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Biodegradation of hydrocarbons exploiting spent substrate from *Pleurotus ostreatus* in agricultural soils

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In Acatzingo, Puebla, Mexico (east-central), oil spills have mainly affected agricultural fields. *Pleurotus ostreatus* is a white rot basidiomycete and produces extracellular enzymes (lacasses, manganese peroxidases, versatile peroxidases and veratryl alcohol oxidases). The production of edible mushrooms generates spent mushroom substrate that may have a biotechnological application. The aim of this study was to evaluate the mushroom substrate of *P. ostreatus* in a microcosm for the bioremediation of an agricultural soil contaminated with diesel. We evaluated the participation of microbial populations and specific enzymatic lacasses, manganese peroxidases, versatile peroxidases, veratryl alcohol oxidases activities of mushroom substrate in the biodegradation of a soil contaminated with 11030 ppm of diesel in four treatments: E1, E2, E3 and E4. All the experiments were performed in triplicate at 25 and 37°C for 28 days, with a soil:substrate ratio of 4:1. The treatments incubated at 37°C were quantified for diesel-tolerant bacteria, and treatments incubated at 25°C were quantified for diesel-tolerant fungi. Mushroom substrate participated in the biostimulation (91% organic material, 0.56% total nitrogen and 0.3% phosphorus) and bioaugmentation of the microorganisms of the mirocosm. Bacteria-tolerant populations increased significantly (p = 0.000) in all the treatments. Lacasses (8.62 U g^{-1}) activity was stimulated at 25°C and was the only one related to biodegradation; however, the highest biodegradation rate (72%) was at 37°C (bacterial biodegradation) being promising for future research.

Key words: Bioremediation, diesel, laccase, veratryl alcohol oxidase, *Pleurotus ostreatus*.

INTRODUCTION

Mexico is one of the world's important oil producers and the activity has had repercussions on the environment. In Acatzingo, Puebla (east-central Mexico) agricultural soil has been affected by hydrocarbon spills. The region's

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Abbreviations: Lac, Laccases; **MnP,** Manganese peroxidases; **VP,** versatile peroxidases; **VAO,** veratryl alcohol oxidases; **SMS,** spent mushroom substrate; **TPH**, total petroleum hydrocarbons; **PAHs,** polyaromatic hydrocarbons.

main economic activity is farming, producing mainly alfalfa, cabbage, lettuce, corn, nopal, green tomato, prickly pear and carrots.

However, agro-food production and safety has been affected by the dispersion and spillage of recalcitrant pollutants due to the pipelines crossing extensive zones of agricultural fields trans-porting diesel, gasoline, fuel oil and crude oil. The lack of maintenance facilities for the pipelines, fuel theft, vehicular transport and even topographic, orographic and hydrological conditions of the site, cause a high incidence of pollution on agricultural land. These hydrocarbon spills have affected the fertility and structure of agricultural soils, reducing crop yields, and altering biological systems, irrigation systems and the peasant economy in the region (Rivera-Pineda et al., 2012).

Petroleum hydrocarbons are comprised of alkanes (linear or branched), cycloalkanes, polycyclic aromatic hydrocarbons (PAHs) and the most complex heavy fraction such as asphaltenes (Polichtchouk and Yashchenko, 2006). Diesel is a complex mixture of linear (C_{10}-C_{28}), branched and cyclic alkanes and PAHs, which is obtained from the middle distillate during the separation of the petroleum and is recognized for causing health problems (Gallego et al., 2001; Bento et al., 2005) due to its content of benzo[a]anthracene, benzo[a]pyrene and dibenzo[a,h]anthracene which are considered highly toxic and reported as mutagenic and carcinogenic (Das and Chandran, 2011).

Among the different technologies available for the remediation of petroleum hydrocarbons, bioremediation uses living beings such as plants and microorganisms to restore or decontaminate soils, using the enzymatic capacity of bacteria and fungi to mineralize the complex hydrocarbon mixtures (Alexander, 1971).

Ligninolytic fungi like *Phanerochaete chrysosporium*, *Bjerkandera adusta* and *Pleurotus ostreatus*, possess the capacity to express enzymes which are incorporated into the environment to degrade compounds of complex molecular composition, and are known as extracellular. *P. ostreatus* is a white rot basidiomycete; it produces laccases (Lac), manganese peroxidases (MnP), versatile peroxidases (VP) and veratryl alcohol oxidases (VAO) (Bourbonnais and Paice, 1988; Palmieri et al., 2001; Wong, 2009). These enzymes are capable of degrading, and even partially mineralizing, recalcitrant pollutants (dyes, endocrine disruptors, PAHs, halogenated compounds, and various agrochemicals) (Gayosso-Canales et al., 2011).

