



Production of edible mushroom and degradation of antinutritional factors in jatropha biodiesel residues

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ABSTRACT

The elimination of antinutritional factors of the *Jatropha curcas* L. seed cake is important for decreasing environmental damage and adding economic value to this residue of the biodiesel industry. In this study, we analyzed the ability of *Pleurotus ostreatus* to degrade antinutritional factors and produce edible mushrooms using different proportions of the *J. curcas* seed cake as substrate. After 60 d of incubation at 25 °C, we observed 95% phytic acid and 85% tannins reductions, and high mushrooms productivity. There was no evidence of tannins or phytic acid in these mushrooms. Furthermore, the phorbol ester concentration observed in these mushrooms was around 1000-fold lower than that found in the non-toxic variety of *J. curcas*. Thus, *P. ostreatus* can degrade the antinutritional factors found in *J. curcas* seed cake. The jatropha seed cake can potentially be used for mushroom production, with high nutritional value, and animal ration, after treated by *P. ostreatus*, adding economic value to the biodiesel residue and avoiding inadequate disposal in the environment.

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1. Introduction

Phorbol ester, phytic acid and tannins are the main compounds that are found in the *Jatropha curcas* L. seed cake, that make this residue unusable as animal feed. The phorbol esters that are found in the seed and the oil are the major toxic compounds of *J. curcas* L. (Makkar, Becker, Sporer, & Wink, 1997). Due to the formation of an insoluble complex between the polyvalent cation and proteins, the phytic acid decrease the absorption of both mineral and protein in the gastrointestinal tract of animals (Liang, Han, Nout, & Hamer, 2009; Liu et al., 2008). Additionally, tannins also have a high capacity to form insoluble complex and precipitate protein, thereby inhibiting the digestion of proteins and amino acids (Rehman & Shah, 2005).

The elimination of these antinutritional factors (phytic acid and tannins) is important for increasing economic value and for making it possible to be used as animal feed. This elimination has been made by thermal (Deshpande & Damodaran, 1990; Rehman & Shah, 2005) or biological treatment (Batra & Saxena, 2005). The biological

method besides being more specific and efficient than thermal treatment can result in products of economical interest (e.g. enzymes, mushrooms, animal feed).

Pleurotus ostreatus has been used in the bioremediation of pollutants and the degradation of lignocellulosic residue by the action of different enzymes (Dundar, Acay, & Yildiz, 2009; Haritash & Kaushik, 2009), including the lignocellulolytic enzymes, tannase and phytase (Batra & Saxena, 2005; Cavallazzi, Brito, Oliveira, Villas-Bôas, & Kasuya, 2004; Collopy & Royse, 2004). In addition, this fungus produces mushrooms using different lignocellulosic residues (Dundar et al., 2009; Fan, Soccol, Pandey, Vandenberghe, & Soccol, 2006; Nunes et al., 2012). The *P. ostreatus* mushrooms have high nutritional value and are sources of protein, carbohydrates, vitamins (e.g. B1, B2 and B3), calcium and iron (Dundar et al., 2009; Wang, Sakoda, & Suzuki, 2001).

Major agroindustrial residues have in its chemical composition higher fibers content with low availability than protein, minerals and vitamins (Villas-Bôas, Esposito, & Mitchell, 2002). Colonization and solid fermentation by fungi have been used to increase the availability and the nutritional value of these residues (Pereira, 2011; Sánchez, 2009; Villas-Bôas et al., 2002). This procedure has been used with success in cocoa (Alemawor, Dzogbefia, Oddoye, & Oldham, 2009), sawdust (Kwak, Jung, & Kim, 2008) and jatropha seed cake (Pereira, 2011).

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Thus, in this study, we tested the ability of *P. ostreatus* to degrade antinutritional factors and produce edible mushrooms using different proportions of the *J. curcas* seed cake as substrate.

