw was measured using as a carbon and nitrogen source 8 g of cut straw (2–3 cm long) adding 5 ml salts solution (in g/L: 0.3 KH_2PO_4, 0.83 $MgSO_4 \cdot 7H_2O$, 0.3 $CaCl_2$, 0.005 $FeSO_4 \cdot 7H_2O$, 1.56 mg $MnSO_4$, 1.4 mg $ZnSO_4$, 0.2 ml Tween 80, pH 6.0), in tightly sealed glass flasks. A container with 30 ml of 0.2 N NaOH was placed in the center of the flask to capture CO_2 emanating from the respiration process. Flasks were inoculated with 10 ml conidial suspension (10^6 conidia/ml), for T1.1 isolate suspension was adjusted to 10^6 UFC/ml. An abiotic control was left without inoculation. Flasks were incubated for 60 days at 25°C. All assays were performed in triplicates. Every two days the container with NaOH was removed to evaluate CO_2 production by titration, and replaced with a new sterile flask containing NaOH. NaOH excess was titrated with a 0.2 N HCl standard solution until the final reaction point was established with phenolphthalein as a pH indicator (Critter *et al.*, 2004).

Cellulolytic and xylanolytic activity evaluation in selected microorganisms

Evaluation was performed in 250 ml Erlenmeyer with 120 ml Mandel's mineral salts solution (Mandels and Weber, 1969) adding the carbon source to be evaluated at 1% (w/v): carboxymethyl cellulose (CMC), straw or xylan, and 0.5% (w/v) nitrogen source: yeast extract or $(NH_4)_2SO_4$ (Table 1). pH was adjusted to 6.0 and 12 ml inoculum was used at 10^6 conidia/ml for cellulolytic isolates and 10^6 UFC/ml for lignolytic isolate. Erlenmeyer flasks were incubated at 28°C with 100 rpm shaking for 10 days. After the fourth day of incubation, samples were collected every two days to determine enzyme activity with protocol described in enzyme determination. All assays were performed in triplicates.

Rice straw degradation capacity evaluation in soil microcosm

These assays were carried-out in 6 L plastic boxes with lids, in which 600 g of sterile soil was placed with 50 g rice straw cut into 3 to 6 cm long pieces. Treatments were performed in triplicate in the following manner: 1) Lignolytic microorganism, 2) Cellulolytic microorganism, 3) Lignolytic-cellulolytic co-culture, and 4) Abiotic control. All treatments were performed in triplicates. Inoculum for all treatments was 62 ml suspension at a concentration of 10^6 conidia/ml for cellulolytic isolates and 10^6 UFC/ml for lignolytic isolate. The experiment was maintained for 50 days under greenhouse conditions with CO_2 follow-up and enzyme activity evaluation. Scanning electron microscopy (SEM) was carried-out on days 0, 30, and 50. Last, a soil sample was taken at the beginning and at the end of the experiment to analyze nitrogen and organic carbon content by Kjeldahl (Bremner, 1960) and Walkley and Black (1934) methodologies, respectively.

For enzymatic activity 2 g of rice straw was sampled every eight days to which 20 ml citrate buffer pH 5.0 was added (Brijwani *et al.*, 2010), and agitated at 150 rpm for 6 h at 25°C. To obtain an extract, the sample was centrifuged twice at 2,500 × *g* for 20 min. With the resulting extract, endoglucanase, cellobiohydrolase, β-glucosidase, xylanase, laccase, and manganese peroxidase activity were assayed as described in a latter section. To evaluate CO_2 production a polyvinyl chloride tube was placed as a container of a glass vial with 20 ml 1 N NaOH to trap CO_2, following the methodology previously described. 1 N HCl was used for titration, measurements were carried-out every three day during the 50 days of the assay.

