

# Catalase and Phosphatase Activities During Hydrocarbon Removal from Oil-Contaminated Soil Amended with Agro-Industrial By-products and Macronutrients

María del Carmen Cuevas-Díaz · Ángeles Martínez-Toledo · Oswaldo Guzmán-López · Cinthya P. Torres-López · Areli del C. Ortega-Martínez · Lizbeth J. Hermida-Mendoza

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**Abstract** Microbiological activities are essential in the bioremediation of polluted soils. The enzymatic activities of microorganisms are usually used as a biological indicator of soil health. The aim of this work was to observe the catalase, acid phosphatase (AcP), and alkaline phosphatase (AIP) activities in soil that was amended with agro-industrial by-products and macronutrients during the process of total petroleum hydrocarbon (TPH) removal. To this end, microcosm tests were performed with soil and agro-industrial by-products ratios of 100:2:2, for soil:sugarcane bagasse pith:filter cake mud (SSF); 100:2, for both soil:sugarcane bagasse pith (SS); and for soil filter cake mud (SF). The macronutrients—carbon, nitrogen, and phosphorus—in the experimental treatments were adjusted to 100:10:1 with a solution of  $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$ . The best TPH removal (51.4%) was obtained with SSF at 15 days. In addition, a significant

correlation was observed between TPH removal and AIP as well as AcP ( $r = 0.74$ ,  $p < 0.0001$ ;  $r = 0.70$ ,  $p < 0.0107$ , respectively). Fungi growth was also correlated with both AIP ( $r = 0.97$ ,  $p < 0.0001$ ) and AcP ( $r = 0.95$ ,  $p < 0.0001$ ) activities. Besides, bacterial and fungi growth showed a correlation with TPH ( $r = 0.86$ ,  $p < 0.001$ ;  $r = 0.77$ ,  $p < 0.0034$ , respectively). It could be said that the agro-industrial by-products and macronutrients contributed to pollutant removal from the oil-polluted soil at relatively short amount of time. In addition, the enzymatic activities were increased after the treatment; in this study, the high sensitivity enzyme was AIP, and it could be used as an indirect indicator of oil pollutant removal.

**Keywords** Biological indicator · Bioremediation · Filter cake mud · Sugarcane bagasse pith · Total petroleum hydrocarbons

M. del Carmen Cuevas-Díaz · O. Guzmán-López ·  
C. P. Torres-López · A. del C. Ortega-Martínez ·  
L. J. Hermida-Mendoza  
Faculty of Chemical Sciences, University of Veracruz, Avenida  
Universidad Km. 7.5, Coatzacoalcos, Veracruz, Mexico

M. del Carmen Cuevas-Díaz  
Institute of Biotechnology and Applied Ecology, University of  
Veracruz, Av. de las Culturas Veracruzanas 101, Col. E. Zapata,  
CP. 91090 Xalapa, Veracruz, Mexico

Á. Martínez-Toledo (✉)  
Faculty of Engineering, Autonomous University of San Luis  
Potosí, Av. Dr. Manuel Nava No. 8, Zona Universitaria, 78290 San  
Luis Potosí, SLP, Mexico  
e-mail: anmartin\_70@hotmail.com

## 1 Introduction

In recent years, Mexico has been subject to a range of soil, air, and water pollution problems across different ecosystems. This pollution could have been related to either organic or inorganic compounds. Various industrial activities involve the use or processing of petrochemicals, which has caused soil pollution problems due to accidental spills worldwide. Such problems also occur in the state of Veracruz, located in southeastern Mexico (PEMEX 2006). Oil pollution modifies the

physicochemical and biological characteristics of the soil (Wyszkowska et al. 2002).

Veracruz is an important producer of agro-industrial products including 16,674,866 t year<sup>-1</sup> of sugarcane (*Saccharum officinarum*), 1,707,170 t year<sup>-1</sup> of orange (*Citrus sinensis* L.), and 304,231 t year<sup>-1</sup> of coffee cherries (*Coffea arabica* L.) (INEGI 1998). However, these industrial activities also generate large amounts of waste that have a potential use within those industries. Every ton of sugarcane processed produces 340 kg of sugarcane bagasse pith and 35 kg of filter cake mud. A small portion of sugarcane bagasse pith has been used for energy production (as boiler fuel) and to improve the soil agro-industrial characteristics (Serratia et al. 1990; Benedicto-Valdés et al. 2005). These studies showed that the agro-industrial by-products are useful as amendments and texturizers in oil-polluted soil (soils that have received chronic contamination) to enhance pollutant removal.

