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Jatropha seed cake supplementation for improved fungal growth and later use as animal feed

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The use of *Jatropha curcas* as a raw material for biodiesel production results in large amounts of a solid residue called Jatropha seed cake (Jc). This residue is composed of lignin, cellulose, toxic compounds and anti-nutritional factors. Though the monomers that make up lignin and tannins could be used in animal feed, the polymer forms are not easily digestible, which necessitates a processing step. White rot fungi are known to produce enzymes capable of degrading these polymers, as well as anti-nutritional factors and some toxic compounds (for example, phorbol ester). Therefore, we evaluated the degradation of lignocellulosic compounds and biomass production by *Pleurotus ostreatus* in pure Jc or with different levels of added agro-industrial residues. After 45 days incubation, the best performing sample in terms of mycelial growth was pure Jc, followed by those supplemented with 20% eucalypt sawdust (JcEs20), 20% eucalypt bark (JcEb20) and 10% coffee husk (JcCh10). Among them, the JcCh10 presented the lowest lignin content after fungal growth. The content of lignin and cellulose/hemicellulose in this substrate were, respectively, 29.19 and 47.27% lower than pure Jc. Thus, *P. ostreatus* has the potential to degrade lignocellulosic compounds found in Jc, and this degradation increases the possibility of using this residue as animal feed.

Key words: Biofuel, lignocellulosic residues, Jatropha seed cake, *Pleurotus ostreatus*, biodegradation.

INTRODUCTION

The hope of finding a renewable source of energy that causes little environmental damage has led to the use of oilseed plants for oil extraction and biofuels production (Gübitz et al., 1999; Lu et al., 2009; Openshaw, 2000). *Jatropha curcas* Linnaeus (1753) has shown good potential for this purpose due to the high oil content in the seeds (Jongschaap et al., 2007; Openshaw, 2000; Lu et al., 2009). In addition, this species adapts well to climatic variations and different soil compositions (Jongschaap et al., 2007), allowing it to be cultivated in different regions. However, after oil extraction, a large amount of lingocellulosic residue remains, which is called Jatropha seed cake (Jc). The disposal and reuse of this residue is a challenge for the biodiesel industry.

Phorbol ester and anti-nutritional factors (Makkar et al. 1997) limit the direct use of Jc for animal feed (Gübitz et

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al.,1999; Raheman and Mondal, 2012). However, these compounds can be degraded by bacteria (Joshi et al., 2011) or white rot fungi, such as *Pleurotus ostreatus* (da Luz et al., 2014; Kasuya et al., 2012). Similarly, fungi have been efficiently used to increase the digestibility and nutritional value of agroindustrial residue, such as cocoa husk (Alemawor et al., 2009), *Brachiaria* sp. (Bisaria et al., 1996) and Jatropha seed cake (Kasuya et al., 2012).

Jc has been used as a substrate for white-rot fungal growth (de Barros et al., 2011; Kasuya et al., 2012; da Luz et al., 2013; Da Luz et al., 2014). However, previous studies focused on the degradation of phorbol ester to improve the usefulness of this material. Here, we focused on improving mycelial growth in the substrate. Recently, fungal growth was associated with the improved chemical composition of Jc (Kasuya et al., 2012). Therefore, better fungal growth might increase, for example, its protein content, *in vivo* digestibility and palatability, all of which are desirable characteristics for further uses in animal feed.

We combined Jc with different agro-industrial residues in order to look for substrate compositions that better supported fungal growth and that reduced lignin and cellulose/hemicellulose content, as lignocellulosic compounds have an adverse effect on digestibility of feed (Woodman and Stewart, 1932). This biological processing by fungi may be an effective alternative means of adding economic value to Jc by transforming it into animal feed.

MATERIALS AND METHODS

Microorganism

The fungus used was *P. ostreatus* isolate PLO 6 (KC782771, GenBank, 2013) from the Laboratory of Mycorrhizal Associations of the Universidade Federal de Viçosa. It was grown in a Petri dish containing 20 mL of potato dextrose agar culture medium (PDA, Merck, Darmstadt, Germany) at pH 5.5 ± 0.3 and incubated at 25°C.

Substrate composition and growth conditions

Pure Jc or Jc supplemented with agro-industrial residues were used for PLO 6 growth. The type and amount of agro-industrial residues added to Jc was chosen based on substrate compositions that are commonly used for white rot fungi growth and lignocellulolytic enzymes production (Nunes et al., 2012; Wang et al., 2009). Forty-five grams of substrate were combined with 25 mL of tap water in a 250 mL glass beaker and autoclaved at 121°C for 20 min. The substrate was then innoculated using fungi grown in rice (de Assunção et al., 2012) and incubated for 45 days at 25°C. This incubation time was determined based on a previous study (da Luz et al., 2013). Three grams sample from each flask were used to determine the pH, as well as ergosterol, lignin and cellulose/hemicellulose levels.

