Biodegradation kinetics of naphthalene in soil medium using *Pleurotus ostreatus* in batch mode with addition of fibrous biomass as a nutrient[†]

Short title: Degradation of naphthalene in soil via fungus-biomass

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ABSTRACT

The efficiency and kinetics of naphthalene biodegradation in a soil medium using *Pleurotus ostreatus* (a type of white rot fungus) in batch mode with and without the addition of Oil Palm Fiber (OPF) as a nutrient are evaluated in this study. Three batches are considered in the biodegradation study; (i) control – spiked soil, (ii) spiked soil with fungus and (iii) spiked soil with both fungus and OPF. Biodegradation is conducted over a period of 22 days for which soil naphthalene concentrations are determined with respect to microwave extraction and HPLC analysis. The results indicate that inoculation with *Pleurotus ostreatus* significantly enhances soil naphthalene biodegradation to 84%, which is further enhanced upon the addition of OPF to 98% with respect to the degradation rate. The high carbon content in OPF (> 40 %) affords it the capacity to be a viable nutrient supplement for *Pleurotus ostreatus* thereby enhancing the potential of *Pleurotus ostreatus* in the biodegradation of Polycylic Aromatic Hydrocarbons (PAHs), and indicating the potential of OPF as a nutrient for PAH biodegradation. A relationship between OPF mass and the biodegradation rate constant has been determined to be linear according to the following equation; $k = 0.0429 \times \text{OPF} + 0.1291$.

KEYWORDS: Biodegradation, naphthalene, *Pleurotus ostreatus*, oil palm fiber, contaminated soil treatment

INTRODUCTION

Biodegradation is a natural and adaptable process that uses living organisms to neutralize/detoxify specific organic contaminants. Examples of these organisms include naturally occurring or isolated microbes (bacteria) and macrobiological lifeforms (fungi or plants), which may be indigenous to a contaminated medium or isolated from other sources and consequently applied in the bioremediation of a contaminated site, where bioremediation corresponds to the use of living organisms in the degradation of contaminants found in soils or water (Vitali, 2001). Biodegradation is an inherent process in the bioremediation of soils contaminated with organic-based chemicals such as Polycyclic Aromatic Hydrocarbons (PAHs) with the benefits of cost-effectiveness, adaptable *in-situ* treatment capability and ability to convert organic contaminants into carbon dioxide and water (REF). Typical contamination would constitute the infiltration of PAHs, such as naphthalene, anthracene, phenanthrene and pyrene, into a soil matrix at abandoned industrial sites, petrochemical processing sites or wood treatment sites from coal and tar deposits produced during the combustion of fossil fuels or biomass (Baek *et al.* 1991; Cutright and Lee 1994; Ellis 1994). Generally, low-molecular-weight PAHs degrade more readily compared to high-molecular-weight PAHs, which are more recalcitrant due to the presence of multiple benzene rings within their structure, which are resistant to ring opening mechanisms by many degradative enzymatic systems and thus may require the addition of specific microorganisms and nutrients to remediate the contaminant effectively (Silva *et al.* 2009). The recalcitrant nature of PAHs is a consequence of their high hydrophobicity and solid-water distribution ratio, such that they tend to interact with non-aqueous phases and the soil's organic matter thereby complicating their bioremediation (Hamdi *et al.* 2007).

There have been various studies on the biodegradation of PAHs in soil using white rot fungi including *Pleurotus ostreatus*, *Irpex lacteus* (Byss *et al.* 2008) and *Phanerochaete chrysosporium* (Bishnoi *et al.* 2007) using PAHs such as phenanthrene, anthracene, fluoranthene and pyrene. Romero *et al.* (2010) and Valentín *et al.* (2007) reported that the white rot fungi *Aspergillus flavus* and *Paecilomyces farinosus* and *Bjerkandera adusta* are capable of degrading anthracene and benzo(a)pyrene in the presence and absence of a cosubstrate. PAH biodegradation research using white rot fungi is prevalent given their natural propensity to mineralize PAHs via the production of ligninolytic enzymes (Pointing 2001).

In this study, attention is focused upon the biodegradation of naphthalene, resulting in contaminated soil, using *Pleurotus ostreatus*, more commonly known as the oyster mushroom; an edible fungus with good medicinal properties (Opletal *et al.*, 1997), which is suitable for bioremediation, because it can rapidly colonize contaminated soil and decompose PAH rings through the production of high levels of extracellular enzyme laccase; used to mineralize PAHs (Pointing, 2001). Furthermore, *Pleurotus ostreatus* is a non-pathogenic fungus, commonly grown and eaten making it more readily publically acceptable for bioremediation applications. Naphthalene is a low-weight PAH and is a natural constituent of coal tar and crude oil commonly used as a chemical intermediate in many chemical-based industries classified as a potential carcinogen and toxic to marine life (Naphthalene, 2005). The United States Environmental Protection Agency (USEPA, 2003) released a report in 2003 detailing the detrimental public health effects of naphthalene, which included adverse effects on the structural integrity of red blood cell membranes.