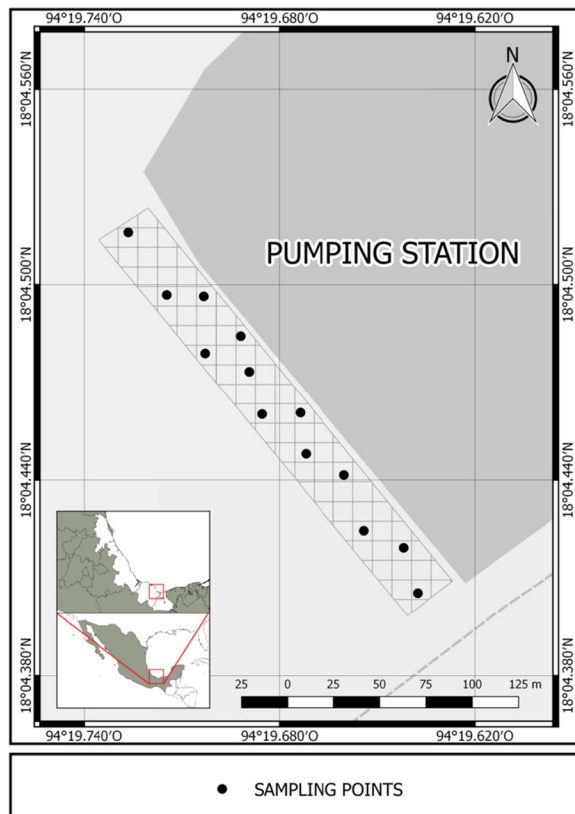
Enzyme activities in soil have been used as a biological indicator of contamination from metals, pesticides, and hydrocarbons (Karaca et al. 2011). Studies have shown the potential of soil enzymes in evaluating the impact of hydrocarbons and fertilizers on soil microorganisms, indicating that soil enzymes represent a useful tool for the early detection and monitoring of soil contamination (Margesin et al. 2000a; Singh et al. 2009; Megharaj et al. 2011; Singh and Ghoshal 2013). While enzymes are primarily of microbial origin, they may also originate from animal or vegetable remains. They may be found under diverse conditions ranging from those associated with living organisms (endoenzymes) to enzyme complexes immobilized by clay and humic substances (exoenzymes or abiotic components) (Paolini 2003; Šarapatka 2003; Burns et al. 2013). These activities are closely related to parameters such as carbon (C) and nitrogen (N) contents, C and N from the microbial biomass, and N mineralization. Metabolic activity in soil is responsible for important processes such as mineralization and humification of organic matter (OM), which in turn will affect a series of processes involving fundamental elements such as C, N, phosphorus (P), and sulfur (S) (García et al. 2003; García-Ruiz et al. 2008). All these reactions can be measured by enzymes that are specific to each substrate. Soil enzymes that have been more widely studied include oxidoreductases (particularly dehydrogenase,

peroxidase, and catalase) and hydrolases (phosphatase, protease, and urease). These play an important role in the soil environment and the C, N, P, and S cycles (García et al. 2003; Cadwell 2005). In this study, the enzyme phosphatase (phosphomonoesterases: acid (EC 3.1.3.2) and alkaline (EC 3.13.1)) and catalase (EC 1.11.1.6) activities in soil were measured to evaluate the improvement of soil health as a result of biostimulation treatment. Phosphatases are good indicators of soil fertility, and these enzymes play critical roles in P cycles because they have been correlated to P stress (Bakshi and Varma 2011). Catalase is an intracellular enzyme in all aerobic bacteria and in most facultative aerobes, and is absent in obligate anaerobic bacteria, facilitating its use as an indicator of the microbial activity in soil and the soil fertility (García et al. 2003). The objective of this study was to remove total petroleum hydrocarbons (TPH) from oil-contaminated soil amended with macronutrients and agro-industrial by-products (sugarcane bagasse pith and filter cake mud) and to monitor phosphatase and catalase activities as a biological indicator of enhanced pollutant removal throughout the experimental period.

## 2 Materials and Methods

### 2.1 Soil Sampling and Obtaining Agricultural By-products

The sampling site was located in Nuevo Teapa in the state of Veracruz, Mexico (18°04' N and 94°19' W) at 80 m elevation. The weather is wet with mean annual temperature of 27 °C and an annual average precipitation of 2456 mm. The types of soil are Acrisol and Vertisol, characterized by the accumulation of clay in the subsoil and its susceptibility to erosion (INAFED 2013). Samples of oil-polluted soil (Fig. 1) were collected at 13 random points in the A1 horizon at depths of 0–30 cm (Mason 1992), and the distance to the contamination source (Industrial activity) was approximately 20–30 m. The filter cake mud and sugarcane bagasse pith were provided by the Cuatrotolapan Sugar Plant located in Hueyapan of Ocampo, Veracruz.



**Fig. 1** Soil sampling map

## 2.2 Soil and Agricultural By-product Analysis

The pH was electronically determined using an Orion 420A potentiometer, USA, using epoxy electrodes; humidity was determined by the gravimetric method as established by NOM-021 (DOF 2002); and OM was determined by the method developed by Walkley and Black in 1949 (Muñoz-Iniestra et al. 2000; DOF 2002). Total N determinations were performed with the Micro-Kjeldahl method, as adapted by Bremner in 1965 (Muñoz-Iniestra et al. 2000; DOF 2002). Available P was determined using the Bray method, as reported in NOM-021 (DOF 2002). All these tests were performed in triplicate and the reagents blank and standards (ACS reagent of Sigma-Aldrich (Sigma, St. Louis, MO, USA)) were used for calibration.

## 2.3 Microbial Analysis

The soil was crushed with a mortar and pestle, passed through a standard sieve of 2-mm mesh size, and kept

at 4 °C in sterile amber glass bottles until its characterization and analysis (Paetz and Wilke 2005). Bacterial growth was evaluated using the plate counting method (Clark 1965) in triplicate. Bacterial groups were analyzed through plating on nutritive agar at 37 °C for 48 h. The Parkinson method (Parkinson et al. 1971) was used to determinate the fungi groups; a  $10^{-2}$ – $10^{-4}$  dilution was performed, and samples were plated on potato-dextrose-agar at 28 °C for 4 days, with rose bengal as the indicator and chloramphenicol as the bactericide. The microorganisms were reported as colony-forming units (cfu) per gram of dry soil ( $\text{g dm}^{-1}$ ). All chemicals (ACS reagent) were purchased from Sigma-Aldrich.