Fungal biomass determination using ergosterol

Ergosterol analysis was used to quantify fungal biomass as described previously (Richardson and Logendra, 1997) but with some modifications. Five grams of substrate was triturated and added to 0.3 g polyvinylpyrrolidone (Sigma) and 15 mL of 95% ethanol. This material was centrifuged for 20 min at 4200 x*g* at 4°C. The supernatant was filtered through a Teflon sieve (200 mm x 53 µm) and stored at 4°C before analysis by high performance liquid chromatography (Shimadzu, CLC-ODS reverse phase and UV detection at 280 nm) using a methanol (Sigma) flow rate of 1.0 mL min^{-1}. A standard curve was prepared using ergostatrien-3β-ol (Sigma) dissolved in 95% ethanol. Fungal biomass was determined by the relationship between the ergosterol content and the dry mass of fungal mycelium cultivated in PDA for 15 days. For dry mass deter-mination, the medium colonized by the fungus was filtered through the Teflon sieve, transferred to a porcelain dish and dried at 60°C until mass was constant (Barajas-Aceves et al., 2002).

Lignin content

For total lignin determination, 1 g sample of each substrate before and after fungal colonization was treated with 10 mL of a mixture of 95% ethanol and 5% toluene and 10 mL hot water (100 ± 10°C) to remove wax and mucilage. This material was filtered, washed and dried at 60°C (Van Soest, 1963; Hatfield et al., 1994). Then, 20 mL of 72% sulfuric acid was added, and the material was autoclaved at 121°C for 1 h. After 12 h at 25°C, the material was filtered (Whatman, GF/D) and washed in hot water until the acid was completely removed.

For insoluble lignin determination, the solid material retained in the filter paper was dried at 105°C until its mass was constant. The soluble lignin content was determined in the acid solution before washing with hot water by measuring the difference between absorbance at wavelengths of 215 and 280 nm (Van Soest, 1963; Hatfield et al., 1994).

Cellulose/hemicellulose content

Cellulose/hemicellulose content was quantified in the same acid solution that was used for lignin content determination (Van Soest, 1963; Hatfield et al., 1994). For this assay, 1 mL of sample was added to 3 mL of sodium hydroxide (2 mol L^{-1}) and 1 mL DNS solution (99.5% dinitrosalicylic acid, 0.4% phenol and 0.14% sodium metabisulfite). This mixture was boiled at 100°C for 5 min. Water was then added to make it 5 mL and the absorbance was measured at 540 nm.

pH determination

The pH of each substrate before and after 45 days of fungal growth was measured as described by Sodré et al. (2001). Five grams of each substrate was placed in Erlenmeyer flasks (125 mL) containing 15 mL water. The flasks were kept in a shaker for 12 h at 220 rpm. The material was then left to stand for 1 h and the pH was measured in the supernatant.

Statistical analysis

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

RESULTS

The percentage of lignocellulosic compounds in the substrate was influenced by the types and amount of

Table 1. Lignin and cellulose/hemicelluloses in substrate before and after growth of *Pleurotus ostreatus*.

Susbtrate	Total lignin (%)		Cellulose/hemicelluloses (%)	
	Before	After	Before	After
Jatropha seed cake	33.14±0.70e	25.73±0.54d	8.63 ± 0.47a	6.94 ± 0.37b
Jatropha seed cake + 10% rice bran	35.88±0.33d	22.28±0.20ef	2.40 ± 0.04c	2.26 ± 0.03f
Jatropha seed cake + 20% rice bran	38.30±0.87c	24.99±0.56d	2.36 ± 0.66c	2.23 ± 0.62f
Jatropha seed cake + 10% eucalypt sawdust	38.74±0.14c	28.84±0.10c	2.83 ± 0.31c	2.64 ± 0.28ef
Jatropha seed cake + 20% eucalypt sawdust	43.72±0.38a	33.9±0.29b	2.60 ± 0.05c	2.44 ± 0.04f
Jatropha seed cake + 10% corn cob	30.33±2.26f	29.13±2.17c	2.92 ± 0.37c	2.66 ± 0.33ef
Jatropha seed cake + 20% corn cob	22.37±0.43h	20.97±0.40f	3.74 ± 0.57bc	3.48 ± 0.53de
Jatropha seed cake + 10% eucalypt bark	38.58±1.95c	29±1.46c	4.44 ± 0.40b	3.85 ± 0.34d
Jatropha seed cake + 20% eucalypt bark	41.75±0.04b	40.27±0.03a	5.68 ± 0.86b	5.09 ± 0.77c
Jatropha seed cake + 10% coffee husk	28.01±0.62g	18.22±0.20g	4.04 ± 0.21b	3.66 ± 0.19d
Jatropha seed cake + 20% coffee husk	29.29±0.11fg	23.96±0.08de	4.06 ± 0.13b	3.68 ± 0.11d
Jatropha seed cake + 0.2% $CaCO_3$+$CaSO_4$	33.14±0.70e	27.57±0.58c	8.63 ± 0.47a	8.05 ± 0.43a

