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Bio-detoxification of *Jatropha curcas* seed cake by *Pleurotus ostreatus*

José Maria Rodrigues da Luz, Mateus Dias Nunes, Sirlaine Albino Paes, Denise Pereira Torres and Maria Catarina Megumi Kasuya*

Departament of Microbiology, Universidade Federal de Viçosa, Campus Universitário, 36570-000, Viçosa, MG, Brazil.

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The detoxification of *Jatropha curcas* seed cake is of major interest for the biodiesel industry to add economic value to this residue and also to reduce the environmental damage caused by its inappropriate disposal. In this context, the treatment of this residue with white rot fungus, *Pleurotus ostreatus,* can be a viable alternative because it produces enzymes capable of degrading different lignocellulosic residues and toxic compounds. In this study, the capacity of *P. ostreatus* to degrade phorbol esters found in *Jatropha* seed cake and the potential to transform this residue in animal feed was evaluated. After 60 days of incubation with the fungus, the phorbol ester concentration was reduced by 99% (final concentration of only 2 x 10^{-4} mg g^{-1} dry mass). This value is lower than the level observed in the non-toxic Mexican variety. Also, we showed that fungal growth improved some features desirable for animal feed, such as, increases the *in vitro* digestibility, decreases lignin and cellulose content and increases the protein content. Therefore, *P. ostreatus* is able to degrade phorbol esters found in *Jatropha* seed cake and has the potential to be used as animal feed.

Key words: Physic nut, toxic compounds, phorbol ester, biodiesel, lignocellulosic residue.

INTRODUCTION

In Brazil, the National Program for Production and Use of Biodiesel published in 2004 inserted the biofuel in the national energy matrix and its production became an alternative to energy self-sufficiency.

Biodiesel is produced from vegetable oils and fats by a transesterification reaction with mono- or dialcohol (Openshaw, 2000). Due to its drought hardiness, easy propagation, low cost of seeds, high oil content and wide adaptation, *Jatropha curcas* has been widely used for biodiesel production (Sujatha et al., 2008). After extracting oil from *J. curcas*, a solid residue, called seed cake, is produced (Openshaw, 2000; Patil and Deng, 2009). This seed cake is rich in lignocelluloses and proteins (Makkar et al., 1997) and has great potential to be used as a biofertilizer, for biogas production or as animal feed (Gubitz et al., 1999). However, toxic compounds, such as phorbol esters and curcin, and antinutritional factors, such as tannins and phytic acid, make this residue

*Corresponding author. E-mail: mkasuya@ufv.br. Tel: +55-031-3899-2970. Fax: +55-031-3899-2573.

improper for direct animal consumption and cause environmental damage if disposed of inappropriately (Goel et al., 2007; Makkar et al., 1997). Curcin and the antinutritional factors can be degraded by thermal or biological treatments (Aregheore et al., 2003; Kasuya et al., 2012; Rehman and Shah, 2005). da Luz et al. (2013) showed the degradation of antinutricional factors by *Pleurotus ostreatus*.

The phorbol esters found in the seed and oil were identified as the main toxic agents of *J. curcas* (Makkar et al., 1997; Sudheer et al., 2009; Salimon and Ahmed., 2009). Animal consumption of these phorbol esters can cause diarrhea and inflammation of the gastrointestinal tract and death (Makkar and Becker, 1997). The most varieties of *J. curcas* cultivated in world are toxic, only one non-toxic variety is found in Mexico (Makkar et al., 1997; Pamidimarri et al., 2009). Thus, detoxification of *Jatropha* seed cake is necessary for their use as animal feed.

White rot fungi (*P. ostreatus, Trametes versicolor, Phanerochaete chrysosporium, Pycnoporus sanguineus*) are able to grow and degrade various pollutants and recalcitrant compounds, such as polycyclic aromatic hydrocarbons (Majcherczyk et al., 1998), tinitrotolueno (Nyanhongo et al., 2006) and phytate (Kasuya et al., 2012) by the action of several lignocellulolytic enzymes, for example laccase, manganese peroxidase (MnP), cellulase and xylanase (Barr et al., 1994; Honggiang and Hongzhang, 2008; Lamarino et al., 2009; Majcherczyk et al., 1998). The above assumptions show that these fungi probably can degrade toxic compounds (e.g. phorbol esters) found in *Jatropha* seed cake. This detoxification would allow the use of seed cake for the production of enzymes and edible mushrooms and as animal feed. Indeed, it has been shown that intake of Jatropha seed cake colonized by *P. ostreatus* is not toxic to goats (Kasuya et al., 2012). This detoxification would allow the use of seed cake for the production of enzymes and edible mushrooms and as animal feed. Thus, the aim of this study was to evaluate the capacity of *P. ostreatus* to degrade phorbol esters found in *Jatropha* seed cake.