PAH biodegradation can be conducted without the addition of co-substrates/nutrients, but their presence can considerably enhance biodegradation efficiency. Eggen and Sveum (1999)

stipulated that the successful degradation of high molecular weight organic compounds using *Pleurotus ostreatus* generally necessitates the addition of suitable carbon co-substrates and there have been numerous studies involving a wide variety of agricultural biomass waste as carbon sources, including potato pulp, wheat straw, wood chips (Lamar and Glaser, 1994) and decayed rice straw (Hamdi *et al.*, 2007). In this study, OPF has been proposed and investigated as a natural organic co-substrate capable of providing nutrients to stimulate PAH biodegradation. OPF is the fibrous waste generated from palm oil milling, approximately 8.56 million tons of which are generated per year in Malaysia alone (Husain *et al.*, 2003). The application of OPF as a co-substrate/nutrient for PAH biodegradation in this study is novel and furthermore would contribute in alleviating the agricultural waste disposal problems.

The objective of this study is to evaluate the efficiency and kinetics of naphthalene biodegradation in a soil medium using *Pleurotus ostreatus* with and without the addition of OPF. Thus far, the biodegradation kinetics of an integrated fungus and biomass soil remediation system has not yet been established. Biodegradation kinetics is essential in order to enable a scale-up from a laboratory-scale PAH biodegradation system to an industrial-scale system, which could be tailored for *in-situ* or *ex-situ* applications.

MATERIALS AND METHODS

Growth and preparation of *Pleurotus ostreatus*

Two plugs of *Pleurotus ostreatus* obtained from C & C Mushroom Cultivation Farm located in the southern state of Johor, Malaysia, cultured on potato dextrose agar (Merck, Malaysia)

plates were placed in two 250 mL Erlenmeyer flasks containing 100 mL of malt extract (Merck, Malaysia) and agitated in an oscillary shaker (MAX Q 2000, Barnstead Lab-Line, USA) at 125 rpm and 25°C for 13 days to establish a stationary growth phase (with respect to constant mass). The resulting mycelium (thread-like, vegetative part of *Pleurotus ostreatus*) samples were filtered and dried in an oven at approximately 100ºC for one day and used to determine the rate of mycelium growth as opposed to the bioremediation rate.

Naphthalene degradation

Soil purchased from a nursery in Shah Alam, Malaysia was sieved to obtain a particle size of less than 2 mm and sterilized using an autoclave at 121ºC for 20 mins prior to being spiked with naphthalene in accordance with the method described by ? (YEAR). The soil pH prior to spiking was determined to be 7.36, which is comparable to that (7.5) reported by Eggen and Majcherczyk (1998). Soil samples (20 g) were individually spiked with naphthalene (Merck, Germany) to a concentration of 2000 mg/kg of soil using acetone (Merck, Malaysia) as a carrier solvent in 250 ml Erlenmeyer flasks. The soil water content of the samples prior to bioremediation was determined to be 60% (w/w) at the beginning of the experiment in accordance with the work of Antizar-Ladislao *et al.* (2009) and maintained as such throughout the duration of the bioremediation through the addition of deionized water as and when required. The flasks were agitated at 125 rpm and 25ºC for a period of three days to ensure the complete vaporization of the volatile acetone. Triplicate batches of the synthesized contaminated soil samples were considered in the degradation studies, namely (i) control – spiked soil, (ii) spiked soil with fungus and (iii) spiked soil with both fungus and OPF, thereby enabling evaluation of

the effect of fungus and OPF addition on the biodegradation kinetics. With respect to the third set of triplicates; 0.1 g of OPF, obtained from a palm oil mill located in Labu-Nilai, Malaysia, which had been finely powdered using a blender to promote homogeneous mixing, was added and mixed with the soil. For characterization purposes, the OPF underwent elemental analysis using a Flash EA 1112 ThermoFinnigan elemental analyzer and both *Pleurotus ostreatus* and OPF were subjected to ThermoGravimetric Analysis (TGA) using TA Instruments Q-500, USA.