Enzyme activity determination

All enzyme activities were spectrophotometrically determined (Evolution 60 UV-VIS Thermo Scientific Co.). Endo-1,4-β-glucanase activity was assayed from 500 µl sample mixed

Table 1. Treatments according to combination of carbon and nitrogen source

Treatment	Carbon source (10 g/L)	Nitrogen source (5 g/L)
1	Carboximetilcellulose (CMC)	None
2		Yeast extract
3		$(NH4)_2SO_4$
4		$(NH4)_2SO_4$ + Yeast extract
5	Rice straw (RS) or xylan beechwood	None
6		Yeast extract
7		$(NH4)_2SO_4$
8		$(NH4)_2SO_4$ + Yeast extract

with 500 µl 2% (w/v) low viscosity CMC in 50 mM citrate buffer (pH 5.0), at 40°C for 60 min (Ghose, 1987). The reaction was stopped by 5 min incubation on ice, followed by 5 min centrifugation at 2,500 × *g* (Qinnghe *et al.*, 2004). Quantification reducing sugars equivalent to glucose was determined by 3,5 dinitrosalicylic acid (DNS) (Miller, 1959). A unit of endoglucanase was defined as the quantity of enzyme required to release 1 µmol reducing sugars per min under assay conditions.

Cellobiohydrolase (exo-1,4-β-glucanase) was evaluated employing ρ-nitrophenyl-β-D-cellobioside as substrate (PNPC, Sigma-Aldrich). The reaction contained 320 µl PNPC in 50 mM sodium acetate buffer (pH 5.0) and 100 µl sample. This mix was incubated at 40°C for 60 min. The reaction was stopped adding 250 µl 0.5 M sodium carbonate. Absorbance was read at 400 nm (Valásková and Baldrian, 2006). Enzyme activity was calculated from ρ-nitrophenol standard curve. One unit cellobiohydrolase activity was defined as the quantity of enzyme required to release 1 µmol ρ-nitrophenol per min.

1-4-β-Glucosidase enzyme activity was quantified using as substrate ρ-nitrophenyl-β-D-glucoside (PNPG, Sigma-Aldrich), following the same method as for cellobiohydrolase.

Endoxylanase activity was determined mixing 500 µl sample with 500 µl 2% (w/v) beech tree xylan in 50 mM citrate buffer (pH 5.0). The reaction was incubated at 40°C for 60 min (Driss *et al.*, 2011). A unit of xylanase was defined as the quantity of enzyme releasing 1 µmol reducing sugars equivalent to xylose per min.

Laccase activity was measured monitoring the change of absorbance at 436 nm (ξ_{436} = 29,300 L/mol/cm) caused by ABTS oxidation [2,2' azino-*bis*-(3 ethylbenzthiazoline sulphonic) acid] in 60 mM sodium acetate buffer (pH 4.5). The assay was carried out by mixing 100 µl buffer, 100 µl ABTS, and 800 µl sample at 20°C for 3 min. One unit laccase activity was defined as the quantity of enzyme oxidizing 1 µmol ABTS per min (Tinoco *et al.*, 2001).

Manganese peroxidase activity was determined by oxidation of 2,6-dimethoxyphenol (2,6-DMP) (Sigma-Aldrich) measured at 468 nm (ξ_{468}= 49,600 M/cm). Reaction mixture contained 450 µl culture supernatant, 500 µl 10 mM 2,6-DMP in 100 mM sodium acetate buffer (pH 5.0), 50 µl 0.4 mM $MnSO_4$, and 30 µl 22 mM H_2O_2. The mixture was incubated for 3 min (Santoyo *et al.*, 2008). Given that both manganese peroxidase and laccase can oxidize 2,6-DMP, laccase activity was subtracted using data from control experiments performed in the absence of H_2O_2 and manganese (Quevedo-Hidalgo *et al.*, 2012). One unit of manganese peroxidase activity was defined as 1 µmol of 2,6-DMP oxidized per min.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA), with Tukey *post-hoc* test (α = 0.05), using IBM SPSS-Statistics (version 19, IBM Corp.).

Results and Discussion

Microorganism selection

Qualitative evaluation results in CMC agar evidenced the highest responses were obtained from isolates S7.1 (13.93 ±