2. Materials and methods

2.1. Microorganism, fungal growth conditions and inoculum production (spawn)

The isolate Plo 6 of *P. ostreatus*, which were used in this study, belong to collection of the Department of Microbiology of Federal University of Viçosa, MG, Brazil. This isolate was grown in a Petri dish containing potato dextrose agar culture medium (Merck) at pH 5.8 and incubated at 25 °C. After 7 days, the mycelium was used for inoculum production (spawn) in a substrate made of rice grains with peel (de Assunção et al., 2012). The rice grains were cooked for 30 min in water at a 1:3 (rice grains:water, w/w). After cooking, the grains were drained and supplemented with 0.35 (g/100 g) CaCO₃ and 0.01 (g/100 g) CaSO₄. These grains (70 g) were 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 25 ± 2 °C for 15 d.

2.2. substrate and inoculation

The *J. curcas* seed cake used in this study was obtained from an industry of biodiesel (Fuserman Biocombustíveis, Barbacena, Minas Gerais State, Brazil).

The proper substrate composition for optimal growth and enzyme production by *P. ostreatus* was chosen based on previously experiments with jatropha seed cake and different agroindustrial residues (Da Luz, 2009). In these experiments, *P. ostreatus* was cultivated in substrates with seed cake added to different proportions of eucalypt sawdust, corncob, eucalypt bark and coffee husk. The purpose of adding these agroindustrial residues was to balance the carbon and nitrogen ratio, which may stimulate the mycelial growth (Nunes et al., 2012). The substrate compositions that were selected for this study were based on the results of these previous experiments were jatropha seed cake (Sc), Sc + 10 (g/100 g) of eucalypt sawdust (ScEs), Sc + 10 (g/100 g) of eucalypt bark (ScEb) and Sc + 30 (g/100 g) of coffee husk + 30 (g/100 g) of rice bran (ScCh). In these substrates, the isolate Plo 6 had better biomass production and greater degradation rate of lignocellulosic compounds when compared to other tested substrates (Da Luz, 2009).

The substrates were humidified with water at 75% of the retention capacity and 1.5 kg of each substrate was placed in polypropylene bags. Next, the bags containing the substrates were autoclaved at 121 °C for 2 h. After sterilization, the substrates were inoculated with 70 g of spawn and incubated at 25 °C for 60 d. Samples for analyses were taken at intervals of 15 d.

2.3. Enzymatic assays

Phytase activity (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) was determined using Taussky–Schoor reagent according to Harland and Harland (1980). To extract the enzyme, 3 g of the substrate was transferred to Erlenmeyer flasks (125 mL) containing 10 mL of sodium chloride (1 g/100 mL). The flasks were kept in a shaker for 1 h at 100 rpm, and the extracts were filtered through Millipore membranes (Whatman 1). The filtrate was centrifuged for 5 min at 2000 × g. The reaction to determine phytase activity contained 100 µL of the filtrate and 1 mL of sodium phytate solution (0.5 g/100 g, Sigma). This reaction was incubated

in a water bath at 60 °C for 10 min, and then 1 mL of trichloroacetic acid (10 g/100 mL) and 5 mL of Taussky–Schoor reagent were added. The phosphorus content was determined with a spectrophotometer (Thermo, Evolution 60) at 500 nm. The standard curve for phosphorus quantification was made using dibasic potassium phosphate (Sigma) with concentrations ranging from 0.004 to 0.02 g/100 mL.

One unit of phytase was defined as the amount of enzyme required to release 1 µmol of inorganic phosphate per min from sodium phytate at 37 °C.

2.4. Determination of phytic acid concentration

To determine phytic acid content, 3 g of each substrate was transferred to Erlenmeyer flasks (125 mL) containing 25 mL hydrochloric acid (4 g/100 mL, Vetec). These flasks were kept in a shaker for 16 h at 220 rpm. The supernatants were transferred to centrifuge tubes (50 mL) containing 1 g of sodium chloride (Vetec), centrifuged at 1000 × g for 20 min and frozen at –20 °C for 30 min.