Sampling, extraction and naphthalene concentration determination

Samples were taken at two-day intervals, whereby a 0.5 g soil sample was placed in a microwave digester and combined with 17.5 ml of *n*-hexane (Merck, Malaysia) and 7.5 ml of acetone (Merck, Malaysia). The extraction of naphthalene from soil samples was performed at 120ºC for 20 minutes using a pressurized microwave extraction system (Multiwave 3000, Anton Paar). The relative concentration of naphthalene present in the soil samples was determined using a Perkin-Elmer Series 200 HPLC with a 150×3.2-mm Brownlee 4-µm PAH reversed-phase column combined with an ultraviolet-visible spectrophotometer scanning at λ_{MAX} = 254 nm, which is the optimum wavelength for naphthalene detection. The mobile phase was 60:40 (v/v) acetontrile/water (HPLC grade, Merck, Malaysia) flowing at 0.5 mL/min.

RESULTS AND DISCUSION

Optimized *Pleurotus ostreatus* growth

Figure 1 presents the growth of *Pleurotus ostreatus* mycelium, which exhibits a typical S-shaped growth curve (Wu *et al.* 2003) and from the insert it is evident that the mycelium surface is rough and sponge-like, which is typical of biomass. In this study, the mycelium exhibits minimal growth for the first four days followed by six days of exponential growth, after day-10 growth asymptotes and no further growth is observed. Mycelium harvesting is thus performed at day-10 once maximum mass is attained. The maximum growth rates for the first four days and the exponential phase are 0.183 and 0.578 g/L.day, respectively, and are of importance with respect to bulk fungus production and identifying when the mycelium mass is maximized and it is suitable for cultivation and consequent use in the biodegradation of naphthalene contaminated soil.

TG analysis of *Pleurotus ostreatus* and OPF

Dried mycelium (20 mg) and OPF (16 mg) samples were heated under nitrogen from 30ºC to 750ºC at a rate of 10ºC/ min. The TGA curves for *Pleurotus ostreatus* and OPF are presented in Figure 2, according to which approximately 10% of the OPF sample's weight is lost as the temperature increases from 75ºC to 100ºC, which corresponds to the evaporation of adsorbed of water. Significant structural (cell wall) weight loss occurs at around 110ºC. The fungus initially undergoes a more rapid weight loss with respect to the increasing temperature than the OPF,

which may be attributed to direct structural weight loss. Over the range 100-350ºC, both the fungus and OPF lose approximately 60% of their initial mass, which according to Monte (2003), would correspond to the decomposition of carbohydrates.

Biodegradation study

Figure 3 presents the biodegradation of naphthalene expressed in terms of the relative percentage present in the soil with respect to time. It is of note that naphthalene biodegrades in the absence of the fungus and nutrient (control samples); such biodegradation is termed "natural attenuation" and is most likely due to the influence of chemical, physical, and biological processes unrelated to the fungus or nutrient, such as external biodegradation caused by microbes present in the added deionized water used to maintain the soil moisture content at 60% (w/w) or as a consequence of the natural volatility of naphthalene. Irrespective of this apparent unpromoted decrease in naphthalene concentration, the batches inoculated with both *Pleurotus ostreatus* and OPF evidently exhibit higher naphthalene biodegradation rates than the control samples. The samples treated with only *Pleurotus ostreatus* exhibit an initial plateau for about ten days, after which rapid degradation appears to occur, achieving a relative naphthalene percentage of approximately 10% (w/w). The samples to which OPF is added attain a similar relative naphthalene percentage two days earlier, thereby inferring a positive effect of the presence of OPF as a nutrient. The final relative naphthalene percentages after 22 days clearly indicate that the presence of fungus and OPF promote naphthalene degradation, with respect to the control samples, yielding final concentration percentages less than 3% for both the fungus and fungus/OPF samples. Additional experimental studies using a Perkin-Elmer GC/MS Clarus

600 (not shown) have been conducted to confirm that biodegradation occurred and findings are found to be consistent with the abovementioned HPLC analysis.

In conjunction with this; according to elemental analysis OPF is composed of 41.3% carbon, 0.7% hydrogen and 5.1% nitrogen, which is significant in that carbon is a macronutrient typically found in large proportions in organism cells (Liebeg and Cutright 1999) and its significant presence in OPF may explain why naphthalene biodegradation appears to be promoted.