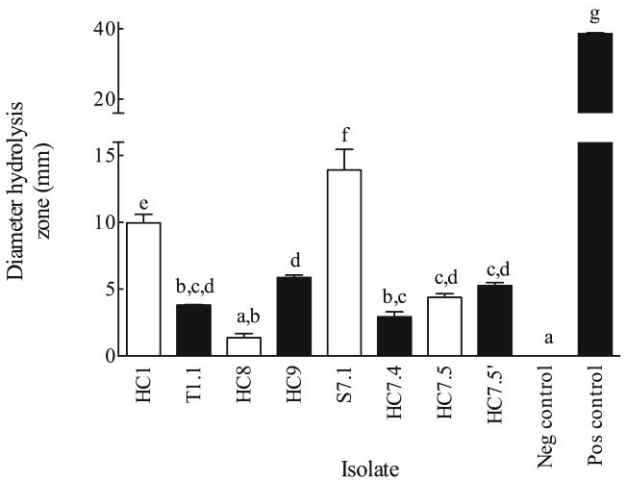

Fig. 1. Zone of clearance diameter in CMC agar for eight fungal isolates evaluated. Negative control: *Escherichia coli* ATCC 25922. Positive control: commercial enzyme *Trichoderma reesei* ATCC 26921. Bars with different letters indicate groups that are significantly different in *post hoc* testing (α = 0.05).

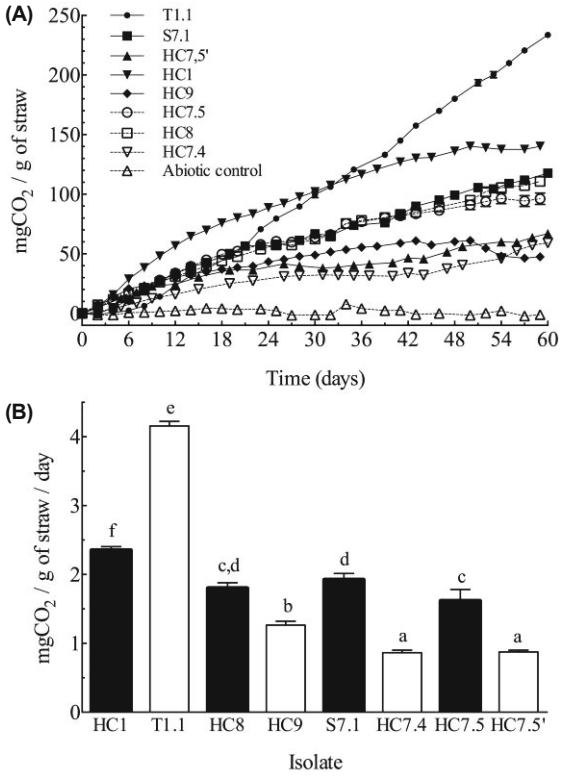

Fig. 2. CO_2 production results using rice straw as carbon and nitrogen source in the laboratory for eight fungal isolates evaluated. Production kinetics (A) and rate of production (B). Bars with different letters indicate groups that are significantly different in *post hoc* testing (α = 0.05).

2.64 mm halo) identified as *Streptomyces* sp. and isolate HC1 (9.97 ± 1.11 mm halo) identified as *Penicillium* sp. (Fig. 1). Isolate HC8, *Trichoderma* sp., presented the lesser response (1.37 ± 0.55 mm halo). The rest of the evaluated isolates corresponding to *Penicillium* sp. and *Paecilomyces* sp. had intermediate zone of clearance formation. In contrast to CMC agar results, CO_2 production results using rice straw as carbon and nitrogen source demonstrated isolate T1.1 (*P. ostreatus*) had the highest CO_2 production, 233.64 ± 2.03 mg CO_2/g rice straw on day 60, with a production rate of 4.15 ± 0.05 mg CO_2/g rice straw/day, followed by isolate HC1 (*Penicillium* sp.), 140.33 ± 2.60 mg CO_2/g rice straw on day 60 and 2.35 ± 0.05 mg CO_2/g rice straw/day and isolate S7.1 (*Streptomyces* sp.), 117.69 ± 2.19 mg CO_2/g rice straw on day 60 and 1.92 ± 0.09 mg CO_2/g rice straw/day (Fig. 2A and B).