## 2.4 Hydrocarbon Extraction and Quantification

The micro Soxhlet extraction method (USEPA 2002) was employed to obtain the TPH concentrations. The test was conducted in triplicate for each sample. In brief, 1 g of soil with 2 g of  $\text{Na}_2\text{SO}_4$  was placed in a cartridge and dichloromethane was used as a solvent (50 mL), at a constant reflux (60 °C) for 4 h. After that, the solvent was evaporated to near dryness in a rotary evaporator, the organic extraction was kept in a weighed amber flask with a Teflon lid, and then the solvent was evaporated until dryness using a nitrogen stream. The final weight of the flask represented the weight fraction of TPH concentrations (DOF 2006). All chemicals (ACS reagent) were purchased from Sigma-Aldrich.

## 2.5 Enzymatic Assays

### 2.5.1 Catalase Activity (EC 1.11.1.6)

The Johnson and Temple (1964) method (García et al. 2003) was used to determinate the catalase activity in the soil. This involved adding 40 mL of distilled water to 1 g of soil (2-mm mesh size) and shaking for 30 min, then 5 mL of a 1:100 dilution of  $\text{H}_2\text{O}_2$  in distilled water was added and shaken for 30 min at 20 °C, followed by the addition of 5 mL of 1.5 M  $\text{H}_2\text{SO}_4$  to stop the enzymatic activity. The solution was filtered and a 25 mL aliquot was evaluated with 0.01 M  $\text{KMnO}_4$ . Control tests were processed in the same manner as the samples but the 5 mL of  $\text{H}_2\text{O}_2$  was replaced by distilled water. A blank was conducted with a mix of 40 mL of distilled water, 5 mL of  $\text{H}_2\text{O}_2$ , and 5 mL of 1.5 M  $\text{H}_2\text{SO}_4$ , and 25 mL

of this mixture was evaluated with  $\text{KMnO}_4$ . The assay was carried out in triplicate for each sample. All substrates (ReagentPlus®) and chemicals (ACS reagents) were purchased from Sigma-Aldrich.

### 2.5.2 Phosphatase Activity: Alkaline (EC 3.13.1) and Acid (EC 3.1.3.2)

Phosphatase activity was determined using the Tabatabai and Bremner (1969) method. In brief, 1 g of soil (2-mm mesh size) was added to MUB<sup>1</sup> (4 mL) buffer solution (with a pH 6.5 or pH 11, for acid phosphatase or alkaline phosphatase, respectively) and p-nitrophenyl phosphate (p-NP; 1 mL) 0.025 M, and shaken for 1 min at 120 rpm. Next, the sample was incubated for 1 h at 37 °C and the reaction was stopped by placing the sample in a cool bath at 4 °C for 5 min. Following this, 1 mL of  $\text{CaCl}_2$  0.5 M and 4 mL of NaOH 0.5 M were added and this was shaken at high speed for 1 min. The sample was then promptly filtered with Whatman no. 42 filter paper. Finally, the absorbance was measured with a UV-vis spectrophotometer at 400 nm. A calibration curve was constructed with p-NP between 0 and 250  $\mu\text{g mL}^{-1}$ ; 1 mL of each dilution was mixed with 4 mL of distilled water, 1 mL of  $\text{CaCl}_2$  0.5 M, and 4 mL of NaOH 0.5 M; then filtered and the absorbance was measured. Control tests were processed with the same procedure as samples but the p-NP was added after the addition of  $\text{CaCl}_2$  0.5 M and NaOH 0.5 M. This assay was performed in triplicate for each sample. All substrates (ReagentPlus®) and chemicals (ACS reagent) were purchased from Sigma-Aldrich.

## 2.6 Experimental Procedure

A microcosm test was performed with 10 g of soil and agro-industrial by-products were added at a soil:by-product ratio of 100:2 for soil:sugarcane bagasse pith (SS) and soil:filter cake mud (SF) and 100:2:2 for soil:sugarcane bagasse pith:filter cake mud (SSF). The moisture was adjusted to 60% of holding capacity. Next, the physicochemical analyses were conducted and the nutrients were adjusted with a solution of  $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$  to obtain a C:N:P ratio of 100:10:1. All the

<sup>1</sup> Modified universal buffer (MUB) consisted of 3.025 g trishydroxymethylaminomethane, 2.9 g maleic acid, 3.5 g citric acid, 1.57 g boric acid, 1 M sodium hydroxide (NaOH) solution (122 mL), and distilled water added to give a final volume of 250 mL (Skujins et al. 1962).

treatments were conducted in triplicate. These conditions were established according to previous reports of the optimal conditions to remove hydrocarbons from oil-contaminated soil using macronutrients and agro-industrial by-products (García-Torres et al. 2011; Antonio-Ordaz et al. 2011). The four experimental treatments are described in Table 1. The study was performed for 15 days at 28 °C and samples were taken on days 5, 10, and 15. Aeration was conducted every 72 h under sterile conditions. Moreover, experimental controls were soil that was sterilized with  $\text{N}_3\text{Na}$  at 5% (sterile soil (S)) and soil without treatment (control soil (C)). All chemicals (ACS reagent) were purchased from Sigma-Aldrich.

## 2.7 Statistical Analyses of Data

A one-way analysis of variance and Pearson's correlation with a 0.05 significance level and three replicates ( $n = 3$ ) were performed using Statistical Analysis Systems (SAS) software for Windows version 9.