Biodegradation kinetics

Biodegradation kinetics have been determined by assuming that degradation is first order with respect to the naphthalene concentration in accordance with LaGrega *et al.* (2001):

$$S = S_0 e^{-k.t} \quad (1)$$

where S is the relative naphthalene percentage, S_0 is the initial relative naphthalene percentage, k is the biodegradation rate constant (day^{-1}) and t is the time (day). Plotting the natural logarithm of the relative naphthalene percentage with time yields a straight line of gradient k (Figure 4). It is evident that the smallest rate constant value corresponds to that for the control sample, which in comparison to the other determined rate constants indicates promotion of naphthalene biodegradation in the presence of the fungus and OPF.

Regression analysis of the experimental data indicates that there is a greater scatter in the fungus-only samples with respect to the R^2 values, but in general the experimental data is well described with respect to first order biodegradation kinetics (Table 1). The p- and F-values for

the time coefficients are lower than 0.05, implying that the time is significant in the physical interpretation of naphthalene biodegradation.

The half-life of the naphthalene is determined with respect to the following equation:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \quad (2)$$

The half-life values for the (i) control, (ii) spiked soil with fungus and (iii) spiked soil with both fungus and OPF are 9.8, 5.3 and 5.0 days, respectively, which further corroborates the conclusion that there is a pronounced effect on biodegradation by the addition of fungus and OPF. The perceived improved biodegradation rates may be attributed to the production of oxidative extracellular enzymes by the white rot fungus, which non-specifically oxidize naphthalene through the abstraction of an electron or a hydrogen atom (Eggen and Majcherczyk, 1998). Pozdnyakova *et al.* (2006) stated that yellow laccase, an enzyme produced by white rot fungi, is not responsible for the oxidation of naphthalene, although naphthalene derivatives, such as α-nitroso-β-naphthol may be susceptible to this enzyme. However to date, there is no definitive consensus as to which enzymes produced by *Pleurotus ostreatus* are capable of oxidizing naphthalene, though Patel *et al.* (2009) suggest that ligninolytic enzymes (manganese peroxidase and laccase) produced by white rot fungi are capable of biodegrading fluoranthene, which is another type of PAH. As already indicated, the enhanced biodegradation rate is evidently a consequence of the presence of fungus, but also OPF, which comprises of a significant quantity of the macronutrient carbon.

Table 2 presents the determined first-order degradation rate constants along with values obtained from similar studies. The obtained *k* values are comparable with other reported biodegradation rate constants for PAHs and petroleum-based hydrocarbons using bacteria and other fungi. The inclusion of OPF with *Pleurotus ostreatus* yields a biodegradation rate on par

with that for the fastest rate, which has been attributed to K_2HPO_4 (approximately 0.13 day^{-1}), and implies that OPF is a viable and cost-effective nutrient suitable for PAH biodegradation.

Quantification study on OPF enhanced biodegradation

The evidence that OPF promotes biodegradation is undeniable, however the degree to which this occurs is of interest. To further demonstrate the relationship between OPF and biodegradation, experiments were performed with OPF quantities varying from 0.1 to 3.0 g added to 20 g of naphthalene-contaminated soil. Using OPF masses greater than 3 g is inadvisable, because the nutrient volume will be in excess of the biodegradation medium. Figure 5 presents the effect of OPF quantity on the biodegradation rate constant, thereby offering an opportunity to optimize biodegradation with respect to the OPF mass. The linear relationship between the biodegradation rate constant, k, and OPF mass, m, of the form: $k = 0.0249 \times m + 0.1291$, with an R^2 value of 0.9843 indicates that the data is well described by the chosen equation. This simple correlation could be useful to quickly estimate the OPF mass required to achieve a particular biodegradation rate constant.

CONCLUSIONS

This study presents a viable and effective batch method for the biodegradation of naphthalene, a type of PAH, in a soil medium. The results indicate that inoculation using *Pleurotus ostreatus* in the presence and absence of OPF significantly enhances the rate of naphthalene biodegradation in contaminated soils from 80% in the control to 84 and 98% with

respect to the degradation rate, respectively. The high carbon content in OPF affords it the potential to be a viable nutrient for *Pleurotus ostreatus* biodegradation and it is likely that the high carbohydrate content makes it an ideal carbon substrate for *Pleurotus ostreatus* and other white rot fungi. Furthermore, lignin degrading enzymes likely allow fungi preferential access to OPF carbohydrate. A linear relationship between OPF mass and the biodegradation rate constant: $k = 0.0249 \times m + 0.1291$, demonstrates its utility in fungal degradation of naphthalene. The biodegradation kinetics determined in this study may be of interest with respect to scaling-up from laboratory-scale PAH biodegradation and also in the tailoring of *in-* and *ex-situ* contaminated soil remediation systems.

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Figure Legends

FIGURE 1. Growth of *Pleurotus ostreatus*. Inset shows its mycelium in potato dextrose agar.