Differences in results obtained could be due to substrate nature. Enzyme production in the cellulolytic complex is highly regulated. In addition, this mechanism depends on microorganism and substrate type (Gutiérrez-Rojas *et al.*, 2015). It was expected a microorganism such as *P. ostreatus*, isolate T1.1, in addition to cellulases, would produce xylanases, laccase, and manganese peroxidase (Membrillo *et al.*, 2008), thus, becoming more efficient in lignocellulosic substrate degradation, measured as CO_2 production on rice straw. Even though, it did not present the highest CMC agar activity. On the other hand, *Streptomyces* sp. S7.1 demonstrated an opposite behavior, i.e. better CMC agar activity than CO_2 production on rice straw. MacKenzie and collaborators (1987) evaluated cellulases and xylanases production in *Streptomyces flavogriseus* and *Streptomyces olivochromogenes*, culturing on different substrates. They found for both microorganisms growth on cellulose primarily induce cellulase activity, but failed to produce appreciable levels of xylanases. Whereas, growth on xylan and wheat bran induced high levels of endoxylanase; suggesting enzyme expression mechanisms of regulation are independent between the two enzymes, but substrate dependent (Mackenzie *et al.*, 1987).

Regarding *Penicillium* sp. HC1 isolate, consistent results were obtained for CMC agar activity as well as CO_2 production on rice straw. It has been reported a great number of species in the *Penicillium* genus are capable of producing a

Table 2. ***Penicillium*** **sp. HC1 and** ***Pleurotus ostreatus*** **T1.1 highest enzyme production depending on carbon and nitrogen source in the medium**

Microorganism	Carbon source	Nitrogen source	Enzyme	Highest activity	
				U/L	Time of culture (days)
Penicillium sp.	CMC	None	β-Glucosidase	45.9 ± 1.8^{c}	8
			Cellobiohydrolase	266.2 ± 28.76^{b}	8
			CMCase	83.4 ± 21.71^{b}	10
	CMC	YE	β-Glucosidase	174 ± 3.89^{a}	10
			Cellobiohydrolase	246.2 ± 46.96^{b}	6
			CMCase	90.3 ± 10.94^{ab}	6
	CMC	$(NH4)_2SO_4$	β-Glucosidase	86.5 ± 7.10^{b}	8
			Cellobiohydrolase	364.0 ± 2.9^{a}	10
			Endo-glucanase	136.9 ± 7.25^{a}	10
	CMC	YE + $(NH4)_2SO_4$	β-Glucosidase	174.7 ± 3.9^{b}	10
			Cellobiohydrolase	279.1 ± 45.15^{b}	6
			Endo-glucanase	74.13 ± 15.57^{b}	10
	Xylan	None	Xylanase	644.6 ± 2.3^{a}	4
		YE		520.1 ± 16.12^{a}	6
		$(NH4)_2SO_4$		514.9 ± 78.36^{a}	4
		YE + $(NH4)_2SO_4$		636.9 ± 73.43^{a}	8
Pleurotus ostreatus	CMC	None	β-Glucosidase	114.2 ± 14.84^{b}	10
			Cellobiohydrolase	56.6 ± 38.47^{a}	10
			CMCase	62.73 ± 18.51^{a}	8
	CMC	YE	β-Glucosidase	158.2 ± 22.33^{a}	8
			Cellobiohydrolase	68.01 ± 4.18^{a}	6
			CMCase	94.7 ± 21.23^{a}	4
	CMC	$(NH4)_2SO_4$	β-Glucosidase	92.9 ± 7.12^{bc}	10
			Cellobiohydrolase	11.8 ± 4.5^{a}	6
			CMCase	29.46 ± 3.3^{a}	8
	CMC	YE + $(NH4)_2SO_4$	β-Glucosidase	99.13 ± 24.16^{c}	4
			Cellobiohydrolase	0^{a}	
			CMCase	60.1 ± 11.10^{a}	4
	Rice straw	None	Xylanase	554.43 ± 40.69^{b}	10
		YE		114.7 ± 37.92^{c}	10
		$(NH4)_2SO_4$		874.51 ± 2.7^{a}	10
		YE + $(NH4)_2SO_4$		523.06 ± 168^{bc}	8

Means with different letters in each enzyme activity (for each fungi) were significantly different in *post hoc* testing (α = 0.05).

multienzymatic extracellular complex efficiently degrading cellulose and hemicellulose in plant biomass. For some cases this complex has resulted more efficient than *Trichoderma reesei* mutants traditionally used in industry. The reasons for such efficiency are high β-glucosidase activity, high specific cellobiohydrolase activity, low sensitivity to product inhibition of the later one, and low bond between these enzymes to biomass lignin component (Gusakov and Sinitsyn, 2012). However, our results suggest these characteristics are distinct of the species, since not all *Penicillium* sp. isolates evaluated demonstrated the same efficiency on CMC agar activity as well as CO_2 production on rice straw.