## 3 Results

### 3.1 Soil and By-product Characteristics

Table 2 lists the physicochemical characteristics of the soil, the agricultural by-products, and the initial enzymatic activity. The initial pH (8.02) of the soil was good for the bioremediation process (Gómez-Romero et al. 2008). Nevertheless, the initial OM of the soil was less than 3%, also, initial N and P were 0.39% and 0.76  $\text{mg kg}^{-1}$ , respectively, and these conditions limited the microbial activity in the soil (5.8 Ln cfu  $\text{g dm}^{-1}$  of bacteria and 7.0 Ln cfu  $\text{g dm}^{-1}$  of fungi, both of them at initial conditions). The initial enzymatic activities in

**Table 1** Soil experimental treatments

Treatment	Soil/by-products ratio	Macronutrients	
		$\text{NH}_4\text{NO}_3$ (g)	$\text{KH}_2\text{PO}_4$ (g)
SSF	100:2:2	0.07	0.02
SS	100:2	0.07	0.04
SF	100:2	0.10	0.07
C	100	0.00	0.00

SSF soil/sugarcane bagasse pith/filter cake mud, SS soil/sugarcane bagasse pith, SF soil/filter cake mud, C control soil

**Table 2** Physicochemical characteristics of soil and agro-industrial by-products

Parameter	Soil	Filter cake mud	Sugarcane bagasse pith
pH	8.02	8.20	5.80
Organic matter (%)	2.82	93.43	97.40
Nitrogen (%)	0.39	0.79	0.23
TPH (mg kg <sup>-1</sup> )	55, 954	ND	ND
Available phosphorus (mg kg <sup>-1</sup> )	0.76	44.90	5.22
Humidity (%)	36.50	0.96	78.59
AcP activity (μmol p-NP g <sup>-1</sup> h <sup>-1</sup> )	0.17	ND	ND
AIP activity (μmol p-NP g <sup>-1</sup> h <sup>-1</sup> )	0.39	ND	ND
Catalase activity (μmol H <sub>2</sub> O <sub>2</sub> g <sup>-1</sup> h <sup>-1</sup> )	0.19	ND	ND
Texture	50.8% sand, 27.2% clay, and 22% silt		

ND no determined

the soil were as follows: catalase 0.19 μmol H<sub>2</sub>O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup>, acid phosphatase (AcP) 0.39 μmol p-NP g<sup>-1</sup> h<sup>-1</sup>, and alkaline phosphatase (AIP) 0.17 μmol p-NP g<sup>-1</sup> h<sup>-1</sup>. Humidity measurements remained constant throughout the experimental period in all the experimental units, and this condition supported microbial activity in the soil. The pH ranged from 6.60–7.35 in all experimental treatments with these conditions favoring the bacterial growth.

### 3.2 TPH Removal, Microbial Progress, and Enzymatic Activities

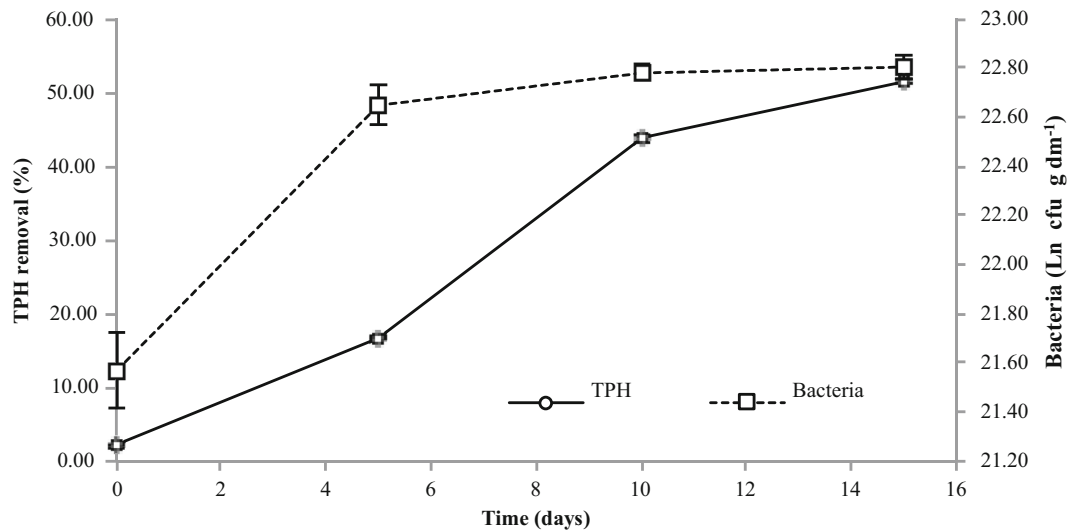
The results of fungal growth, bacterial growth, pH, OM, TPH, N, P, and enzymatic activities of the experiments are presented in Table 3. The initial concentration of the TPH in the soil was 55,954 mg kg<sup>-1</sup>; the SSF treatment showed that the best TPH removal was at 51.4% (Fig. 2) and the second-best result (46.4%) was obtained with the SS treatment and the lowest TPH removal (34.2%) was obtained with the SF treatment. Bacteria played an important role in the bioremediation process, indicating that they were more reactive than the fungi population. Nevertheless, the bacterial and fungal growth were observed to have similar significant correlations with TPH removal (Table 4): for SSF treatment, the bacteria and fungi correlations were  $r = 0.86$ ,  $p < 0.0003$  and  $r = 0.77$ ,  $p < 0.0034$ , respectively; for SS, the correlations were  $r = 0.80$ ,  $p < 0.0017$  and  $r = 0.75$ ,  $p < 0.005$ , respectively; and for SF were  $r = 0.82$ ,  $p < 0.001$  and  $r = 0.81$ ,  $p < 0.0013$ , respectively. For treatments with SSF and SS, the TPH had a negative correlation with N content ( $r = -0.94$ ,  $p < 0.0001$  and  $r = -0.97$ ,  $p < 0.0001$