FIGURE 2. TGA curves for *Pleurotus ostreatus* and OPF

FIGURE 3. Biodegradation of naphthalene in soil.

FIGURE 4. First-order biodegradation kinetics of naphthalene in soil.

FIGURE 5. Effect of OPF amount used on the biodegradation rate constant.

TABLE 1. Regression analysis result

Parameter	Batch (i): Control	Batch (ii): *Pleurotus ostreatus*	Batch (iii): *Pleurotus ostreatus* and OPF
k (day^{-1})	0.0707	0.1304	0.1399
R^2	0.9759	0.8932	0.9524
Adjusted R^2	0.9729	0.8813	0.9464
Standard error	0.0891	0.3507	0.2126
Significance F	9.32×10^{-08}	1.15×10^{-05}	1.43×10^{-06}
p-value	< 0.05	< 0.05	< 0.05

TABLE 2. Comparison of the obtained first-order degradation rate constants with values obtained from other bioremediation studies.

Contaminant(s)	Biodegradation organisms	Nutrient(s)	k (day^{-1})	R^2	References
Total petroleum hydrocarbon	*Bacillus mycoides* (bacteria)	Urea and potassium dehydrogenate	0.036	0.972	Yudono *et al.* 2009
Total petroleum hydrocarbon[a]	*α-Proteobacteria* (bacteria)	KNO_3 and K_2HPO_4	0.009 – 0.137	–	Vinas *et al.* 2005
Total petroleum hydrocarbon	*Cunninghamella echinulata var. elegans* (fungus)	K_2HPO_4, Na_2HPO_4, $MgCl_2$, NH_4Cl $CaCl_2$, Na_2SO_4 and $FeCl_3$	0.134	–	Cutright 1995
Total petroleum hydrocarbon	*a-proteobacteria and Flexibacter-Cytophaga-Bacteroides phylum* (bacteria)	$NaNO_3$ and $Na_5P_3O_{10}$	0.026	0.83	Macnaughton *et al.* 1999
Naphthalene	-	-	0.071	0.9759	This study
Naphthalene	*Pleurotus ostreatus* (fungus)	-	0.130	0.8932	This study
Naphthalene	*Pleurotus ostreatus* (fungus)	Oil palm fiber	0.140	0.9524	This study

[a]TPH included acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene and benzo(*a*)anthracene.