MATERIALS AND METHODS

Microorganism, fungal growth conditions and inoculum production (spawn)

The isolate Plo 6 of *P. ostreatus* (GenBank accession number KC782771, 2013) was grown in a Petri dish containing potato dextrose agar culture medium (Merck, Darmstadt, Germany), at pH 5.8, and incubated at 25°C. After seven days, the mycelium was used for inoculum production (spawn) in a substrate made of rice grains. The rice was cooked for 30 min in water at a 1:3 ratio of rice: water (w/w). After cooking, the rice was drained and supplemented with 0.35% $CaCO_3$ and 0.01% $CaSO_4$. This rice (70 g) was packed into small glass jars and sterilized in an autoclave at 121°C for 1 h. After cooling, each jar was inoculated with 4 agar discs (5 mm diameter) containing mycelium and incubated in the dark at room temperature for 15 days.

Substrates used and inoculation

To select the most suitable substrates for lignocellulolytic enzyme production, preliminary experiments were conducted with *Jatropha* seed cake and different lignocellulosic residues. *P. ostreatus* was grown in *Jatropha* seed cake with different percentages of eucalypt sawdust, corn cob, coffee husk and eucalypt husk. The addition of these agroindustrial residues was designed to balance the carbon and nitrogen relationship, which might benefit mycelial growth (Elisashvili et al., 2008; Giardina et al., 2000; Shashirekha et al., 2005).

Based on the results of these preliminary experiments, the compositions selected for biological detoxification are as shown in Table 1. The substrates were humidified with water at 75% of retention capacity, and 1.5 kg of each substrate was placed in polypropylene bags and was autoclaved at 121°C for 2 h. After cooling, the substrates were inoculated with 75 g of spawn and incubated at 25°C. Samples of non-inoculated bags were also kept.

Enzymatic assays

After 15, 30, 45 and 60 days of incubation, 10 g of each substrate was placed in Erlenmeyer flasks (125 mL) containing 25 mL of sodium citrate buffer (50 mM, pH 4.8). The flasks were kept in a shaker for 30 min at 150 rpm, and extracts were filtered with Millipore membranes (Cavallazzi et al., 2004). Enzyme assays were performed in triplicate, and enzyme activity was calculated as the difference in absorbance between non-inoculated and inoculated samples.

Laccase and manganese peroxidase (MnP) activities were measured using 2,2'-azino-bis-3-etilbenzotiazol-6-sulfonic acid (Buswell et al., 1995) and phenol red solution (Kuwahara et al., 1984) as substrates, respectively. Xylanase and cellulase activities were calculated by measuring the levels of reducing sugars that were produced by the enzymatic reactions (Bailey et al., 1992; Mandels et al., 1976).

One unit of enzyme activity was defined as the amount of enzyme required to catalyze the production of one µmol of colored product or reducing sugars per mL per min.

Chemical composition of the substrates

Lignin, cellulose and hemicellulose content were determined as described by Hatfield et al. (1994).

Tannins and phytic acid were quantified by a colorimetric method as described by Makkar et al. (1995) and Gao et al. (2007), respectively.

The manganese, copper and zinc content found in *Jatropha* seed cake that was not inoculated, were evaluated by plasma emission spectrometry (Perkin Elmer, M Optima 3300 DV optical Inductively Coupled Plasma Emission Spectrometer) after acid digestion.

The level of reducing sugars was determined by the DNS method (99.5% dinitrosalicylic acid, 0.4% phenol and 0.14% sodium metabisulfite) and the standard curve was made with D-glucose (Merck, Darmstadt, Germany) with the concentrations of the standard ranging from 0.5 to 1.5 g L^{-1}.

Phorbol ester determination

Phorbol ester content was analyzed by high performance liquid

Table 1. Substrate compositions used for *P. ostreatus* growth.