for SSF and SS, respectively). For all the treatments, a maximum bacterial growth was presented on the tenth day, at the same time catalase activity decreased, while AcP and AIP activities remained constant. Higher catalase, AcP, and AIP activities were present on day 5 for all treatments: the day on which hydrocarbon removal began to increase. The maximum catalase activity observed was with the SSF treatment (0.49 μmol H<sub>2</sub>O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup>) on day 5 (Table 3), but there was no significant difference between this result and that obtained with SS. The lowest activity occurred under the SF treatment. In the same experimental period, with regard to AcP activity, the highest was observed with the SF treatment (1.80 μmol p-NP g<sup>-1</sup> h<sup>-1</sup>), followed by SS (1.33 μmol p-NP g<sup>-1</sup> h<sup>-1</sup>), and finally SF (1.33 μmol p-NP g<sup>-1</sup> h<sup>-1</sup>). The AIP activity for the SSF treatment was 1.83 μmol p-NP g<sup>-1</sup> h<sup>-1</sup> on day 5 and no significant differences were observed between this result and those for the SS treatment; the lower value was for the SF treatment (1.12 μmol p-NP g<sup>-1</sup> h<sup>-1</sup>). The Pearson's correlation tests for the SSF treatment showed that P consumption had a significant correlation with bacterial and fungal growth ( $r = 0.81$ ,  $p < 0.0014$  and  $r = 0.86$ ,  $p < 0.0003$ , respectively), as well as with AIP, AcP, and catalase activities ( $r = 0.92$ ,  $p < 0.0001$ ;  $r = 0.94$ ,  $p < 0.0001$ ; and  $r = 0.97$ ,  $p < 0.0001$ , respectively), as shown in Fig. 3. The best results were observed on day 15 under the SSF treatment:  $7.98 \times 10^9$  cfu g dm<sup>-1</sup> for bacterial growth,  $1.05 \times 10^7$  cfu g dm<sup>-1</sup> for fungal growth, 0.35 μmol H<sub>2</sub>O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> for catalase activity, 1.30 μmol p-NP g<sup>-1</sup> h<sup>-1</sup> for AcP activity, and 1.80 μmol p-NP g<sup>-1</sup> h<sup>-1</sup> for AIP activity. Finally, with this treatment,