After thawing, the supernatants were centrifuged under the same conditions and filtered through Millipore membranes (Whatman GF/D, 4.7 cm). The filtrate (1 mL) was diluted in 24 mL of deionized water. Then, 3 mL of this solution was transferred to another centrifuge tube (50 mL) containing 1 mL of Wage reagent (0.03 g/100 g of ferric chloride hexahydrate, 0.3 g/100 mL of sulphosalicylic acid, 2.4 g/100 mL hydrochloric acid 0.65 mol/L). This mixture was again centrifuged (1000 × g) at 10 °C for 10 min, and the absorbance of the supernatant was detected at 500 nm using a spectrophotometer (Latta & Eskin, 1980). Sodium phytate (Sigma) concentrations ranging from 0.03 to 1.6 g/100 mL were used to make a standard curve.

2.5. Determination of tannins concentration

For extraction of the tannins in 3 g of substrate were added 10 mL methanol (Sigma) and 0.5 g of polyvinylpyrrolidone (Makkar, Bluemmel, & Becker, 1995). This material was homogenized in a shaker at 220 rpm for 1 h, and 5 mL barium hydroxide (0.1 mol/L) and 5 mL of zinc sulfate were added. The reaction to determine the tannins content (tannic acid equivalent) contained 2 mL of the supernatant, 5 mL of sodium carbonate (2 g/100 mL) in sodium hydroxide (0.1 mol/L) and 1 mL of Folin–Ciocalteu's reagent. This reaction was incubated in a water bath at 37 °C for 10 min, and the absorbance was detected at 765 nm using a spectrophotometer (Makkar et al., 1995). The standard curve was made using a solution of tannic acid (Sigma) with concentrations ranging from 0.01 to 1 g/100 mL.

2.6. Mushroom production

Polypropylene bags containing substrates with mycelial growth after 28, 43 and 58 d of incubation were transferred to a cold chamber at 10 °C for 48 h. This procedure was performed to induce the formation of the primordial of fruit bodies. Mushrooms fructification was performed in a chamber with temperature controlled at 18 ± 2 °C.

The biological efficiency (BE) was calculated according to Wang et al. (2001): BE = 100 × (fresh mass of mushroom (g)/dry mass of substrate (kg)).

2.6.1. Mushroom chemical analysis

The mushrooms were chemically analyzed to verify the concentrations of antinutritional factors, phosphorus, ergosterol, phorbol ester, soluble protein and reducing sugars. Mushrooms produced in each substrate were mixed, and from this mixture,

200 g of fresh mushrooms were triturated using a blender (Walita) for 10 min with addition 5 mL of deionized water. For each analysis, 10 g of crushed mushrooms was used.

The content of the tannins, phytic acid and phosphorus were determined according to described previously for substrate samples.

The ergosterol content was quantified using high-performance liquid chromatography (HPLC) according to Richardson and Logendra (1997). We use ergosta-5.7.22-trien-3 β -ol (Sigma) as standard.

The phorbol ester concentration was determined using HPLC as described by Makkar et al. (1997). To do so, 10 mL of methanol (Sigma) was added to 10 g of the crushed mushrooms, and this mixture was centrifuged at 4000 \times g for 10 min. The supernatant was filtrated using Millipore membranes (Whatman GF/D, 2.5 cm). An additional 10 mL of methanol was added to the solid material retained in the membranes, which were 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 a Rotavapor (Büchi, 461 Water Bath) 175 and resuspended in 5 mL of tetrahydrofuran (Sigma). Then, 20 μ L 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 (g/100 mL) orthophosphoric acid (Makkar et al., 1997). We use phorbol-12-myristate 13-acetate (Sigma) as standard.

The soluble protein and reducing sugar contents were determined according to the colorimetric methods described by Bradford (1976) and Miller (1959). For this analysis, 10 g of the crushed mushrooms was placed in Erlenmeyer flasks (125 mL) containing 25 mL sodium citrate buffer (0.05 mol/L and pH 4.8). These flasks were kept in a shaker for 30 min at 150 rpm, and the extracts were filtered using Millipore membranes (Cavallazzi et al., 2004).