Substrate	Mass substrates (kg)	
	J. curcas	Agroindustrial residue
Jatropha seed cake (Jc)	20	0
Jc + 10% eucalypt sawdust (JcEs)	18	2
Jc + 10% eucalypt husk (JcEh)	18	2

chromatography (HPLC), as described by Makkar et al. (1997). Three grams of the substrate were triturated for 10 min in a porcelain mortar. These samples were transferred to centrifuge tubes (50 mL) containing 20 mL of methanol (Sigma) and centrifuged at 4000 x*g* for 10 min at 4°C. The supernatant was filtered on paper filter (Whatman GF/D, degree 2.5 cm). An additional 10 mL of methanol was added to the solid material retained in the membranes, which was again centrifuged and filtrated. The supernatant of the first and second filtrations were transferred to Erlenmeyer flasks (125 mL), dried in a vacuum (40°C) in rotavapor (Büchi, 461 Water Bath) and resuspended in 5 mL of tetrahydrofuran (Sigma). Twenty microliters of this suspension was injected into the HPLC (Shimadzu, C18 reverse phase and UV detection at 280 nm) and eluted with a gradient of acetonitrile and 0.175% orthophosphoric acid (Makkar et al., 1997). The standard curve was generated using phorbol-12-myristate 13-acetate (Sigma) with concentrations ranging from 0.005 to 0.5 mg mL^{-1}. The retention time of the standard was between 41 and 52 min, with four characteristic phorbol ester peaks (Makkar et al., 1997).

Biomass determination

The fungal biomass was determined by ergosterol content according to Richardson and Logendra (1997). Five grams of substrate colonized by fungus was triturated for 10 min in a porcelain mortar containing 0.3 g polyvinylpyrrolidone (Sigma) and 15 mL ethanol 95%. Then, this material was centrifuged during 20 min at 4.200 *g* and 4°C. The supernatant was filtered on Teflon sieve (200 mm x 53 µm) and stored at 4°C. Twenty microliters of this suspension was injected into the HPLC (Shimadzu, CLC-ODS reverse phase and UV detection at 280 nm) and eluted with methanol (Sigma) at 1.0 mL min^{-1}. Standard curve was prepared with ergostatrien-3β-ol (Sigma) dissolved in ethanol 95%.

Dry mass of the substrates

To determinate the dry mass, 1.5 kg of the substrate was dried at 105°C until a constant weight was obtained (Kasuya et al., 2012; Silva and Queiroz, 2002).

Chemical composition of Jatropha seed cake

To determine the dry mass (DM), organic matter (OM), crude protein (CP) and mineral matter (MM) we used the methodology describe by Silva and Queiroz (2002). The content of non-fibre carbo-hydrates (NFC), lignin, hemicellulose and cellulose were evaluated as describe by Van Soest et al. (1991). Ether extract (EE) was evaluated as describe by the American Oil Chemistry Society (AOCS, Official procedure Am 5-04, Ankon technology). The crude protein was measured with the Bradford method (Bradford, 1976).

In vitro digestibility

To measure the dry matter *in vitro* digestibility, we used the method describe by Tilley and Terry (1963) with minor modifications. We collected ruminal liquid of fistulated cattle from the department of animal husbandry (Universidade Federal de Viçosa), about two hours after feeding. The samples were incubated at 39°C for 30 min for anaerobic bacteria selection. We incubated 350 mg of each samples in anaerobic jars with continuous flow of CO_2 and a solution containing 4 mL ruminal liquid and 32 mL of McDougall buffer. The jars were incubated at 39°C for 48 h at 120 rpm. After the incubation, we filtered the samples in porcelain crucibles and washed with hot water until complete removal of the McDougall buffer. Then, we added 70 mL of neutral detergent solution and autoclaved at 121°C for 15 min. After this, the samples were washed again with hot water until complete removal of neutral detergent solution and washed once with 10 mL of acetone. The crucibles were maintained in 105°C for 16 h. The samples were put in the desiccator and their masses were measured.

Statistical analysis

The experiment was a completely randomized design with 5 replicates. The data were subjected to analysis of variance and mean values were compared by Tukey's test ($p < 0.05$) using Saeg software (version 9.1, Universidade Federal de Viçosa).

RESULTS AND DISCUSSION

The chemical composition of the substrates was significantly altered after heat treatment and, generally, there was a decrease in lignin, cellulose, antinutritional factor and phorbol ester content (Table 2). Several authors have shown a decrease in lignocellulosic compounds and antinutritional factors after heat treatment (Alvarez and Vázquez, 2004; Makkar et al., 1997; Rehman and Shah, 2005).

Dry mass, biomass and lignocellulosic compounds degradation

The dry mass loss (Figure 1A) correlated positively with the percentage of lignin degradation (significance = 0.154) (Figure 2A) and negatively with biomass (significance = 0.354) (Figure 1B), as also reported by Berg (2000). Furthermore, in the degradation of *Quercus petraea* leaves by the basidiomycetes, *Hypholoma fasciculare*

Table 2. Compounds found in substrate used for *P. ostreatus* growth.