The isolation of these enzymes in *in vitro* cultures for bioremediation purposes is costly and therefore limits their application. SMS is a source of mycelium and ligninolytic enzymes produced during the growth of the fungus; unused lignocellulosic substrate is inexpensive and readily available (Singh et al., 2011), and its reuse minimizes the adverse impact on the environment (Pardo-Giménez and Pardo-González, 2008). In addition, 62,374 ton of fresh edible mushrooms are produced in Mexico every year, and *P. ostreatus* represents 4.86% of domestic production (Martínez-Carrera et al., 2010). Crude extracts of SMS from white rot fungi have been used for the biodegradation of PAHs (Lau et al., 2003), bleaching of textile dyes (Singh et al., 2010; Singh et al., 2011), and immobilization of heavy metals (Garcia-Delgado et al., 2013), as well as the application of SMS in the biodegradation of PAHs in soil (Eggen, 1999).

In addition, the hydrocarbon degrading enzymes from SMS as well as laccase and manganese peroxidase have been evaluated to biodegrade total petroleum hydrocarbons (TPH) and PAHs. The SMS of mushroom *P. pulmonarius* were evaluated at a concentration of laccases of 1.7-2.0 U mg^{-1} and 880 U g^{-1} and a concentration of manganese peroxidases of 1.8-1.9 U mg^{-1} and 580 U g^{-1}; these enzymes could act directly and immediately to degrade 40% TPH (at 1.2 ±0.2 g kg^{-1} initial concentration) (Chiu et al., 2009), and completely biodegrade naphthalene, phenanthrene, benzo[α]pyrene and benzo[*g,h,i*]perylene (200 ppm of PAHs initial concentration) (Lau et al., 2003).

The aim of this investigation, then, was to evaluate the SMS of *P. ostreatus* under microcosm conditions for the bioremediation of an agricultural soil contaminated with diesel, through microbial characterization, bioaugmentation (microorganisms present in the SMS) and biostimulation (co-substrate of the lignocellulosic material of the SMS).

MATERIALS AND METHODS

Sampling

A non-contaminated soil sample was taken from the following geographic coordinates: 18° 57' 01" N 97° 43' 40" W in the municipality of Acatzingo, Puebla, Mexico. The spent *P. ostreatus* substrate samples were donated by local producers from the Puebla region.

Physicochemical and microbiological characterization in soil and spent mushroom substrate (SMS) of *P. ostreatus*

The physicochemical characterization of the agricultural soil and of *P. ostreatus* SMS was done according to Arshad and Coen (1992).

The microbiological characterization of the soil and substrate was performed using serial dilution and the plate count technique in culture media suitable for quantification of cultivable populations of bacteria, fungi, actinomycetes and diesel-tolerant bacteria (Taylor et al., 2002). The different microorganisms were quantified using differential culture media and different growth conditions, additionally were differentiated morphologically. The bacteria population was quantified by pour plate technique using 0.1 ml aliquots of appropriate dilution into nutrient agar (Bioxon Mexico) plates with pH 7 and incubated at 37°C for 24 h. The fungi populations were quantified by plating out the diluents on potato dextrose agar (Bioxon Mexico) added with streptomycin (0.05 g l^{-1}), pH 5.5 and were incubated at 25°C for five days. The actinomycetes were quantified using congo-red medium (Fisher-Scientific, Mexico), grown at 25°C for five days.

The determination of bacteria diesel-tolerant were grown in basal medium (g l^{-1}): NH_4NO_3, 1; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_47H_2O$, 0.409; $CaCl_2$, 0.02; $FeCl_3$, 0.00005, supplemented with 100 µl of sterile diesel and pH 7.0, incubated at 37°C for 24 h. The microorganisms were reported as Log cfu g^{-1} of dry soil.

Experiments in microcosms to evaluate the enzymatic activities of SMS of *P. ostreatus* in the biodegradation of diesel