2.7. Statistical analysis

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

3. Results and discussion

3.1. Degradation of antinutritional factors

The tannin concentrations observed in the jatropha seed cake (Fig. 1) were similar to those found in the fruit peel of *J. curcas* (Makkar, Aderibigbe, & Becker, 1998). The greatest concentration of this compound was observed in the substrate with coffee husk and eucalypt bark (Fig. 1). This may have been due to the presence of tannins in the eucalypt bark (Vázquez, González-Alvarez, Santos, Freire, & Antorrena, 2009) and in the coffee husk (Barcelos, Paiva, Pérez, Santos, & Cardoso, 2001).

The thermal treatment of the substrates decreased the tannin concentration by 46% (Fig. 1). This result was similar to that observed in vegetables after cooking or autoclaving at 121 °C and 128 °C for different periods of time (Rehman & Shah, 2005).

Regardless of the substrate, tannin degradation by *P. ostreatus* Plo 6 increased as a function of the incubation time, and the highest rate was observed between 15 and 30 d in the substrate with coffee husk and eucalypt bark (Fig. 1). The high degradation rate of this compound was also observed in *Pleurotus* sp. cultivated in coffee husk for 60 d (Fan et al., 2006) and this tannin degradation may be related to tannase activity (tannin acyl hydrolase, EC 3.1.1.20). The

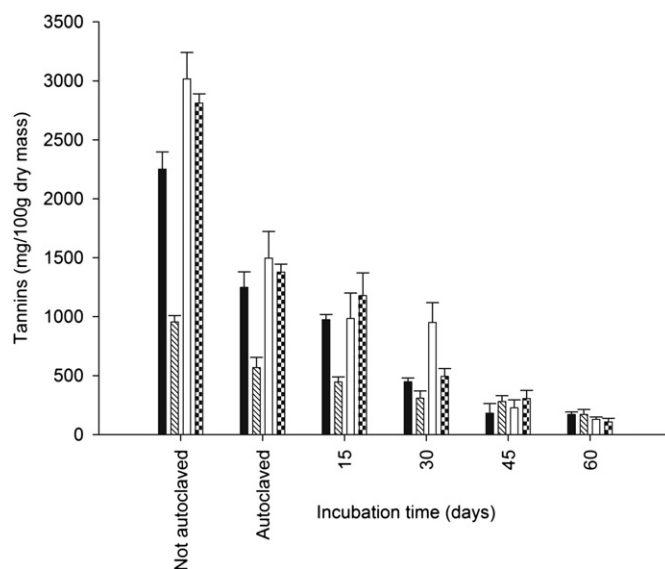


Fig. 1. Tannin degradation for *Pleurotus ostreatus* Plo 6, for 60 days of incubation in substrates with different proportions of *Jatropha curcas* seed cake. (■) = Seed cake; (▨) = Seed cake + 10 (g/100 g) of eucalypt sawdust; (▤) = Seed cake + 10 (g/100 g) of eucalypt bark; (▩) = Seed cake + 30 (g/100 g) of coffee husk + 30 (g/100 g) of rice bran.

activity of this enzyme in the polyphenols degradation has been reported in *Aspergillus* and *Penicillium* (Batra & Saxena, 2005). Thus, *P. ostreatus* can degrade the tannins in *J. curcas* seed cake and the other tested substrates.

The acid phytic content in the jatropha seed cake (Fig. 2A) was lower than the percentage this acid found in the seed of *J. curcas* by Makkar et al. (1998). This result shows that a percentage this acid was in the oil.

Although phytic acid is considered to be heat-stable (Deshpande & Damodaran, 1990), the sterilization of the substrates decreased in 20% the content of phytic acid (Fig. 2A). A 50% decrease in this antinutritional factor was also observed in legumes subjected to autoclaving at 121 °C for 90 min (Rehman & Shah, 2005).