Compound	Substrate (mg g^{-1})					
	Thermal treatment					
	Before			After		
	Jc	JcEs	JcEh	Jc	JcEs	JcEh
Lignin		See Figure 2			See Figure 2	
Cellulose		See Figure 3			See Figure 3	
Monosaccharides	8.25 ± 1.14	7.51 ± 0.11	6.30 ± 0.67	15.88 ± 5.64	14.96 ± 3.16	19.91 ± 1.49
Tannin*	2.50 ± 0.12	0.95 ± 0.44	3.01 ± 0.70	1.24 ± 0.34	0.56 ± 0.09	1.49 ± 0.11
Phytic acid	3.08 ± 0.28	2.31 ± 0.02	2.33 ± 0.17	1.08 ± 0.007	0.77 ± 0.009	0.77 ± 0.001
Phorbol ester	1.07 ± 0.02	0.63 ± 0.01	0.72 ± 0.01	0.73 ± 0.01	0.47 ± 0.01	0.56 ± 0.001
Manganese	0.044	nd	nd	nd	nd	nd
Copper	0.022	nd	nd	nd	nd	nd
Zinc	0.036	nd	nd	nd	nd	nd

* Contents are reported as equivalents of tannic acid; nd- not determined; Jc- *Jatropha* seed cake, JcEs – *Jatropha* seed cake + 10% eucalypt sawdust, JcEh10- *Jatropha* seed cake + 10% eucalypt husk (see Table 1).

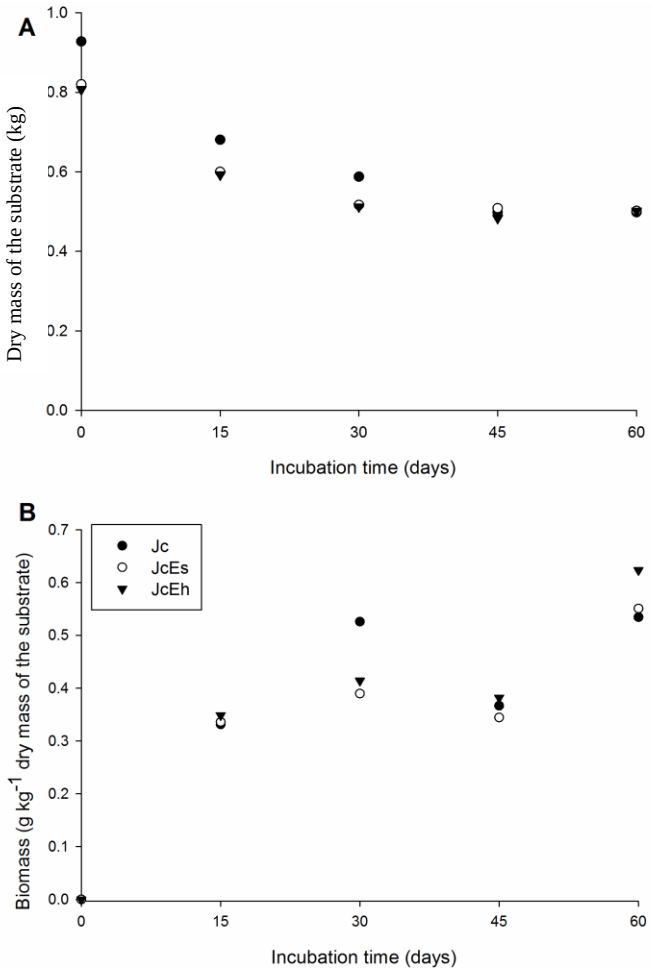

Figure 1. Dry mass (A) and mycelial biomass production (B) during 60 d of incubation with *P. ostreatus* in substrates with different proportions of *Jatropha* seed cake.

and *Rhodocollybia butyracea*, similar correlations were observed between dry mass and lignocellulolytic enzyme activity (Valáskova et al., 2007).

The reduction of total dry mass was, on average, 46% in seed cake and 39% in the other substrates (Figure 1A). However, these total losses dry mass were lower than that obtained by *P. ostreatus* in wheat straw after 50 days of incubation (Baldrian and Gabriel, 2003) and in lingocellulosic residues after 98 days (Baldrian and Gabriel, 2002). According to those authors, the high loss of dry mass was due to presence of copper, manganese and zinc in the substrate that stimulated the activity of the lignocellulolytic enzymes (Cavallazi et al., 2004). In this context, the high laccase and MnP activities (Figure 2B and C), which resulted in high lignin degradation (Figure 2A), can also be explained by the availability of these element in *Jatropha* seed cake (Table 2). The presence of copper in the laccase binding center stimulates the activity of this enzyme (Koroleva et al., 2001). MnP is a glycoprotein that has iron as prosthetic group and dependent on the hydrogen peroxide and manganese concentration for lignin oxidation (Brown et al., 1991; Hofrichter, 2002).