Given the results obtained on both substrates did not show an association, i.e., isolates with the best CMC agar activity did not correspond to the best CO_2 production on rice straw, it was decided to prioritize activity on rice straw, thus the selected microorganisms for the following stages of this study were isolates T1.1 (*Pleurotus ostreatus*) and HC1 (*Penicillium* sp.).

Cellulolytic and xylanolytic activity evaluation on selected isolates

Selected microorganisms cellulolytic activity was evaluated on CMC and for xylanolytic activity on oat-xylan or rice straw. Additionally, since enzyme expression can also be regulated by nitrogen source (Qinnghe *et al.,* 2004), carbon sources were combined with nitrogen sources: none, yeast extract, ammonium sulphate, and a mixture of both. Results of this evaluation are described in Table 2.

The highest values for *Penicillium* sp. HC1 β-glucosidase (174.7 ± 3.9 U/L) were achieved when a mixture of organic and inorganic nitrogen source was used. In contrast, the highest values for cellobiohydrolase (364.0 ± 2.9 U/L) and endoglucanase (136.9 ± 7.25 U/L) were observed when ammonium sulphate was the nitrogen source. Xylanases were not affected by the source of nitrogen and the highest value was 644.6 ± 2.3 U/L without any nitrogen source added. Even though, during the last years an increase in reports describing cellulases and xylanases produced form different *Penicillium* genus has been observed, result comparison is difficult due to distinct conditions for each culture. Substrate, culture condition, and enzyme activity assay conditions, all represent determinant factors. As a case in point, β-glucosidase values have been reported from 200 U/L, when using a mix of lactose (0.25%) and cellulose (0.75%) as a substrate, up to 500 U/L, when only lactose at 1% is used for *P. echinelatum* 9A02S1 (Sehnem *et al.*, 2006). Krogh and collaborators (2004) evaluated β-glucosidase activity and endoglucanase using 2% Solka-Floc cellulose with reported values of 970 U/L (β-glucosidase) and 12,000 U/L (endoglucanase) for *P. verruculosum* IBT 18366, 1,700 U/L (β-glucosidase) and 900 U/L (endoglucanase) for *P. minioluteum* IBT 21486, 1,090 U/L (β-glucosidase) and 980 U/L (endoglucanase) for *P. brasilianum* IBT 20888, and 2,450 U/L (β-glucosidase) and 600 U/L (endoglucanase) for *P. pinophilum* IBT 10872. These same authors evaluated xylanase production in 12 *Penicillium* isolates, using as a substrate oat xylan finding activities ranging from 1,500 to 105,000 U/L, with *P. persicinum* IBT 13226 presenting the highest activity (105,000 U/L) and *P. funiculosum* IBT 5816 the second highest activity (42,000 U/L) (Krogh *et al.*, 2004), all of which were higher compared with the ones reported in this work.

Even though, the values are not comparable for the aforementioned reasons, their ratio β-glucosidase:Endoglucanase is comparable. For the results reported by Krogh *et al.* (2004) they were as follows: 0.080 for *P. verruculosum* IBT 18366, 1.89 for *P. minioluteum* IBT 21486, 1.11 for *P. brasilianum* IBT 20888, and 4.08 for *P. pinophilum* IBT 10872. For our case 1.28, however we did not find the same behavior for the β-glucosidase:Cellobiohydrolase ratio, our was 0.48. For most cases the ratio was greater than one, characteristic of the *Penicillium* genus, representing an advantage over other cellulolytic microorganisms such as *Trichoderma reesei*. *T. reesei* is identified by a low β-glucosidase secretion, resulting in a β-glucosidase:Endoglucanase or β-glucosidase:Cellobiohydrolase ratio lower than one (Gusakov and Sinitsyn, 2012). A high level of β-glucosidase is necessary to promote a rapid and complete conversion of cellobiose to glucose. Cellubiose is an intermediate metabolite of cellulose hydrolysis and is an endoglucanase inhibitor, and to a greater extent of cellobiohydrolases. Thus, it represents a bottleneck in the cellulose enzymatic hydrolysis (Gruno *et al.*, 2004).