An experimental design in microcosm was used to evaluate the participation in microbial populations and enzymatic activities (laccases, veratryl alcohol oxidases, manganese peroxidases, versatile peroxidases) present in the study system. The treatments in the design were: sterile contaminated soil and sterile SMS (E1); sterile contaminated soil and non-sterile SMS (E2); non-sterile contaminated soil and sterile SMS (E3); non-sterile contaminated soil and non-sterile SMS (E4). The soil and SMS were sterilized three times at 10 PSI for 2 h every other day. The soil used was spiked with 11030 ppm diesel and weathered for three months. For each treatment, 120 ml serological flasks were used, with 30 g of the soil-substrate mixture and adjusted to a C:N:P ratio of 100:10:1, the initial values of the organic matter and nitrogen from mixture were used for the adjustment, using sterile solutions of NH_4SO_4 1 N and K_2HPO_4 1 N. Humidity was maintained between 23.8 and 25.6% ± 5.23. The atmosphere of the flasks was changed every third day with an air flow (1.8 ml air/sec) through a sterile membrane (0.22 µm). The treatments were incubated for 28 days at 25 and 37°C. Diesel's biodegradation (%), the initial and final population of bacteria and fungi tolerant to diesel were determined for the treatments incubated at 25 and 37°C respectively. All the treatments were performed in triplicate. The % of biodegradation was determined according to the following equation:

The % of biodegradation = [(initial diesel concentration – final diesel concentration) / initial diesel concentration] * 100.

Microbiological analysis

The quantification of the initial and final population of diesel-tolerant bacteria and fungi was done by direct plate count. The determination of bacteria was done for the treatments incubated at 37°C grown in basal medium (g l^{-1}): NH_4NO_3, 1; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_47H_2O$, 0.409; $CaCl_2$, 0.02; $FeCl_3$, 0.00005; pH 7.0. The determination of fungi was done for the treatments incubated at 25°C using minimal medium with the following chemical composition (g l^{-1}): $(NH_4)_2SO_4$, 7; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_47H_2O$, 0.409; dextrose, 0.1; Sol. E 100x, 100 µl; rose bengal, 0.05; Streptomycin sulfate, 0.05; pH 5.5. diesel, previously sterilized by filtration, was used as carbon source, adding 100 µl over the Petri dish. The microorganisms were reported as Log cfu g^{-1} of dry soil.

Quantification of diesel

Initial and residual diesel was quantified based on the EPA method 8015 C (nonhalogenated organics by gas chromatography) using a gas chromatograph-mass spectrometry (GC-MS Mainframe, HP Model 6890 System-FID) with an HP-5 (30 m x 0.25 mm x 0.25 µm) capillary column, with the following parameters: the injector temperature was 250°C, column was set at an initial temperature of 50°C for one minute followed by a 14°C increment per minute to 275°C and the isothermal held for 8 min. Carrier gas (He) flow was 1.0 ml/min, and makeup N_2 gas was used with a total run time of 25 min.

Quantification of enzymes

An extract was prepared from the sample of the SMS of *P. ostreatus* and from the mixture soil: substrate (5 g) with 20 ml sodium acetate buffer (50 mM, pH 5.0), by shaking for 2 h at 80 rev/min and at 4°C, and then filtered through Whatman 1 paper. The extracts were stored at 4°C to determine the enzymatic activities (Isikhuemhen and Mikiashvilli, 2009).

Lac activity was determined by oxidation of 2,2'-azinobis, 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) at 25°C and read at 420 nm (ε_{420}= 36000 $M^{-1}cm^{-1}$). MnP was determined by the formation of Mn^{3+}-malonate complex at 25°C in malonate buffer (sodium malonate) 50 mM (pH 4.5), with 1 mM $MnSO_4$ as substrate, and 0.1 mM H_2O_2; they were determined at an absorbance of 270 nm (ε_{270}= 11590 $M^{-1}cm^{-1}$). VP activity was monitored by the formation of Mn^{3+}-tartrate complex at 25°C and read at 238 nm (ε_{238}= 6500 $M^{-1}cm^{-1}$) during the oxidation of 0.1 mM Mn^{2+} ($MnSO_4$) in 0.1 M of the sodium tartrate buffer (pH 5), and 0.1 mM H_2O_2. For the determination of VAO, the reaction mixture contained 1 mM veratryl alcohol, 0.25 M sodium tartrate buffer pH 5.0, and was monitored at an absorbance of 310 nm for the formation of veratraldehyde (ε_{310}= 9300 M^{-1} cm^{-1}). Therefore, one unit of enzymatic activity is defined as the amount of enzyme activity that transforms 1 μmol min^{-1} of substrate; the enzymatic activities were reported with specific activities (U g $soil^{-1}$) (Bollag and Leonowicz, 1984; Bourbonnais and Paice, 1988; Palmieri et al., 2001; Gayosso-Canales et al., 2011).

Statistical analysis

A variance analysis (means test) was made to compare the different treatments and Tukey's multiple comparison test ($p \leq 0.05$) using the Minitab statistical package Versión 16.1.0 (licensed to the UPAEP).