The high lignin degradation (Figure 2A), associated with greater dry mass loss (Figure 1A) and high ligninase activity (Figure 2B and C), suggesting that this polymer was the main source of carbon used for biomass production in Jatropha seed cake and in the substrate containing eucalypt sawdust (Figure 1B). This result could be due to a higher initial lignin concentration than cellulose and monosaccharide in these substrates (Table 2).

The higher cellulose degradation (Figure 3A) and also higher xylanase activity (Figure 3B) were observed in substrate containing eucalypt husk. The fungal biomass production in these substrates was due to, mainly, high

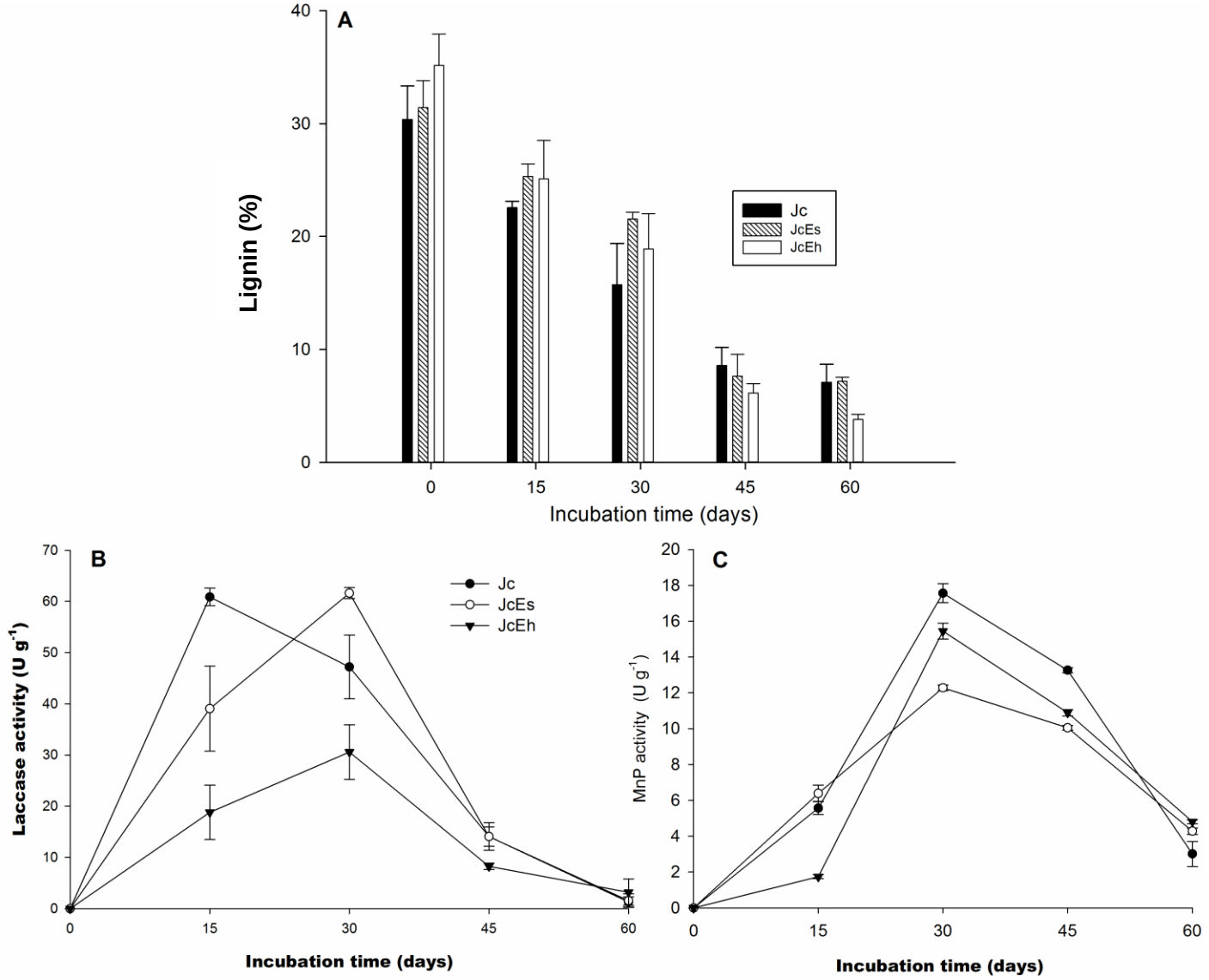

Figure 2. Lignin percentage (A) and laccase (B) and manganese peroxidase activity (C), during 60 days of incubation with *Pleurotus ostreatus* in substrates with different proportions of *Jatropha* seed cake.