The phytic acid concentration decreased (Fig. 2A) and the inorganic phosphorus percentage increased (Fig. 2B) in function of the incubation time, thus resulting in a negative correlation ($r = 0.84$). The degradation of this acid with phosphorus liberation in the medium was accompanied by phytase activity during incubation time (Fig. 2C). Ullah and Phillippy (1994) showed that phytic acid degradation by phytase can be monitored by changes in the inositol or inorganic phosphate concentrations liberated in the culture medium. The activity of this enzyme caused a 95% decrease of phytic acid in the substrates (Fig. 2A and C). A high degradation rate of this antinutritional factor by microbial phytase was also observed in culture medium containing rapeseed meal that has phytic acid content between 2 to 4 g/100 g of the dry mass (El-Batal & Karem, 2001). The presence of this enzyme was also observed in *Aspergillus* sp. (Ullah & Phillippy, 1994), *Agaricus* sp., *Lentinula* sp. and *Pleurotus* sp. (Collopy & Royse, 2004). Thus, *P. ostreatus* degrades the phytic acid that is present in jatropha seed cake and increases the potential to use this residue in animal feed. Phytase is added to animal feed to increase the mineral bioavailability, e.g. phosphorus, calcium, zinc and iron (Liang et al., 2009). Therefore, phytase production by *P. ostreatus* in *J. curcas* seed cake could make it usable in animal feed.

Thus, these results show the importance of the biological treatment to degrade the toxic compound and antinutritional factors (Figs. 1 and 2) found jatropha seed cake for animal feed. The