**Table 3** Results obtained (average;  $\pm$ standard deviation) of the experimental treatments

Time (day)	pH	OM	N	P	TPH	Bacteria	Fungi	AIP	AcP	Catalase
<b>SSF (<math>n = 3</math>)</b>										
0	6.60 $\pm$ 0.01	4.14 $\pm$ 0.00	0.39 $\pm$ 0.01	0.81 $\pm$ 0.04	2.15 $\pm$ 0.27	21.57 $\pm$ 0.15	15.41 $\pm$ 0.05	1.38 $\pm$ 0.02	0.96 $\pm$ 0.01	0.21 $\pm$ 0.04
5	6.75 $\pm$ 0.01	5.40 $\pm$ 0.10	0.37 $\pm$ 0.01	2.02 $\pm$ 0.03	16.86 $\pm$ 0.17	22.65 $\pm$ 0.08	16.14 $\pm$ 0.10	1.83 $\pm$ 0.02	1.33 $\pm$ 0.01	0.50 $\pm$ 0.01
10	7.14 $\pm$ 0.01	5.86 $\pm$ 0.10	0.33 $\pm$ 0.01	1.73 $\pm$ 0.07	43.70 $\pm$ 0.52	22.78 $\pm$ 0.03	13.32 $\pm$ 0.08	1.81 $\pm$ 0.01	1.30 $\pm$ 0.01	0.38 $\pm$ 0.01
15	7.35 $\pm$ 0.01	6.03 $\pm$ 0.10	0.29 $\pm$ 0.01	1.51 $\pm$ 0.03	51.43 $\pm$ 0.24	22.80 $\pm$ 0.05	16.17 $\pm$ 0.06	1.81 $\pm$ 0.01	1.30 $\pm$ 0.04	0.36 $\pm$ 0.07
<b>SS (<math>n = 3</math>)</b>										
0	6.60 $\pm$ 0.01	4.37 $\pm$ 0.10	0.38 $\pm$ 0.01	0.78 $\pm$ 0.03	3.65 $\pm$ 0.33	15.49 $\pm$ 0.11	15.38 $\pm$ 0.17	1.10 $\pm$ 0.02	1.10 $\pm$ 0.02	0.20 $\pm$ 0.01
5	6.88 $\pm$ 0.01	5.35 $\pm$ 0.17	0.35 $\pm$ 0.00	2.01 $\pm$ 0.01	14.81 $\pm$ 0.42	22.64 $\pm$ 0.02	16.12 $\pm$ 0.04	1.82 $\pm$ 0.01	1.33 $\pm$ 0.02	0.50 $\pm$ 0.00
10	6.95 $\pm$ 0.01	5.81 $\pm$ 0.01	0.31 $\pm$ 0.01	1.76 $\pm$ 0.04	40.19 $\pm$ 0.12	22.73 $\pm$ 0.02	13.36 $\pm$ 0.13	1.80 $\pm$ 0.00	1.23 $\pm$ 0.02	0.36 $\pm$ 0.03
15	7.35 $\pm$ 0.01	5.98 $\pm$ 0.10	0.29 $\pm$ 0.01	1.54 $\pm$ 0.05	46.35 $\pm$ 0.50	22.75 $\pm$ 0.00	16.13 $\pm$ 0.02	1.80 $\pm$ 0.01	1.11 $\pm$ 0.03	0.34 $\pm$ 0.03
<b>SF (<math>n = 3</math>)</b>										
0	6.60 $\pm$ 0.01	4.08 $\pm$ 0.10	0.39 $\pm$ 0.01	0.94 $\pm$ 0.02	3.31 $\pm$ 0.21	21.19 $\pm$ 0.13	15.26 $\pm$ 0.11	0.61 $\pm$ 0.02	1.15 $\pm$ 0.01	0.20 $\pm$ 0.05
5	6.80 $\pm$ 0.00	5.18 $\pm$ 0.00	0.36 $\pm$ 0.00	1.56 $\pm$ 0.03	12.54 $\pm$ 0.13	22.58 $\pm$ 0.06	15.77 $\pm$ 0.09	1.12 $\pm$ 0.04	1.80 $\pm$ 0.01	0.39 $\pm$ 0.02
10	6.94 $\pm$ 0.02	5.36 $\pm$ 0.10	0.36 $\pm$ 0.01	1.51 $\pm$ 0.03	28.32 $\pm$ 0.75	22.66 $\pm$ 0.03	12.44 $\pm$ 0.09	0.95 $\pm$ 0.00	1.74 $\pm$ 0.01	0.34 $\pm$ 0.01
15	7.28 $\pm$ 0.01	5.64 $\pm$ 0.10	0.31 $\pm$ 0.00	1.16 $\pm$ 0.02	34.21 $\pm$ 0.41	22.67 $\pm$ 0.03	15.84 $\pm$ 0.05	0.88 $\pm$ 0.02	1.75 $\pm$ 0.01	0.34 $\pm$ 0.02
<b>C (<math>n = 3</math>)</b>										
0	8.02 $\pm$ 0.01	2.81 $\pm$ 0.10	0.39 $\pm$ 0.01	0.76 $\pm$ 0.03	3.43 $\pm$ 0.06	5.84 $\pm$ 10.12	7.06 $\pm$ 6.12	0.51 $\pm$ 0.01	0.19 $\pm$ 0.01	0.19 $\pm$ 0.06
5	8.21 $\pm$ 0.01	2.93 $\pm$ 0.00	0.39 $\pm$ 0.01	0.78 $\pm$ 0.04	4.34 $\pm$ 0.44	12.27 $\pm$ 10.63	8.83 $\pm$ 7.66	0.48 $\pm$ 0.02	0.20 $\pm$ 0.02	0.19 $\pm$ 0.00
10	8.23 $\pm$ 0.06	2.99 $\pm$ 0.01	0.39 $\pm$ 0.00	0.77 $\pm$ 0.03	5.57 $\pm$ 0.47	11.67 $\pm$ 10.11	7.30 $\pm$ 6.33	0.48 $\pm$ 0.04	0.21 $\pm$ 0.03	0.19 $\pm$ 0.00
15	8.27 $\pm$ 0.06	2.93 $\pm$ 0.01	0.38 $\pm$ 0.02	0.72 $\pm$ 0.05	5.51 $\pm$ 0.07	5.84 $\pm$ 10.11	8.60 $\pm$ 7.45	0.47 $\pm$ 0.02	0.21 $\pm$ 0.02	0.19 $\pm$ 0.00
<b>S (<math>n = 3</math>)</b>										
0	9.28 $\pm$ 0.03	2.93 $\pm$ 0.00	0.39 $\pm$ 0.01	0.53 $\pm$ 0.03	2.89 $\pm$ 0.71	0.000	0.000	0.31 $\pm$ 0.01	0.13 $\pm$ 0.01	0.20 $\pm$ 0.01
5	9.30 $\pm$ 0.00	2.99 $\pm$ 0.10	0.39 $\pm$ 0.01	0.62 $\pm$ 0.05	2.94 $\pm$ 0.42	0.000	0.000	0.28 $\pm$ 0.02	0.14 $\pm$ 0.01	0.21 $\pm$ 0.00
10	9.23 $\pm$ 0.03	2.99 $\pm$ 0.01	0.39 $\pm$ 0.02	0.56 $\pm$ 0.05	3.07 $\pm$ 0.15	0.000	0.000	0.29 $\pm$ 0.02	0.14 $\pm$ 0.00	0.20 $\pm$ 0.03
15	8.87 $\pm$ 0.06	3.05 $\pm$ 0.00	0.38 $\pm$ 0.00	0.54 $\pm$ 0.06	3.63 $\pm$ 0.08	0.000	0.000	0.26 $\pm$ 0.01	0.14 $\pm$ 0.01	0.20 $\pm$ 0.01