monosaccharide availability (Table 2, Figure 1B) and xylanase activity (Figure 3B), because cellulase activity (Figure 3C) and laccase activity (Figure 2B) were low. This high monosaccharide availability (Table 2) may have influenced the differential expression of lignocellulolytic enzymes. This differential expression and also the role of these enzymes in the enzymatic degradation complex of lignocellulosic compounds have also been shown in *L. edodes* and *P. ostreatus* cultivated in different substrates (Cavallazi et al., 2004; Elisashvili et al., 2008).

High cellulase activity in Jatropha seed cake and substrate containing eucalypt sawdust shows a synergistic effect of this enzyme with ligninases (Figure 2 and 3C). Therefore, the use of lignin as a source of carbon and energy requires substrates containing cellulose or other carbon sources, as suggested by Ruggeri and Sassi (2003). It is important to observe that there was fungal biomass production (Figure 1B) and enzymatic activity (Figure 2 and 3) in Jatropha seed cake without addition of none lignocellulosic residues. With these results we reached the following conclusions: a) for the mycelial growth and fungal enzyme production, there is no need for the addition of lignocellulosic residues and b) the phorbol ester and antinutritional factors concentration found in Jatropha seed cake was not toxic for *P. ostreatus* (Table 2). Thus, this fungus can be used to