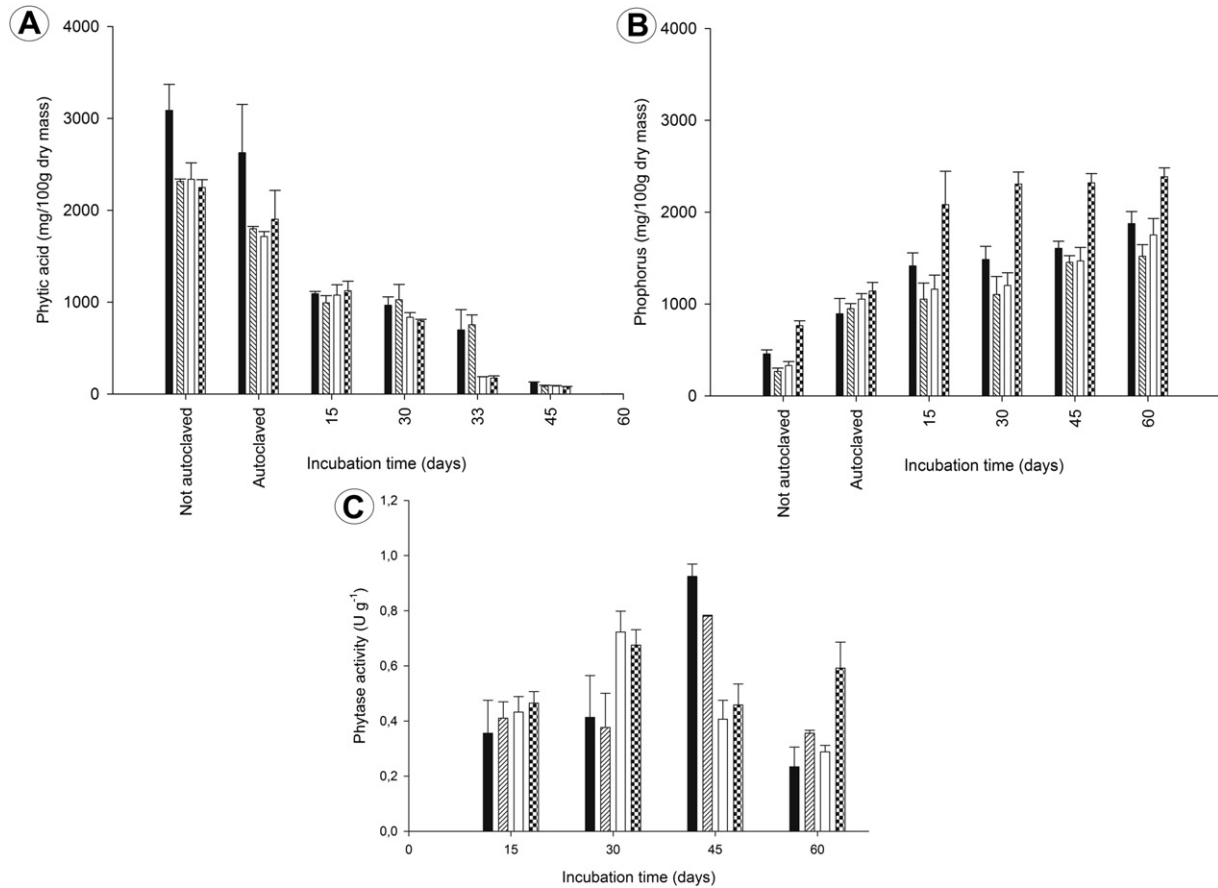


Fig. 2. Phosphorus percentage (A), phytic acid concentration (B) and phytase activity (C) in *Pleurotus ostreatus* Plo 6 mushrooms grown in substrates with different proportions of *Jatropha curcas* seed cake. (■) = Seed cake; (▨) = Seed cake + 10 (g/100 g) of eucalypt sawdust; (□) = Seed cake + 10 (g/100 g) of eucalypt bark; (▩) = Seed cake + 30 (g/100 g) of coffee husk + 30 (g/100 g) of rice bran.

reduction of 99% de phorbol ester was show by the treatment of the jatropa seed cake with *P. ostreatus* for 60 d (Universidade Federal de Viçosa, 2012). Pereira (2011) observed that goats fed during 60 d with different percentage of those substrates (Sc, ScEs, ScEb and ScCh) colonized by *P. ostreatus* increased dry matter intake and weight, without any clinical symptom of intoxication. The author concluded that jatropa seed cake colonized by *P. ostreatus* can be used with safely in up to 20% of dry matter in the diet of goats.

3.2. Mushroom production and chemical analysis

The *P. ostreatus* mushroom production in each substrate was observed after 30, 45 and 60 days of incubation. Biological efficiency was influenced by substrate composition and incubation time (Fig. 3). These influences were also observed in *P. ostreatus* cultivated in coffee husk (de Assunção et al., 2012; Silva et al., 2012) and in different agroindustrial residues (Nunes et al., 2012).

The EB was greater in the substrates with addition of agroindustrial residues than in the pure jatropa seed cake (Fig. 3). This data show the importance of the addition of those residues in the jatropa seed cake to balance the carbon and nitrogen ratio that increase the bioconversion of the substrate in mushrooms (Fig. 3). Furthermore, independent of the addition of agroindustrial residues, the EB was higher than observed in eucalypt sawdust, corncob, eucalypt bark, coffee husk or sugarcane bagasse inoculated with *P. ostreatus* (Nunes et al., 2012). Thus, the contents of phorbol ester and antinutritional factors found in jatropa seed cake do not inhibit the fungal growth and mushrooms production.

Besides, we did not observe any morphological changes in the mushrooms.

The nutritional composition of the mushrooms produced in *J. curcas* seed cake showed that this food is a source of protein,

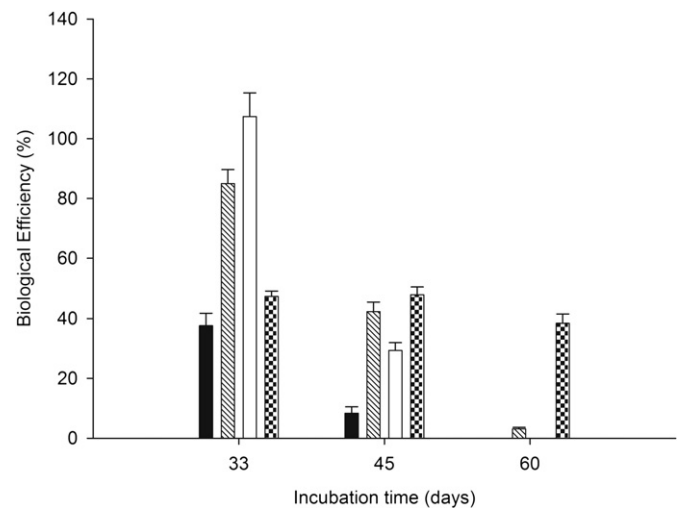


Fig. 3. Biological efficiency of *Pleurotus ostreatus* Plo 6 grown in substrates with different proportions of *Jatropha curcas* seed cake. (■) = Seed cake; (▨) = Seed cake + 10 (g/100 g) of eucalypt sawdust; (□) = Seed cake + 10 (g/100 g) of eucalypt bark; (▩) = Seed cake + 30 (g/100 g) of coffee husk + 30 (g/100 g) of rice bran.