In a column, means with different letters differ by analysis of variance and Tukey test at 5% probability.

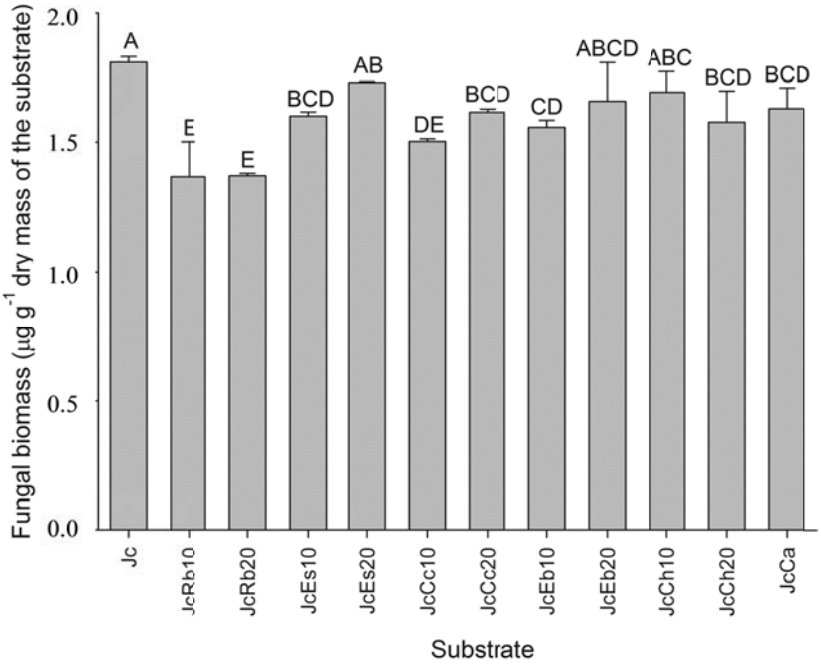

Figure 1. Biomass of *Pleurotus ostreatus* grown at 25°C for 45 days in substrates with different proportions of Jatropha seed cake. Bars followed by the same letters do not differ by Tukey's test ($p<0.05$). Substrate components are described in Table 1.

agroindustrial residue added in Jc (Table 1; $P < 0.05$). The cellulose/hemicelluloses content was higher in Jc than in the other substrates (Table 1).

The addition of agroindustrial residues to Jc influenced fungal growth (Figure 1; $P < 0.05$). Except when rice bran and corncobs were added, any other residue was suitable for fungal growth (Figure 1). The best substrates for *P. ostreatus* growth were Jc-supplemented with 20% eucalypt sawdust, 20% eucalypt bark or 10% coffee husk (Figure 1).

Lignocellulosic compound degradation varied with substrate composition (Figures 2 and 3), leading to differences in the final content of these compounds (Table 1). Coffee husk showed the lowest levels of lignin ($P < 0.05$), and eucalypt sawdust, the lowest level of cellulose/hemicellulose after fungal growth ($P < 0.05$).