RESULTS

Physicochemical characterization and microbiological analysis of non-contaminated soil and spent substrate (SMS) of *P. ostreatus*

The agricultural soil presented a sandy loam texture, moderately alkaline (pH= 8.01), with 2.04% organic material, 0.032% total nitrogen and 0.00256% phosphorus. In addition, the humidity percentage (17.35%) was low compared to the spent mushroom substrate (SMS) of *P. ostreatus* (76.77%). With regard to the characterization of SMS, it presented an acid pH (pH= 5.68) and a high content of organic material (91%), total nitrogen (0.56%) and phosphorus (0.3%) compared to agricultural soil.

Bacteria were the most abundant cultivable microorganisms in the non-contaminated soil and in the SMS (6.02 and 4.8 Log cfu g^{-1}, respectively); however, diesel-tolerant bacteria were reduced in these samples, being more populous in soil (5.05 Log cfu g^{-1}) than in SMS (4.71 Log cfu g^{-1}). The group of actinomycetes in soil was about 2.9 Log cfu g^{-1} and was absent in the SMS, but the group of fungi remained in the same order in these samples (3.45 to 3.68 Log cfu g^{-1}).

The SMS of *P. ostreatus* presented different ligninolytic

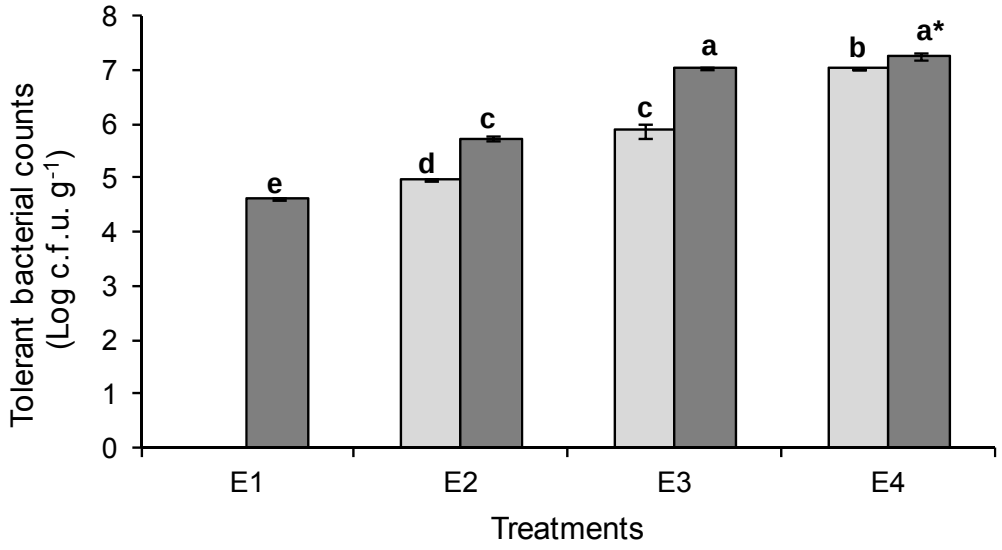

Figure 1. Population dynamics of tolerant diesel bacteria in microcosm using farm soil contaminated with diesel and SMS (4:1): treatments were E1, contaminated sterile soil and sterile substrate; E2, contaminated sterile soil and non sterile substrate; E3, contaminated non sterile soil and sterile substrate; E4, contaminated non sterile soil and non sterile substrate. Values were the mean of triplicate treatments incubated at 37°C at the beginning (light bars) and end (dark bars) of the experiment. *Different letters represent highly significant differences (p = 0.000) between treatments (Tukey's test).

enzymes such as Lac with a specific enzymatic activity of 263 U g^{-1}, VP (5.6 U g^{-1}) and VAO (9.5 U g^{-1}); MnP was not detected.

Evaluation in a microcosm of the enzymatic activities of SMS in the biodegradation of diesel in agricultural soil (11030 ppm)

In the microcosm, the tolerant bacteria increased significantly in all the treatments evaluated, the highest being E4 with 7.26 Log cfu g^{-1} (Figure 1). In contrast, the tolerant fungi populations presented a significant reduction (p = 0.000) according to system conditions and ranged from 2.82-5.22 Log cfu g^{-1} (Figure 2). However, biodegradation at 25°C was significantly lower (p = 0.000) than at 37°C, and E4 achieved the highest biodegradation of diesel (72%) (Table 1).