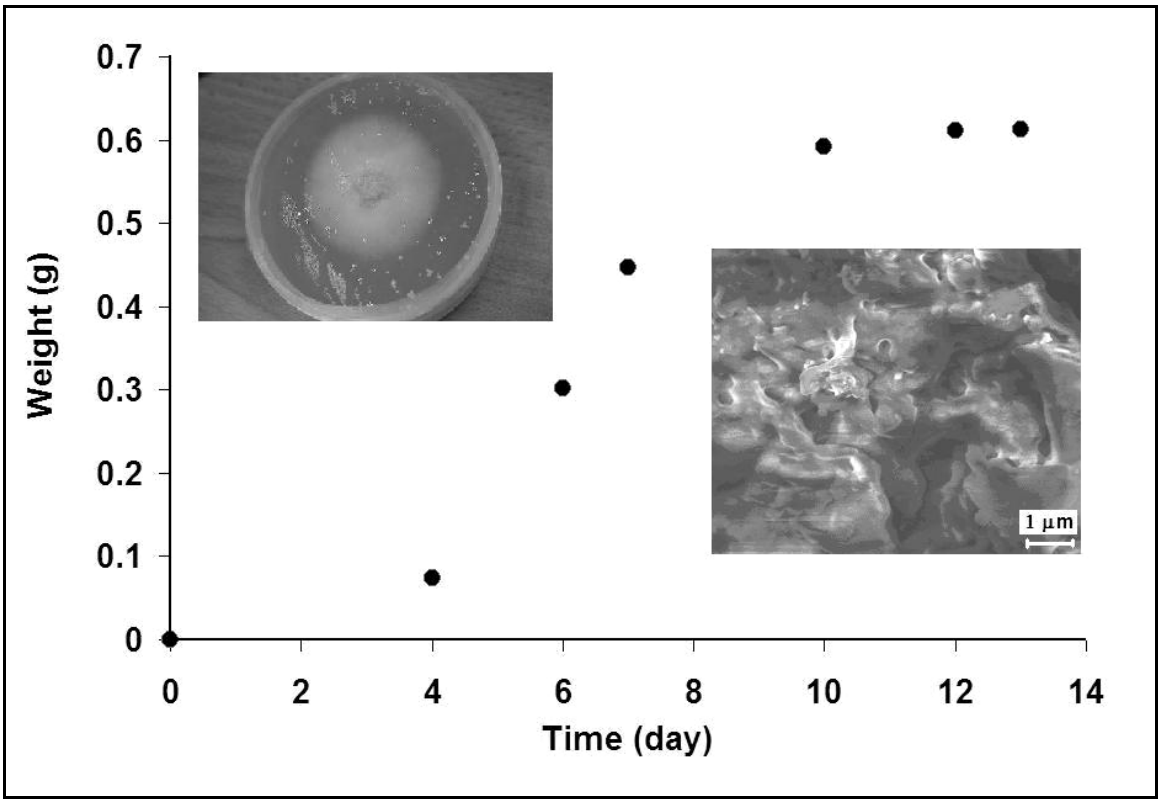

FIGURE 1.

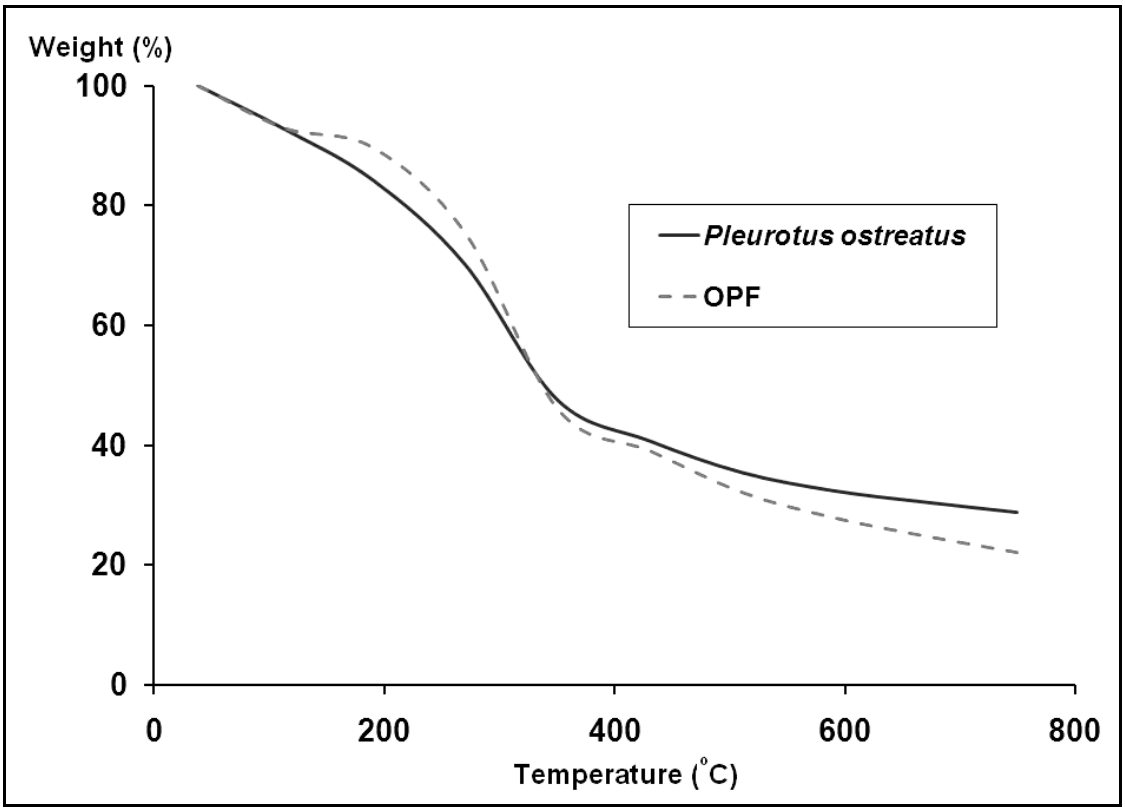
Weight (%)
100
80
60
40
20
0
0
200
400
600
800
Temperature (°C)
Pleurotus ostreatus
OPF

FIGURE 2.