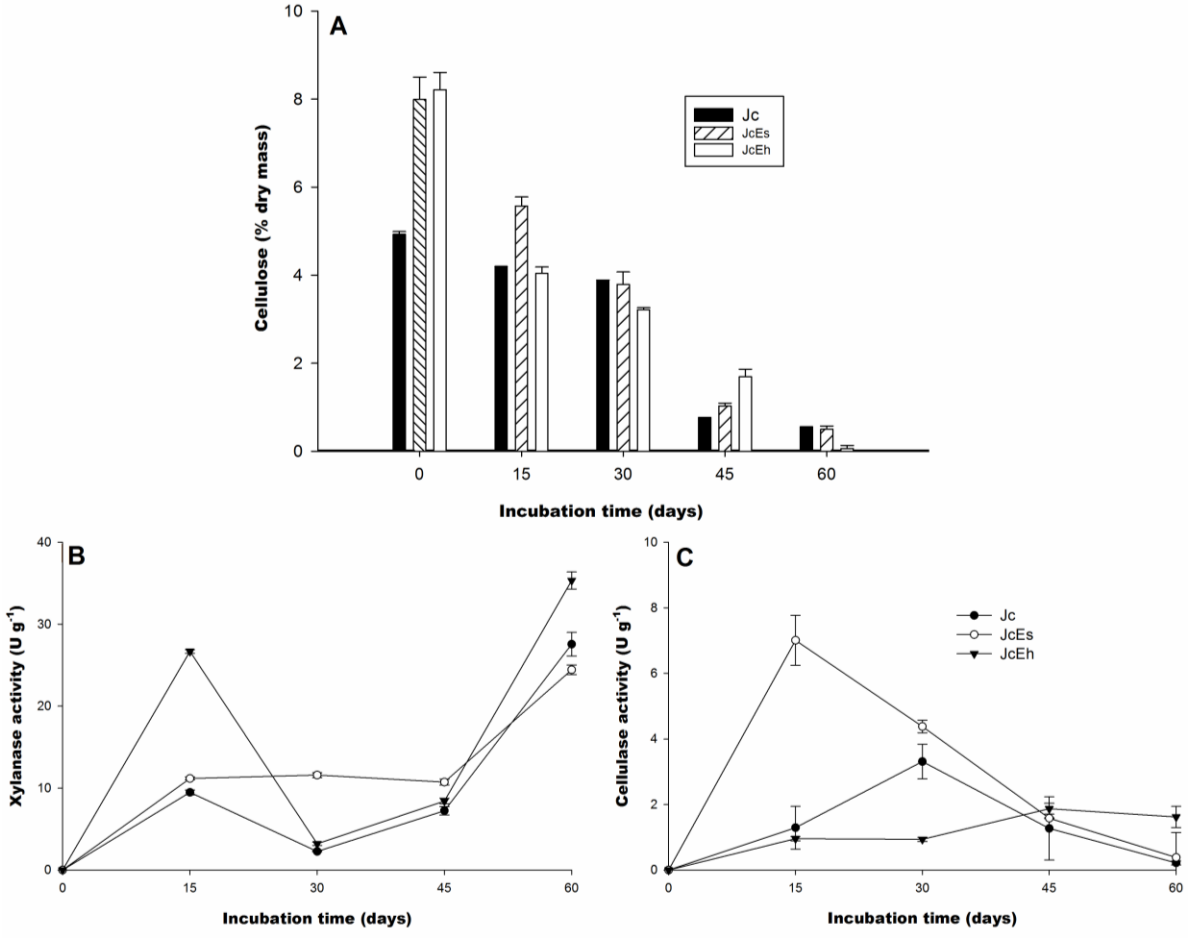

Figure 3. Cellulose percentage (A) and xylanase (B) and cellulase activity (C), during 60 days of incubation with *Pleurotus ostreatus* in substrates with different proportions of *Jatropha* seed cake.

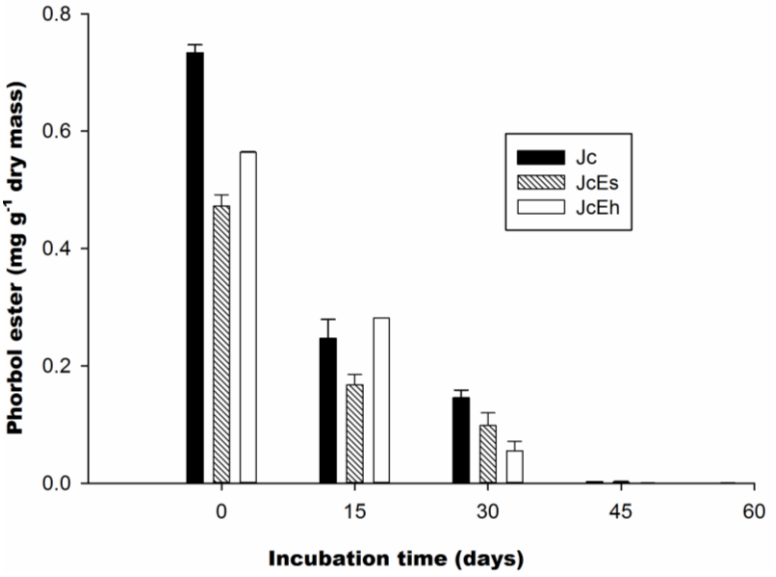

Figure 4. Phorbol ester degradation for *Pleurotus ostreatus* in substrates with different proportions of *Jatropha* seed cake.

Table 3. Chemical composition (%) of *Jatropha* seed cake added with agro-industrial residues colonized or not by *P. ostreatus* for 45 days.

Components	Jc		JcEs		JcEb	
	non-inoculated	colonized	non-inoculated	colonized	non-inoculated	colonized
Dry mass (DM)	95.03	96.24[a]	94.83	96.31[a]	95.03	96.08[a]
Organic matter (OM)	93.30	91.14[a]	93.97	92.49[a]	92.74	91.97[a]
Crude protein (CP)	11.44	13.16[a]	10.74	12.15[a]	11.08	11.26[a]
Ether extract (EE)	17.93	7.56[a]	16.82	7.08[a]	16.21	7.10[a]
Non-fibre carbohydrates (NFC)	63.94	70.92[b]	66.42	73.26[a]	65.26	73.80[a]
Mineral matter (MM)	6.70	8.86b	6.03	7.51[b]	7.26	8.03[b]
In vitro digestibility dry mass	54.90[b]	77.92	60.06[a]	80.44	60.31[a]	83.90

Jatropha curcas seed cake (Jc), Jc + 10% of eucalyptus sawdust (JcEs), Jc + 10% of eucalyptus bark (JcEb); See Table 1. Means followed by the same letter in the same line, do not differ by analysis of variance and Tukey test (P<0.05).

degrade lignocellulosic compounds of the Jatropha seed cake (Figure 2A and 3A) and to increase the organic matter and non-fibre carbohydrate content and digestibility of this residue as observed by Kasuya et al. (2012), using different percentages of Jatropha seed cake biodetoxified in the ration of goats.

Phorbol ester degradation

The initial phorbol concentration found in *Jatropha* seed cake was between 0.63 and 1.07 mg g^{-1} (Table 2). This concentration was higher than that found in the non-toxic variety from Mexico (Makkar et al., 1997) and lower than that found in varieties of Cape Verde and Nicaragua (Makkar and Becker, 1997; Martínez-Herrera et al., 2006). Therefore, these results demonstrate that phorbol concentration in the *J. curcas* seed can be influenced by the growing region and variety.