In the evaluation of extracellular enzymes in the microcosm, Lac activity was significantly stimulated (p = 0.000) in treatments E2, E3, and E4 with contaminated soil (11030 ppm diesel) at 25°C (0.92, 7.62 and 8.62 U g^{-1}, respectively) (Figure 3); no activity was detected in treatments at 37°C. VAO activity was also significantly stimulated (p = 0.000) at 25 and 37°C. The highest VAO activity was in treatment E4 at 25°C with 0.55 U g^{-1}, and at 37°C with 0.83 U g^{-1} (Figures 4 and 5). Manganese peroxidase and versatile peroxidase were undetectable in any treatment. In treatment E1 (sterile soil and sterile SMS) no enzymatic activity was detected, as shown in Figures 3, 4 and 5; but the tolerant bacterial and fungal population was stimulated at the end of the experiment with 4.63 and 2.82 Log cfu g^{-1} respectively (Figures 1 and 2).

DISCUSSION

The agricultural soil from Acatzingo, Puebla, Mexico presented a low nutritional content that is inadequate for farming activity according to Arshad and Coen (1992), and the SMS, being composed mainly of wheat straw, contains high doses of total nitrogen (0.56%) and organic material (91%); the physicochemical characteristics vary depending on the origin of the substrate and can contribute from 0.11 to 10.85% total nitrogen and 18.66 to 51.00% organic carbon for the development of the mushroom according to López-Rodrigez et al. (2008). The total nitrogen (0.56%), organic carbon (52.78%) and phosphorus (0.3%) content of the SMS of *P. ostreatus* was similar to that reported by García-Delgado et al. (2013) with 42% organic carbon and 0.7% total nitrogen.

The role of SMS, besides being a soil texturizer, is as co-substrate because of the incorporation of C, N and P, necessary for microbial metabolism whereby the treatment E3 increased native populations of tolerant diesel bacteria. In addition, the SMS of *P. ostreatus* bioaugmented the diesel-tolerant bacterial populations to 5.74 Log cfu g^{-1} in treatment E2 (Figure 1). Chiu et al. (2009) reported similar results during a bioremediation

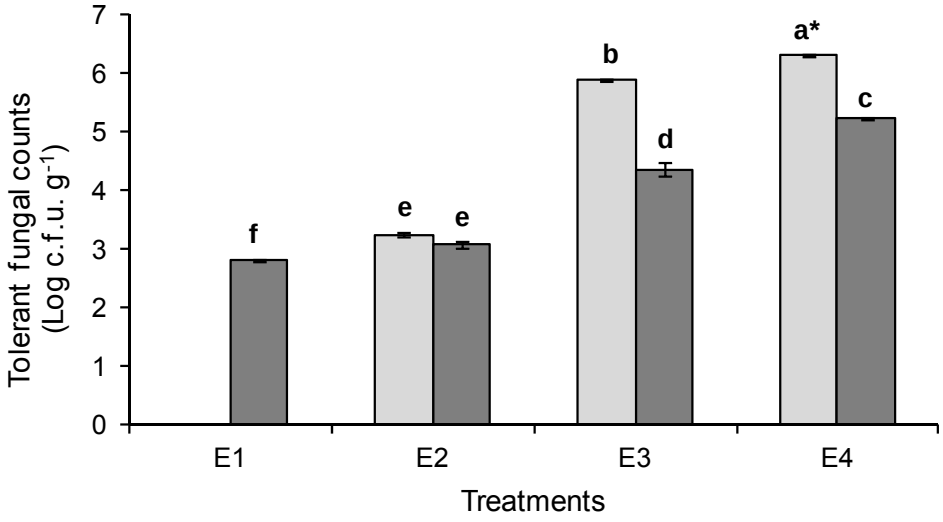

Figure 2. Population dynamics of tolerant diesel fungus in microcosm using farm soil contaminated with diesel and SMS (4:1), treatments were E1, contaminated sterile soil and sterile substrate; E2, contaminated sterile soil and non sterile substrate; E3, contaminated non sterile soil and sterile substrate; E4, contaminated non sterile soil and non sterile substrate. Values were the mean of triplicate treatments incubated at 25°C at the beginning (light bars) and end (dark bars) of the experiment. *Different letters represent highly significant differences (p = 0.000) between treatments (Tukey's test).

Table 1. Evaluation of diesel´s biodegradation in microcosm using farm soil contaminated and SMS (4:1).