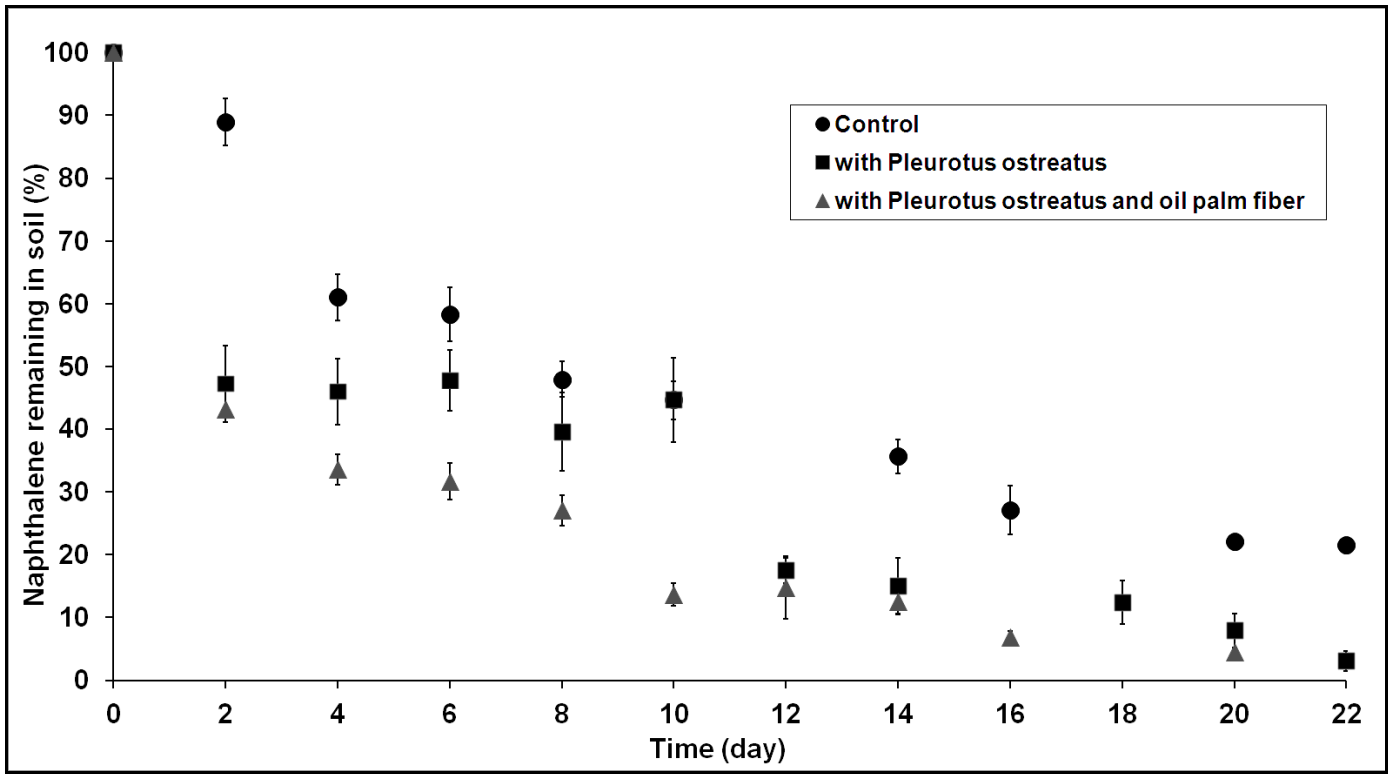
Naphthalene remaining in soil (%)
Time (day)
Control
with Pleurotus ostreatus
with Pleurotus ostreatus and oil palm fiber
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14
16
18
20
22

FIGURE 3.