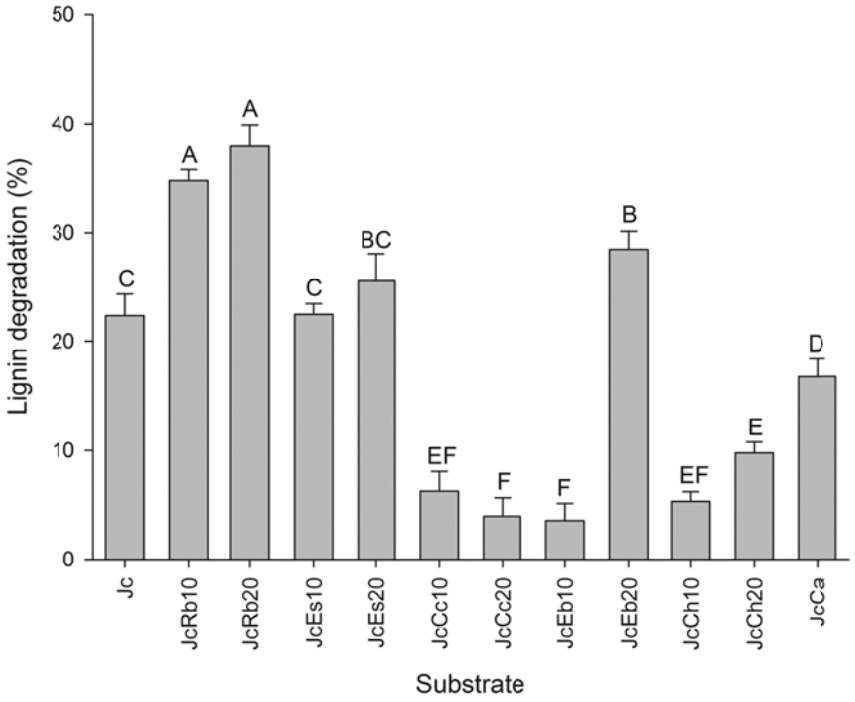

Figure 2. Percentage of lignin degraded by *Pleurotus ostreatus* after growth in substrates with different proportions of Jatropha seed cake at 25°C for 45 days. Bars followed by the same letters do not differ by Tukey's test ($p<0.05$). Substrate components are described in Table 1.

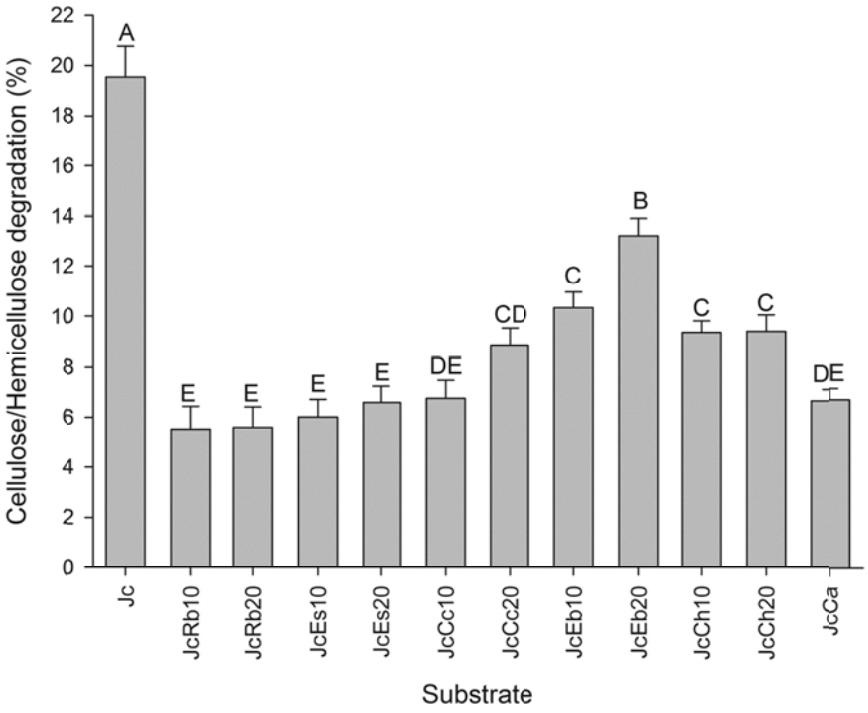

Figure 3. Percentage of cellulose/hemicellulose degraded by *Pleurotus ostreatus* after growth in substrates with different proportions of Jatropha seed cake at 25°C for 45 days. Bars followed by the same letters do not differ by Tukey's test ($p<0.05$). Substrate components are describe in Table 1.

DISCUSSION

In this study, the addition of agroindustrial residues to Jc affected fungal growth (Figure 1), perhaps by altering some physicochemical characteristics of the substrate. The addition of low granulometry substrates, for example, rice bran, likely decreased internal empty spaces available in the substrate (Rossi et al., 2003), reducing gas exchange with the atmosphere and oxygen availability, which is fundamental for aerobic microorganisms. Additionally, the substrate C:N ratio is important for mycelial growth (Mwangi et al., 2012) and alterations in this parameter can significantly change fungal growth rates (Couto et al., 2012; Mwangi et al., 2012). The addition of corncobs, a substrate with lower nitrogen content than Jc, decreased the fungal growth rate (Figure 1). It is worth noting that for better fungal growth, the substrate C:N ratio must be approximately 25:35 (Mwangi et al., 2012). Moreover, the presence of toxic compounds (e.g., caffeine found in the coffee husk) can inhibit or decrease *P. ostreatus* growth (Pandey et al., 2000; Fan et al., 2003). However, this problem seems to be attenuated by performing a prior boil of the substrate (Houdeau et al., 1991; da Silva et al., 2012; de Assunção et al., 2012). Therefore, substrates should be carefully selected to favor fungal growth.