Treatment	Temperature (°C)	Residual diesel (mg/kg)[1, 2]	± SD (mg/kg)	Diesel's biodegradation (%)	*F*-value
E1	25	10044[a]	30.3	8.9	3442.77
E1	37	9368.1[b]	56.2	15.1	
E2	25	7012.6[c]	66.6	36.4	
E2	37	4835.4[d]	49.9	56.2	
E3	25	4776.6[d]	40.2	56.7	
E3	37	3454.6[e]	79.6	68.7	
E4	25	3695.0[e]	37.6	66.5	
E4	37	3086.2[f]	117.5	72.0	

[1]Each value represents the average of triplicate with initial concentration of 11030 ppm diesel, treatments: E1, contaminated sterile soil and sterile substrate; E2, contaminated sterile soil and non sterile substrate; E3, contaminated non sterile soil and sterile substrate; E4, contaminated non sterile soil and non sterile substrate. [2]Different letters in the column indicate statistically significant differences according to the Tukey test (p = 0.000). SD, Standard deviation.

process when using SMS of *P. pulmonarius;* the substrates contributed macronutrients for the biostimulation of microbial populations giving, in addition, a bioaugmentation effect. Furthermore, Martens and Zadrazil (1998) suggest that organic substrates contribute nutrients that stimulate native microflora in the soil through products formed by the lysis of such substrates increasing degradation rates.

In this work, a reduction in the fungal population (Figure 2) was observed at the end of the experiment, associated with less biodegradation of diesel. Despite evidence of the biodegradation of contaminants by native fungi (Pérez-Armendáriz et al., 2010), the metabolic activity of native microorganisms is unpredictable; it can be affected by, among other things, the presence of the contaminant (Bento et al., 2005). However, the bacterial populations were significantly stimulated (p = 0.000) (Figure 1) and the highest biodegradation obtained (72%) (Table 1). Under the conditions studied in this work, the bacteria showed more ability to biodegrade diesel compared to fungi. It is possible that bacterial metabolism, the system of oxidation by monooxygenase and dioxygenase enzymes is more efficient than that of fungal enzymes (Das and Chandran, 2011). The biodegradation of diesel in treatment E1 (Table 1) was due to the loss and chemical degradation of diesel, and to the microorganisms

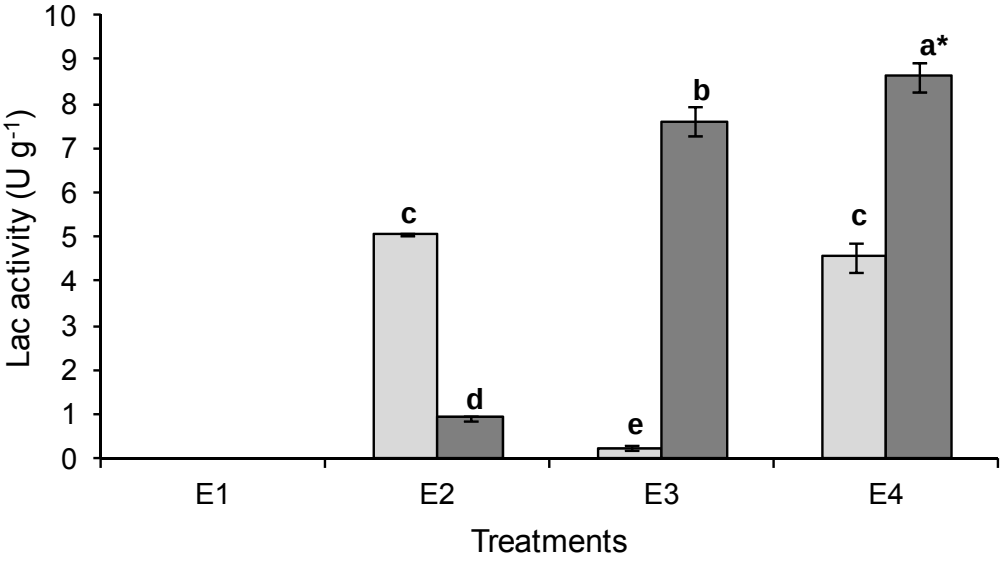

Figure 3. Laccase activity in microcosm using farm soil contaminated with diesel and SMS (4:1). Treatments correspond E1, contaminated sterile soil and sterile substrate; E2, contaminated sterile soil and non sterile substrate; E3, contaminated non sterile soil and sterile substrate; E4, contaminated non sterile soil and non sterile substrate. Values were the mean of triplicate treatments incubated at 25°C at the beginning (light bars) and end (dark bars) of the experiment. *Different letters represent highly significant differences (p = 0.000) between treatments (Tukey's test).