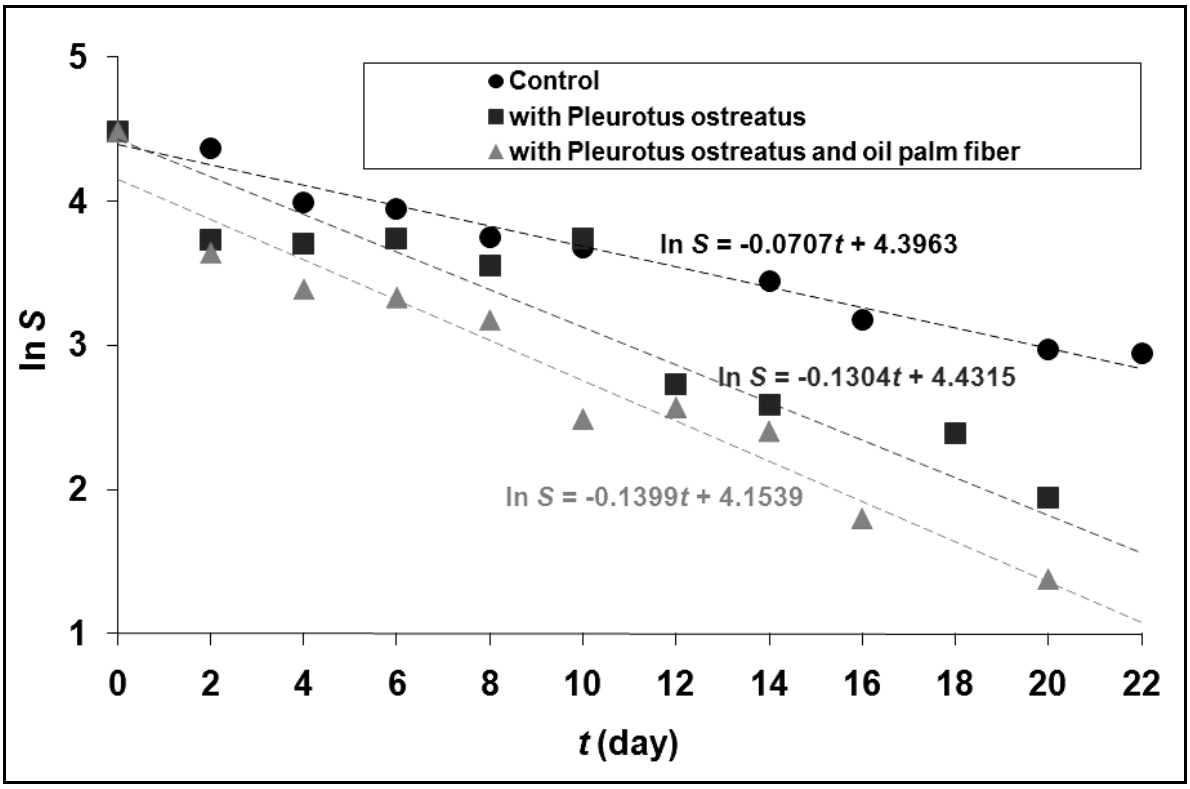
Control
with Pleurotus ostreatus
with Pleurotus ostreatus and oil palm fiber
ln S = -0.0707t + 4.3963
ln S = -0.1304t + 4.4315
ln S = -0.1399t + 4.1539
ln S
t (day)
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22

FIGURE 4.

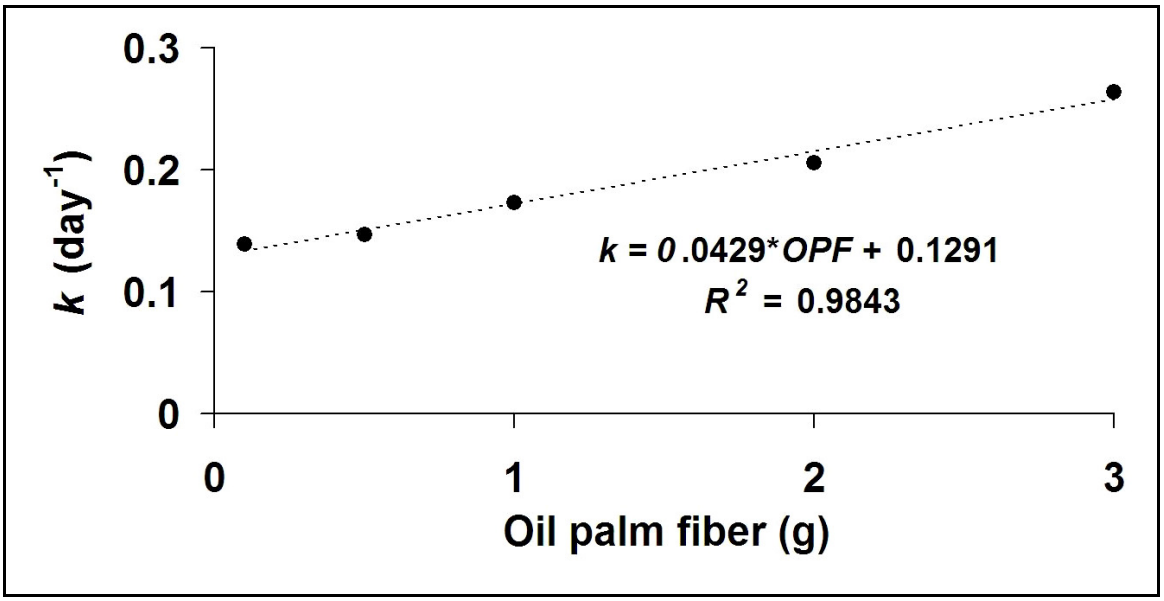
0.3
0.2
0.1
0
k (day-1)
k = 0.0429*OPF + 0.1291
R2 = 0.9843
0
1
2
3
Oil palm fiber (g)

FIGURE 5.