In regards to *P. ostreatus* the highest values for β-glucosidase (158.2 ± 22.33 U/L), cellobiohydrolase (68.01 ± 4.18 U/L), and endoglucanase (94.7 ± 21.23 U/L) can be obtained using yeast extract as the nitrogen source. A similar behavior was observed for xylanases, a maximum activity value of 874.51 ± 2.7 U/L was obtained when yeast extract was the sole nitrogen source. These results agree with those reported by Qinnghe *et al.* (2004), who evaluated *P. ostreatus* SYJ042 xylanase production in submerged fermentation using different nitrogen sources including ammonium nitrate (NH_4NO_3), ammonium sulfate ($(NH_4)_2SO_4$), phosphatic diamine ($(NH_4)_2HPO_4$), yeast extract, tryptone, peptone, and meat extract. They found an activity range between 1,900 to 1,980 U/L with inorganic sources. In addition, activities increased when organic sources were employed (6,530 to 8,430 U/L), with peptone yielding the highest activity (Qinnghe *et al.*, 2004). On the other hand, it has been reported nitrogen source does not affect *P. dryinus* IBB 903 endoglucanase or xylanases production when tangerine peelings were used as a carbon source. Furthermore, the maximal endoglucanase activity was revealed after 10 days growth in medium containing ammonium sulphate (53,700 U/L), while maximal xylanase activity (95,200 U/L) was recorded after 8 days of submerged fermentation (Elisashvili *et al.*, 2006). Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes (Songulashvili *et al.*, 2007). However, nitrogen source influence is related to wood-rotting basidiomycetes and/or the source of carbon and/or the condition of the culture. Therefore, it is not possible to generalize regarding this aspect.

Results obtained in this evaluation allow to conclude both native isolates evaluated *Penicillium* sp. HC1 and *Pleurotus ostreatus* T1.1 are capable of producing enzymes necessary for rice straw degradation, although the evaluated values are subject to optimization if either one would be used for enzyme production.

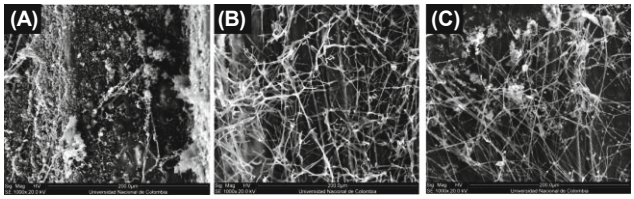

Fig. 3. Rice straw residue SEM at day 30 of inoculation. *Penicillium* sp. HC1 (A), *Pleurotus ostreatus* T1.1 (B), and co-culture (C).

Evaluation of microorganism capability to degrade rice straw

Scanning electron micrographs during residue degradation process illustrate for monoculture or co-culture microorganism growth and colonization on rice straw residue (Fig. 3). As it was expected, treatment exclusively inoculated with *Penicillium* sp. HC1 displayed scarce mycelium growth and abundant conidia production (Fig. 3A). In contrast, mycelium growth was observed for *Pleurotus ostreatus* T1.1 inoculated treatment (Fig. 3B). Last, co-culture treatment exhibited copious mycelium and limited conidia (Fig. 3C).

Moreover, CO_2 measurements evidenced *Penicillium* sp. HC1 treatment had the lowest CO_2 production (30.34 ± 0.94 mg CO_2/g straw for day 47 during evaluation) compared with *P. ostreatus* T1.1 with a CO_2 production of 68.76 ± 3.73 mg CO_2/g rice straw (Fig. 4). Likewise, rate of CO_2 production for *Penicillium* sp. HC1 was the lowest (0.72 ± 0.09 mg CO_2/g rice straw/day) compared with *P. ostreatus* T1.1 (1.47 ± 0.16 mg CO_2/g straw/day), behavior comparable to evaluations performed in the laboratory (Fig. 2A and B). Contrary to what was expected, microorganism co-culture did not produce an increase in CO_2 production. This remained similar to the *P. ostreatus* T1.1 treatment (65.81 ± 3.37 mg CO_2/g straw; rate of production 1.32 ± 0.08 mg CO_2/g straw/day), indicating microorganism co-culture did not increase straw degradation. In regards to enzyme activity, no differences were observed among the three treatments for endoglucanase, cellobiohydrolase, and xylanase maximum values. Although, β-glucosidase activity was greater for *Penicillium* sp. HC1 treatment, no differences were evidenced between *P. ostreatus* T1.1 and microorganism co-culture treatment (Table 3). With respect to lignolytic activity, *Penicillium* sp. HC1 was unable to produce detectable levels of laccase and manganese peroxidase (Table 3). These results agree with reports in the literature, since the *Penicillium* genus is reported as cellulolytic and xylanolytic, and not capable of generating the aforementioned enzymes (Chávez *et al.*, 2006).