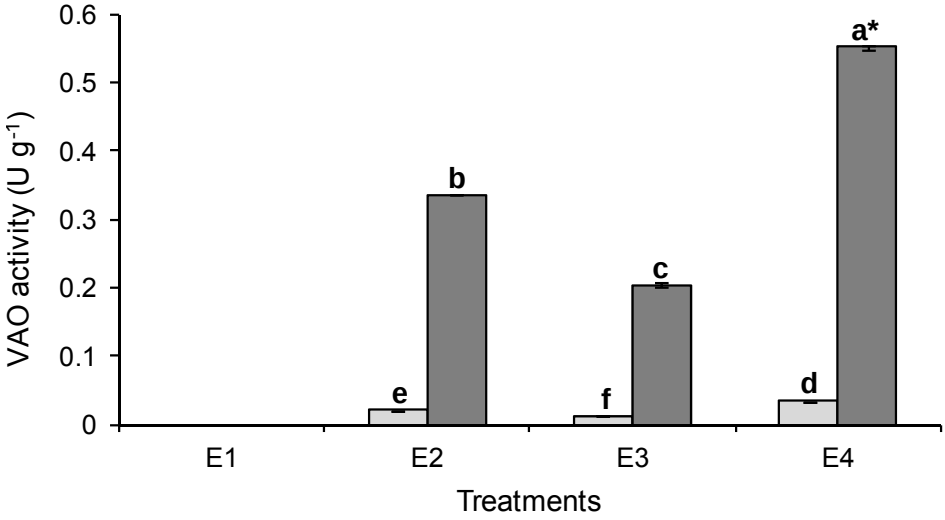

Figure 4. Veratryl alcohol oxidase activity in microcosm using farm soil contaminated with diesel. Treatments correspond E1, contaminated sterile soil and sterile substrate; E2, contaminated sterile soil and non sterile substrate; E3, contaminated non sterile soil and sterile substrate; E4, contaminated non sterile soil and non sterile substrate. Values were the mean of triplicate treatments incubated at 25°C at the beginning (light bars) and end (dark bars) of the experiment. *Different letters represent highly significant differences (p = 0.000) between treatments (Tukey´s test).

that withstood the soil sterilization process (Figures 1 and 2). With the results obtained, the SMS of *P. ostreatus* enabled high (72%) percentages of diesel biodegradation (11030 ppm initial concentration) at 37°C for 28 days (Table 1) which is promising for an escalation and subsequent physiological studies, since the application

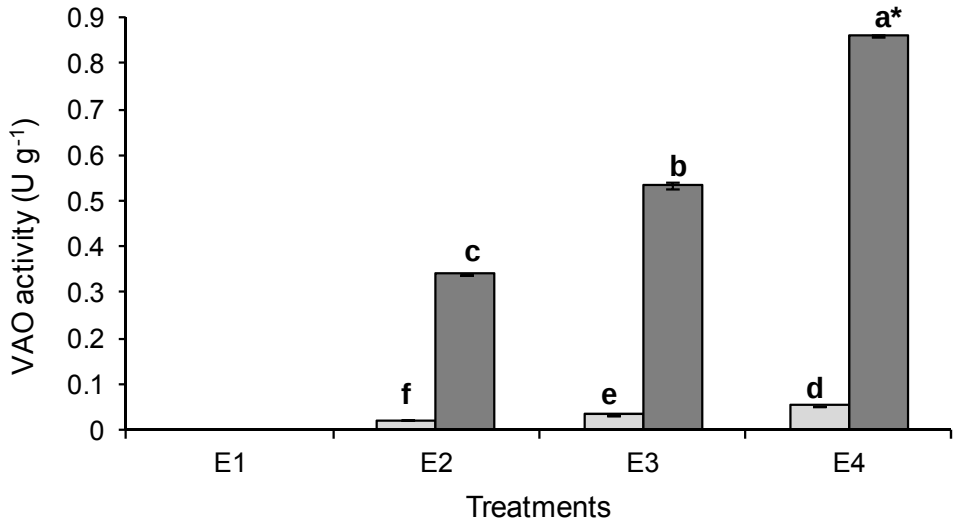

Figure 5. Veratryl alcohol oxidase activity in microcosm using farm soil contaminated with diesel. Treatments correspond E1, contaminated sterile soil and sterile substrate; E2, contaminated sterile soil and non sterile substrate; E3, contaminated non sterile soil and sterile substrate; E4, contaminated non sterile soil and non sterile substrate. Values were the mean of triplicate treatments incubated at 37°C at the beginning (light bars) and end (dark bars) of the experiment. *Different letters represent highly significant differences (p = 0.000) between treatments (Tukey's test).

of other SMS (*P. pulmonarius*) (3%) achieve the biodegradation of 40-45% of TPH (1200 ppm initial concentration) for 22 days in *ex situ* treatments (Chiu et al., 2009).