The decreasing lignin and cellulose/hemicellulose degradation seems to influence fungal growth (Figures 2 and 3). Cavallazzi et al. (2004) noted that carbohydrases in *P. ostreatus* can metabolize new carbon resources from polysaccharides, while its lignocellulosic enzymes can break down lignin polymers and phenolic compounds. As lignin and cellulose/hemicellulose are the main source of carbon in Jc (Kasuya et al., 2012), the activity of these enzymes is highly relevant for fungal growth in this substrate. Therefore, it is expected that substrates with more readily available carbon sources (e.g., monosaccharides, cellulose) should support fast and efficient mycelial growth. Indeed, here we observed that *P. ostreatus* grew well in Jc, the substrate with the highest level of cellulose/-hemicellulose (Table 1). However, the addition of agroindustrial residues decreased the level of these compounds (Table 1), which in turn increases the importance of substrate depolymerization by lignocellulolytic enzymes in order to improve availability of carbon for fungal growth (Mata and Savoie, 1998). However, instead of increasing, lignin degradation also decreased when some residues were added (Figure 2), probably reducing carbon availability for mycelial growth, which may explain the reduction in fungal growth (Figure 1). Thus, the addition of substrates with a high level of cellulose/hemicellulose seems to be an interesting strategy to improve fungal growth and expand the potential uses of Jc.

Lignin and cellulose/hemicellulose degradation (Figures 2 and 3) can increase digestible dry mass, given the natural recalcitrance of these compounds (Pérez et al., 2002). Indeed, an increase in digestibility after lignin degradation was shown when *Phanerochaete chrysosporium* was cultivated on cotton stalks (Shi et al., 2009) and *Pleurotus sajor-caju* was cultivated on agroindustrial residues (Bisaria et al., 1996). Here, supplementation with agroindustrial residues was shown to be an effective way to decrease lignin and cellulose/hemicellulose in the substrate before and after fungal growth (Table 1). Among the treatments that did not influence the fungal growth (Figure 1), Jc supplemented with 10% coffee husk had the lowest lignin content (Table 1). Furthermore, the lignin and cellulose/hemicellulose content was lower than pure Jc (Table 1). This difference is important for animal feed, because a negative relationship between digestibility and lignocellulosic compounds content has been observed for more than 70 years (Woodman and Stewart, 1932).

Recently, *P. ostreatus* growth in Jc was observed to increase its *in vitro* digestibility and the amount of non-fiber carbohydrates and crude protein (da Luz et al., 2014), which are all desirable features for animal feed (Montagne et al., 2003). Moreover, Kasuya et al. (2012) showed that Jc after *P. ostreatus* growth was well-accepted by goats, leading to an increase in dry mass intake. The above authors pointed out that consumption and palatability of the feed was not diminished by the inclusion of detoxified Jc. Here, lignin and cellulose/hemicellulose content after fungal growth (Table 1) were lower than that reported by Kasuya et al. (2012). In this study, Jc supplemented with 10% coffee husk and processed via fungal growth showed good potential for use as an animal feed.

However, some considerations about coffee husk should be made, as coffee husk contains antinutritional compounds, such as tannins and caffeine (Pandey et al., 2000). Both compounds were shown to be degraded by *P. ostreatus* (Fan et al., 2003; da Luz et al., 2013), although the degradation of these compounds should be further evaluated, as tannins have an adverse effect on rumen metabolism (Makkar et al., 1995) and caffeine can cause dependence (Griffiths and Woodson, 1988) and, depending on the concentration, can even be toxic to animals (Hosenpud et al., 1995). Indeed, the intake of feed with 20% coffee grounds by rats has been shown to negatively affect dry matter digestibility by depressing feed intake and increasing urinary output and water intake (Campbell et al., 1976). Thus, from this starting point, further studies should evaluate the degradation of tannins and caffeine by fungi in Jc supplemented with 10% coffee husk, and animal assays should be performed using this substrate.

Conflict of interest

The authors have not declared any conflict of interests.

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