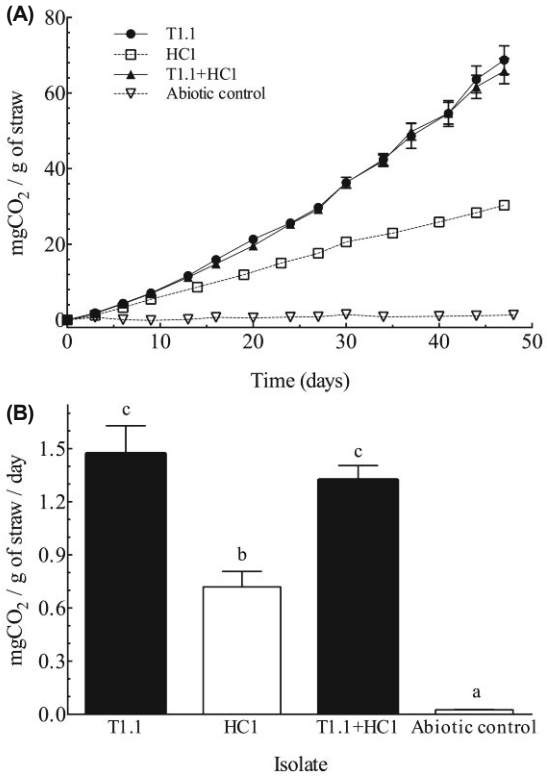

Fig. 4. Greenhouse CO_2 production (A), production kinetics and (B) rate of production. Bars with different letters indicate groups that are significantly different in *post hoc* testing (α = 0.05).

Compatible lignocellulolytic fungal consortium (fungal compatible co-culture) is frequently associated with increased substrate degradation in comparison with activity obtained by each fungus (He *et al.*, 2009). Dwivedi *et al.* (2011) evaluated xylanase and laccase production by a mutant *Penicillium oxalicum* SAUE-3.510 strain in mono-culture or in co-culture with *Pleurotus ostreatus* MTCC 1804, on different lignocellulosic substrates. Their findings evidenced xylanase and laccase activities were higher in co-culture than in axenic cultures (Dwivedi, *et al.*, 2011). Furthermore, Monahan *et al.* (2014) compared fungal monoculture efficiency with co-cultures in simultaneous delignification and saccharification of kitchen waste and *Eichhornia crassipes* leaves. These authors described *Trichoderma* spp. co-culture with other cel-

Table 3. Maximum enzyme activity for different rice straw degradation treatments

Treatment / Enzyme	HC1		T1.1		HC1+T1.1	
	Highest activity time (days)	Activity (U)	Highest activity time (days)	Activity (U)	Highest activity time (days)	Activity (U)
Cellobiohydrolase	21	9.4[a]	49	9.6a	28	9.6a
Endoglucanase	35	49.5[a]	49	50a	49	55a
β-Glucosidase	35	16.9[b]	49	11a	14	10.6a
Laccase	-	0.0[a]	49	24.3b	42	47.9c
Mn peroxidase	-	0.0[a]	28	4.4b	42	4.5b
Xylanase	28	185.5[a]	42	167a	49	154.6a

Means with different letters were significantly different in *post hoc* testing (α = 0.05).