OM (%), N (%), P (mg kg<sup>-1</sup>), TPH (% of removal), bacteria (Ln cfu g dm<sup>-1</sup>), fungi (Ln cfu g dm<sup>-1</sup>), AIP (μmol p-NP g<sup>-1</sup> h<sup>-1</sup>), AcP (μmol p-NP g<sup>-1</sup> h<sup>-1</sup>), and catalase (μmol H<sub>2</sub>O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup>)  
 SSF soil/sugarcane bagasse pith/filter cake mud, SS soil/sugarcane bagasse pith, SF soil/filter cake mud, C control soil, S sterile soil



**Fig. 2** TPH removal (%) and colony-forming units (cfu) of bacteria behavior in the treatment of soil/sugarcane bagasse pith/filter cake mud ( $n = 3$ ). White triangle TPH removal and white square

bacteria growth average values. Error bars show the standard deviation

the AcP was observed to have a significant correlation with bacterial growth ( $r = 0.95$ ,  $p < 0.0001$ ) and TPH removal ( $r = 0.70$ ,  $p < 0.0107$ ).

#### 4 Discussion

The pH ranges in the soil (6.60–7.35) did not significantly change throughout the experimental period in all the treatments. According to some authors (Gómez-Romero et al. 2008; Montejo et al. 2008), it is important to maintain a pH range between 6 and 8 to obtain good results for removal of pollutants from soil. The pH values in the present study resulted in more bacterial growth than fungal growth. The humidity in the soil was another factor that facilitated microbial development, and in our experimental treatments, the constancy of this parameter facilitated heterotrophic microbial growth. Geisseler et al. (2011) reported that moisture was enhanced by the addition of residues, which enables the enzymatic activities in the soil to increase. In the current study, the OM in the experimental treatment increased following the amendments, thereby resulting in the progression of the microbial and enzymatic activities according to the reports of Burns (1982) and Shahsavari et al. (2013). There was a lower consumption of total N than of P; the latter nutrient is important in the mineralization process as well as many metabolic pathways and certain enzymatic activities in soil (Sylvia

et al. 1999; Margesin et al. 2000b). The enzyme activities were also low in the soil during the experimental period compared with the activities observed in other healthy soils (alkaline phosphatase, 6.76–27.34  $\mu\text{mol p-NP g}^{-1} \text{h}^{-1}$ ; acid phosphatase, 0.05–86.33  $\mu\text{mol p-NP g}^{-1} \text{h}^{-1}$ ; and catalase, 61.20–73.90  $\mu\text{mol H}_2\text{O}_2 \text{g}^{-1} \text{h}^{-1}$ ) (Nannipieri et al. 2002). The alkaline phosphatase activity was higher than that of acid phosphatase because the pH remained neutral during experimental period; it was suggested that these proteases are more affected for soil pH than for OM or level of disturbance (Acosta-Martínez and Tabatabai 2011). For the SSF treatment, P concentration was positively correlated with AcP and AIP activities, as well as with fungal growth. Therefore, P availability for this condition was important to reactivate the native micro-flora in order to remove the pollutants, which is consistent with the findings of Margesin et al. (2000b). An inverse relationship was observed between inorganic P availability and phosphatase activity, although this depends on the initial bio-availability of P (DeForest et al. 2012). An increase in enzyme activity as a response to demand of resources results in the release excess of products, which may be due to a positive relationship between enzyme activity and resource availability (Singh and Ghoshal 2013). Nevertheless, the N content was related to TPH removal for the SSF and SF treatments. This suggests that the N content in the filter cake mud is more available to the microorganisms (waste degradation) and allows the

**Table 4** Pearson's correlation between the parameters observed of the experimental treatments

	OM	N	P	TPH	Bacteria	Fungi	AIP	AcP	Catalase
SSF									
pH	0.82**	-0.97***		0.99***	0.81**	0.70*	0.67*	0.63*	
OM		-0.71**	0.69*	0.89***	0.91***	0.87***	0.85***	0.83***	
N				-0.94***	-0.76**	-0.65*	-0.62*	-0.59*	
P					0.82**	0.86***	0.92***	0.94***	0.97***
TPH					0.86**	0.77**	0.74***	0.70*	
Bacteria						0.94***	0.96***	0.95***	0.74**
Fungi							0.97***	0.95***	0.78**
AIP								0.99***	0.87***
AcP									0.88**
SS									
pH	0.63*	-0.93***		0.89***	0.78**	0.73**	0.73**		
OM		-0.83***	0.67*	0.88***	0.85***	0.83***	0.80**		
N				-0.97***	-0.84***	-0.79**	-0.77**		
P					0.89**	0.91**	0.93**	0.81**	0.93***
TPH					0.80**	0.75**	0.72**		
Bacteria						0.98***	0.99***		0.73**
Fungi							0.98***		0.76**
AIP								0.57**	0.81**
AcP									0.84***
SF									
pH		-0.98***		0.94***	0.75**	0.75**	0.66*		
OM			0.67*	0.78**	0.79**	0.78**	0.72**		0.58*
N					-0.73**	-0.71**	0.64*		
P					0.74**	0.70*	0.82***	0.92***	0.83***
TPH					0.82**	0.81**	0.71*		
Bacteria						0.94***	0.98***	0.82**	0.88***
Fungi							0.92**	0.76**	0.79**
AIP								0.91***	0.93***
AcP									0.92***

OM (%), N (%), P (mg kg<sup>-1</sup>), TPH (% of removal), bacteria (cfu g dm<sup>-1</sup>), fungi (cfu g dm<sup>-1</sup>), AIP (μmol p-NP g<sup>-1</sup> h<sup>-1</sup>), AcP (μmol p-NP g<sup>-1</sup> h<sup>-1</sup>), and catalase (μmol H<sub>2</sub>O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup>)