In addition, the SMS of *P. ostratus* was a source of ligninolytic enzymes (263 U g^{-1} Lac, 5.6 U g^{-1} VP and 9.5 U g^{-1} VAO) for the biodegradation of diesel in the microcosm. Singh et al. (2003) reported 7.59 U g^{-1} Lac in SMS of *P. sajor-caju*. Furthermore, Lau et al. (2003) reported Lac activities of 880 ± 40 U g^{-1} and of MnP 580 ± 20 U g^{-1} in SMS of *P. pulmonarius* to be higher than our results, and when applied (5% of SMS) to a contaminated soil (200 ppm of PAHs), presented a biodegradation of 100% after two days under constant agitation (at 80°C). Law et al. (2003) reported 89% biodegradation of pentachlorophenol (100 mg l^{-1} initial concentration) in water system by the SMS of *P. pulmonarius* (5%) after two days of incubation at 250 rev/min in darkness at room temperature. Also, enzymatic extracts from SMS of *A. bisporus* (0.43 ± 0.02 U ml^{-1} Lac) have been used *in vitro* to evaluate the biodegradation of PAHs in the presence of heavy metals (Cd or Pb), which affected degradation with a reduction in Lac activity (García-Delgado et al., 2013). In our study, the application of the SMS of *P. ostreatus* to a contaminated agricultural soil (11030 ppm diesel) contributed extracellular enzymes like Lac (0.92 U g^{-1}) and VAO (0.34 U g^{-1}) in the microcosm (Figures 3, 4 and 5) which suggests that they are part of the bioremediation process.

Lignocellulosic residues also have the role of cosubstrate since they stimulate microbial activity in general (Eggen, 1999); in our study, it was observed that the SMS increased the Lac production 30.85 times and the VAO production 15.83 times (Figures 3 and 5). The highest Lac activity (8.62 U g^{-1}) was at 25°C (E4) with a high diesel biodegradation of 66.5% (Table 1) suggesting Laccase stimulation due to the presence of the contaminant; similar results were reported by Eggen (1999) for a soil contaminated with creosote, showing the Lac activity of 0.57 U g^{-1}. Other studies conducted with different enzymatic extracts of SMS of different basidiomycetes (*Agaricus bisporus*, *P. eryngii, P. ostreatus* and *Coprinus comatus*) have presented the same effect (Li et al., 2010). In addition, in *in vitro* cultures of strains of *P. eryngii, P. ostreatus, P. pulmonarius* and *P. sajor-cajur,* Lac and VP activities were related to biodegradation (Rodríguez et al., 2004).

The stimulation of Lac expression in soils contaminated with recalcitrant substances for *P. ostreatus* has been widely reported; Gayosso-Canales et al. (2011) showed that this basidiomycete was producing 19.1 U mg of Lac $protein^{-1}$ when polychlorinated biphenyls were present in soil.

An interesting result in this work was the concentration of VAO at 37°C (0.83 U g^{-1}), also found in contaminated soils at 25°C (0.55 U g^{-1}) (Figures 4 and 5). These results suggest that the expression of VAO enzyme can be by both bacteria and fungi, due to the presence of intermediary compounds like veratrylic alcohol, resulting from the biodegradation of lignocellulosic substrates and

hydrocarbons. VAO is an enzyme that oxidizes two O_2 electrons to H_2O_2 for the formation of a free radical followed by the loss of a proton, in lignin biodegradation with characteristics similar to the majority of peroxidases but with the ability to biodegrade aromatic alcohol compounds such as veratrylic alcohol (Bourbonnais and Paice, 1988).

The presence of VAO has been reported *in vitro* in fungi such as *P. sajor-caju*, *P. ostreatus*, *P. cornucopiae*, *P. eryngii*, *P. floridanus*, and *P. pulmonarius* (Bourbonnais and Paice, 1988; Gutiérrez et al., 1994; Marzullo et al., 1995), and in the unicellular bacteria *Comamonas* sp*.,* VAO has been purified with the ability to decolorize textile dyes (Jadhav et al., 2009; Chen et al., 2012).

In conclusion, this study investigates different factors associated with the use of spent substrate of *Pleurotus ostreatus* in the bioremediation of an agricultural soil contaminated with diesel. The best treatment was obtained with the non-sterile mixture at 11030 ppm diesel with 72% biodegradation, evaluated after 28 days of incubation at 37°C. Therefore, the SMS of *P. ostreatus* contributed extracellular enzymes such as Lac (0.92 U g^{-1}) and VAO (0.34 U g^{-1}) in the microcosm system used. The SMS also functioned as a biostimulant agent by contributing the residues of ligninolytic origin increasing the production of Lac (30.85 times) and VAO (15.83 times) and as biostimulant by increasing the number of cfu of microorganisms in the bioremediation system.

Conflict of Interests

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

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