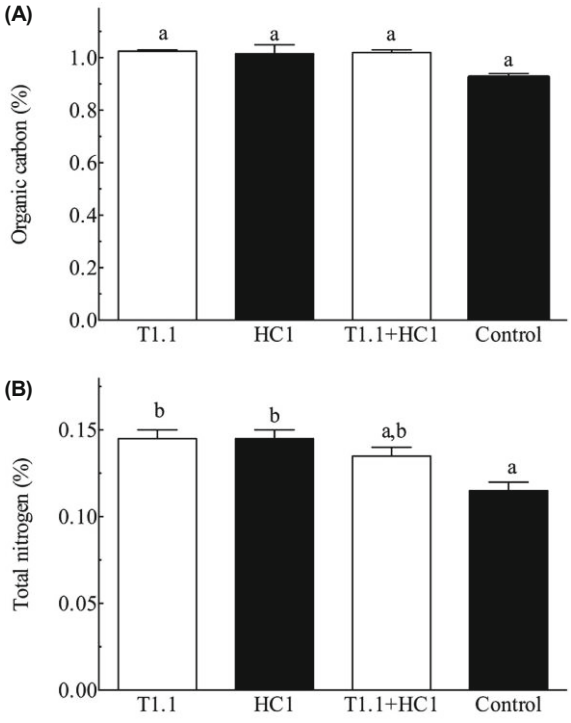

Fig. 5. Percent of soil organic carbon (A) and total nitrogen (B), after rice straw treatment (50 days). Bars with different letters indicate groups that are significantly different in *post hoc* testing (α = 0.05).

lulolytic fungi improved lignocellulose degrading enzyme activity compared with *Trichoderma* monoculture. Nevertheless, co-culture with other fungi (*Aspergillus, Pycnopurus*, and undidentified strain F113) did not result in significant degradation activity compared to corresponding monocultures (Mohanan *et al.*, 2014). Hence, it can be concluded co-culture is not always implied in increased degradation activity. In fact, improved degradation is dependent on the organisms involved and used conditions during the degradation process.

With respect to the results obtained in the present work, these demonstrate behavior of two fungi in co-culture is practically identical to *P. ostreatus* T1.1 monoculture, indicating that *P. ostreatus* T1.1 inhibited *Penicillium* sp. HC1 growth as observed in our SEM results (Fig. 3), even though it had been previously verified these were not antagonistic microorganisms (data not shown). This could be due to *P. ostreatus* T1.1 growth rate being greater than *Penicillium* sp. HC1, generating a negative effect, since both fungi were inoculated at the same time. Future studies should evaluate inoculation conditions such as time and concentrations to grant *Penicillium* sp. HC1 an advantage over *P. ostreatus* T1.1.

Despite the system is still susceptible for optimization, high carbon mineralization rates (measured as CO_2 production, Fig. 4) were observed when comparing to reports in the literature. Devevre and Horwath (2000) evaluated the effects of flooding and temperature on rice straw degradation. Under their best conditions (non-flooded, 25°C) they obtained 2.5 mg CO_2/g dry soil, approximately, after 160 days of observation (Devevre and Horwath, 2000), results far less lower when comparing with the data of the present study. This indicates lignocellulolytic microorganisms efficiently contribute to an increase in the rate of rice straw degradation.

Last, we determined total organic carbon and nitrogen as an indicator of nutrient return to the soil proceeding from rice straw degradation (Fig. 5). In a crop this return of nutrients results in decrease of fertilizers used by the farmers (Garcés and Ospina, 2009). For all samples analyzed no differences in organic carbon content were observed, most likely due to short evaluation time. Nonetheless, differences were observed regarding total nitrogen, since all treatments presented higher values compared with control (Fig. 4B). One of the inconveniences of rice straw incorporation into the soil is nitrogen availability for the plant, due to nitrogen immobilization in microbial biomass as a result of a high C/N ratio in this residue (Shindo and Nishio, 2005). Pathak *et al.* (2006) demonstrated rice straw could be successfully managed *in situ*. Microorganism inoculation avoids problems of net *N*-immobilization and yield reduction of the subsequent crop due to rice straw amendment (Pathak *et al.*, 2006). In this work we found an increase in nitrogen content, even though we cannot distinguish organic (non-available) or inorganic (available), it is an interesting finding that must be proven in future studies in the field.

In conclusion, *Pleurotus ostreatus* T1.1 and *Penicillium* sp. HC1 isolates selected out of eight initially chosen demonstrated their potential to be utilized for *in situ* rice straw degradation, and posterior incorporation into the soil. Both were capable of colonizing the residue and produce high rates of mineralization without diminishing the soil nitrogen content. However, future work should optimize co-culture condition if both microorganisms are to be used at the same time.

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