Table 1

Main nutrients and antinutritional factors found in *Pleurotus ostreatus* Plo 6 mushrooms produced in *Jatropha curcas* seed cake.

Compounds ^a	Quantity (mg) per 100 g of fresh mushroom)
Soluble protein	37.27 ± 6.25
Reducing sugars	41.22 ± 7.49
Phosphorus	1.89 ± 0.62
Phytic acid	0.00
Tannins ^b	0.00
Ergosterol	6.04 ± 0.24

^a Analysis done only on mushrooms produced in seed cake.

^b Equivalent at tannin acid.

Table 2

Phorbol ester concentration found in the *Pleurotus ostreatus* mushrooms produced in the *Jatropha curcas* seed cake.

Incubation time (days)	Phorbol ester ($\mu\text{g g}^{-1}$ of fresh mushroom) ^{a,b}
30	0.081A
45	0.009B
60	0.009B

Means with different uppercase letters differ by analysis of variance and Tukey test at 5% probability.

^a Analysis done only on mushrooms produced in seed cake.

^b Equivalent at phorbol-12-myristate-13-acetate.

carbohydrates, phosphorus and ergosterol (Table 1). The contents of these nutrients were similar to those found in *P. ostreatus* mushroom grown in different agroindustrial residues (Dundar et al., 2009; Nunes et al., 2012; Tewari, 1986; Wang et al., 2001). According to Tewari (1986), the mushrooms contain 85 (g/100 g) to 95 (g/100 g) water, 3 (g/100 g) protein, 4 (g/100 g) carbohydrates, and 1 (g/100 g) minerals and vitamins. However, these nutrient contents, mainly the proteins, depend on the substrate composition (Dundar et al., 2009). Potassium, phosphorus, copper, iron and calcium are the main minerals found in mushrooms (Wang et al., 2001). *P. ostreatus* mushrooms are also rich in amino acids, fibers and vitamins, including thiamine, riboflavin, pyridoxine and niacin (Dundar et al., 2009; Wang et al., 2001). The ergosterol content found in the *P. ostreatus* mushroom (Table 1) was greater than the content of this compound observed in commercial mushrooms by Jasinghe and Perera (2005).

In the *P. ostreatus* mushrooms of this study, neither tannins nor phytic acid were detected (Table 1), but low levels of phorbol ester were found. The concentration of this compound decreased in function of incubation time (Table 2). Furthermore, phorbol ester concentration of the mushrooms (Table 2) was around 1000-fold lower than the concentration of this compound found in the non-toxic variety of *J. curcas* (Makkar et al., 1998). This Mexican variety has 0.11 mg/g of phorbol ester and was not toxic to fish, chickens or rats (Makkar et al., 1997). According to these authors, the seeds of this non-toxic variety were typically consumed by humans and chickens. Phorbol ester concentrations of 0.8 mg/g or higher caused appetite loss, diarrhea and reduced motor activity in rats fed with the seed meal *Jatropha* (Rakshit, Darukeshwara, Raj, Narasimhamurthy, Saibaba, & Bhagya, 2008). As phorbol ester content in the mushrooms was from 0.009 to 0.081 $\mu\text{g g}^{-1}$ (Table 2), it could be used as food, even so, we suggest to test in animals to guarantee the food security.

4. Conclusions

In this study we show the potential to use the residue of biodiesel to produce mushroom, a food with high nutritional value. Also, the antinutritional factors degradation allows using this

residue as animal feed, adding economic value and avoiding inadequate disposal in the environment.

Acknowledgments

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