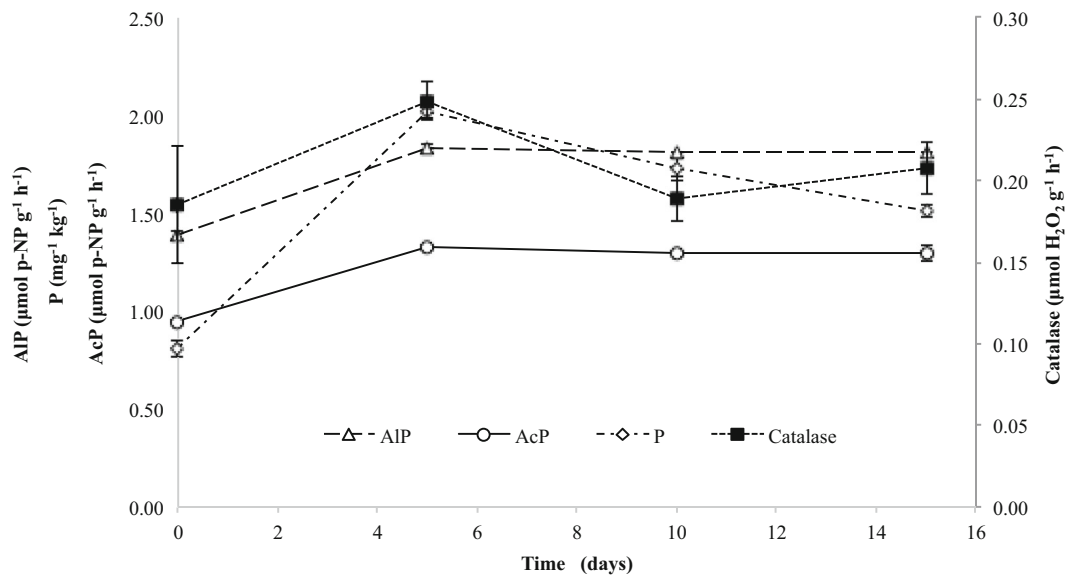
\* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.001$  (significant Pearson's correlation coefficients)

SSF soil/sugarcane bagasse pith/filter cake mud, SS soil/sugarcane bagasse pith, SF soil/filter cake mud

pollutant removal. Similar results were obtained by Adesodun and Mbagwu (2008), Lee et al. (2008), and Ros et al. (2010) in hydrocarbon-contaminated soils amended with poultry pig manure compost and fresh sewage sludge, respectively. The higher hydrolysis rates for organic P with respect to proteins and the increasing ratio of phosphatase to aminopeptidase activity indicated that N cycling was slower than P cycling (Singh and Ghoshal 2013). Although low, fungal growth was

sufficient to promote a synergism with the bacterial population to achieve TPH removal. In a similar report by Shahsavari et al. (2013), 85% removal of TPH from soil amended with pea straw was observed, as compared to 51% for the control case. They also observed a change in the bacterial population in soils amended with plant residues and a weak correlation ( $r = 0.68$ ) between these residues and TPH removal. This confirms that the residues provided a substrate for microorganism growth.





**Fig. 3** Enzymatic activities and phosphorus consumption during the treatment of soil/sugarcane bagasse pith/filter cake mud ( $n = 3$ ). White triangle alkaline phosphatase, AIP; white circle

acid phosphatase, AcP; white diamond phosphorus, P; and black square catalase, average values. Error bars show the standard deviation

The constancy in the enzymatic activity in this study may be due to a protective effect against degradation promoted by the OM from the by-products. Similar results were reported by Pascual et al. (2002). It has also been reported (Chaudhary et al. 2012) that AIP increases more than AcP in soil polluted by kerosene.

The decrease in catalase activity occurred when the TPH began to get established, as reported by Achuba and Peretiemo (2008). This activity was altered as a result of the environmental changes to soil properties such as pH, nutrient availability, hypoxia, and microorganism populations. The mechanisms by which the enzymes are immobilized and consequently become established in soil have not been entirely explained (Gianfreda and Ruggiero 2006). In addition, it has been reported that enzymatic activities could be affected by factors such as sampling methods, storage time, and changes in pollutants (García et al. 2003). For all treatments, it was difficult to attribute the AIP activity to only bacterial or fungal growth because it had a significant correlation with both groups, although the correlation was slightly higher with bacterial growth. Moreover, AIP was correlated with TPH removal in the SS treatment, while AcP was correlated with TPH removal for the SSF treatment. Therefore, phosphatase activities played an important role in the removal of the pollutants. These results are consistent with those obtained by Wyszowska et al. (2002, 2006).

## 5 Conclusions

The results of this study showed that agricultural by-products could be a good option for the bioremediation of oil-polluted soil. Furthermore, a mix of by-products may be optimal to obtain substantial results over a relatively short time, as they serve not only to support microorganisms but also to supply nutrients and OM to immobilize the enzymes. The enzymatic activities for all the treatments increased during the experimental period and could thus be used as an indicator of pollutant removal. Alkaline phosphatase activity was the more representative enzyme in this study, which had a significant correlation with both microbial growth and TPH removal. It is necessary to develop site-specific evaluation criteria for the more accurate use of soil enzyme activities as a soil quality parameter.

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