Autoclaving the substrates at 121°C reduced, on average, 20% the phorbol ester content (Table 2). However, these compounds were not degraded by treatment at 160°C for 30 min (Aregheore et al., 2003). Moreover, the addition of sodium hydroxide and sodium hypochlorite combined with heat treatment was able to reduce the phorbol concentration by 25% (Goel et al., 2007). In this work, *P. ostreatus* was able to degrade 99% of the phorbol ester content after 60 days of incubation (Figure 4). This rate of degradation was higher than that obtained by chemical deodorization, deacidification, or bleaching agents applied to the oil and seed cake of *J. curcas* (Rakshit et al., 2008). The same chemical processes, with the exception of bleaching, when applied to *J. curcas* seed, were not effective in reducing phorbol esters (Salimon and Ahmed, 2009).

The observed phorbol ester degradation (Figure 4) may be explained by the capacity of *P. ostreatus* to deploymerize lignin. As observed by Barr and Aust (1994) and Majcherczyk et al. (1998), the degradation of organic compounds, for example chlorophenols and aromatic hydrocarbons, occurs due to the lignin depolymerization reaction, mainly through the activities of laccase and MnP. The activities of these enzymes from *Phanerochaete* sp. (Perez et al., 1998) and *P. ostreatus* (Lamarino et al., 2009) have also been reported in the decolorization of various dyes in the textile industry and in the elimination of different pollutant compounds.

Maximum phorbol ester elimination did not occur until the 30th incubation day (Figure 4) and coincided with the interval of high laccase and MnP activity (Figures 2B and 2C). However, other enzymes could have influenced this degradation because the degradation of phenols and furfural by cellulase has been observed by Honggiang and Hongzhang (2008). In our study, higher cellulase and xylanase activities (Figure 3) were observed between the 15th and 45th incubation days as indicated by 37 and 60% of phorbol ester degradation, respectively. In the substrate containing eucalypt husk, however, lower phorbol ester degradation and lower ligninases activity were observed on the 15th day of incubation (Figures 2B, C and 4). This evidence supports the hypothesis that phorbol ester degradation occurs by co-metabolism with the enzymes responsible for lignin depolymerization.

Finally, after 60 days of incubation with *P. ostreatus*, the residual phorbol ester concentration was, on average, 2 x 10^{-4} mg g^{-1} dry mass (Figure 4). This concentration is much lower than the 0.09 mg g^{-1} found in the non-toxic Mexican variety of *Jatropha* (Aregheore et al., 2003). Furthermore, Kasuya et al. (2012) observed no clinical symptoms of poisoning in goats fed with *Jatropha* seed cake detoxified by *P. ostreatus* and concluded that biodetoxified *Jatropha* seed cake can be safely used in the goat diet.

Chemical composition of *Jatropha* seed cake

The colonization of some agro-industrial residues by microorganism has been successfully used as strategy to increase their digestibility and nutritional value. The growth

of *P. ostreatus* in *Jatropha* seed cake added or not with agroindustrial residues improved some features desirable to animal feed. We observed an increase in CP, NFC, MM and a decrease in EE, lignin, cellulose, hemicellulose contents (Table 3). Our data is similar to the chemical composition of other goat feeds (Lee et al., 2008; Khan et al., 2009).

The CP is the main source of essential amino acids to the animals (NRC, 2000; Verbic, 2002). This is one of the principal factors used to assess the potential of agro-industrial residues to be used in animal feed. Therefore, the increase in CP due to fungal growth is beneficial to ruminants (Table 3).

The decreases in EE content (Table 3) contributes to the use of *Jatropha* seed cake in animal feed, because is recommended for diet dry matter with less than 10% of EE (Mohammed et al., 2004). According to Palmquist and Mattos (2011), lipid content higher than 5% of dry mass consumption is due to (a) regulatory mechanisms which control food intake or (b) the limited ability of ruminants to oxidize fatty acids.

The fungal growth improves the *in vitro* digestibility of all substrates tested (Table 3). This may be related to the increase in NFC and the decrease in hemicellulose/ cellulose and lignin content, since carbohydrates are a source of carbon and energy more available for animals and microorganism than polymers (Dohme et al., 2001). Thus, is reasonable to conclude that the mycelial growth increases the potential to use *Jatropha* seed cake in animal feed.

Therefore, *P. ostreatus* degrades phorbol esters found in *Jatropha* seed cake detoxifying this residue of bio-diesel manufacture and improves the potential to use this residue as ration.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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