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Biodegradation of pyrene by using earthworm in soil

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ABSTRACT: Pyrene, a toxic four benzene ring Polycyclic Aromatic Hydrocarbons (PAHs) that persists in the environment, is highly resistant to degradation. Therefore, this study aims are to investigate the mechanism and optimum pyrene degradation rate with minimum toxic by-products by using earthworms. The earthworms were collected randomly from soil at Universiti Teknologi Malaysia (Malaysia) as pyrene degrader to eliminate pyrene in soil. Several factor such as soil to earthworm ratio, soil moisture content, effect of soil sterilization and comparison of pyrene degradation using different experimental approaches on pyrene degradation efficiency were examined. In this study was found by using earthworms, 25% of pyrene was removed by earthworms from 200 g of soil containing 100 mg L^{-1} of pyrene after 14 days treatment, suggesting that wild earthworms were able to degrade the pyrene with higher efficiency in the natural soil without sterilization. However, no metabolite was detected during the pyrene removal process. The pyrene might be fully degraded or accumulated in fatty tissue in earthworms. Nevertheless, in the treatment using wild earthworms are suitable to be used as pyrene degrader.

KEYWORDS: Degradation, Wild Earthworm, Pyrene, Soil

1. Introduction

Energy is key factor to support economic growth of the country. Petroleum and coal is most important input for economic growth and development. In fact, use of energy drives economic output and industrial growth and is central to the operation in economy modern. However, during the extracting and production of coal or petroleum are given negative side effect especially to produce polycyclic aromatic hydrocarbons (PAHs) as by-products. In addition, the PAHs by product has possesses more than one benzene ring and widely distributed by represent high molecular weight compound [1]. In coal tar and fossil they are ubiquitous natural constituents [2]. The major factors of PAHs are generally coming from human activities such as forest burning, automobiles exhaust, and incomplete combustion of fossil fuels [3-4].

In total 16 types of PAHs as priority pollutants has listed and differentiated by United States Environmental Protection Agency (US-EPA) based on their characteristic of PAHs,

whereas some of them are considered as human carcinogens [5-7]. Table 1 shown properties of PAHs. The study about degradation of PAHs have begun since few decades ago. However, degradation of efficiency becomes not effective when molecular weight of PAHs increases. Therefore, to improve and develop degradation methods by numerous of type degradation technics are still being carried out to date. There are several methods used for degradation of PAHs by physical-chemical, such as bioreactor treatments [8-10], composting [11,12] and bio-sparging [13]. Summary of conventional method for PAH contaminated soil are presented in Table 2 [14]. There was several limitations of those process such as high maintenance and operational cost, long time treatment, large space operation and other tedious requirements that hinder further development. Therefore, there are a several methods by biodegradation that have specify and emerged a solution to degrade of PAHs. Recently, biodegradation by used several type of biomaterials or organisms were applied to degrade PAHs featuring with fungi [15-20], plant [21-23] and bacteria [24-26].

Table 1. Main physio-chemical and carcinogenic characteristics of US Environmental Protection Agency (EPA) priority PAHs for control [5].

PAHs	**No of Ring**	**Solubility (25°C), mg/L**	**Octanol/ Water Partition coefficient[a]**	**Carcinogenic group[b]**
Naphthalene	2	3.2	2.3×10^3	2B
Naphthylamine	2	4.6	1.4×10^3	1
Acenaphthylene	3	3.4	2.1×10^4	Not reported
Acenaphthylamine	3	3.93	1.2×10^4	Not reported
Fluorene	3	1.9	1.5×10^4	3
Anthracene	3	0.05-0.07	2.8×10^4	3
Phenanthrene	3	1-1.3	2.9×10^4	3
Fluoranthene	4	0.26	3.4×10^5	3
Chrysene	4	0.002	4.0×10^5	3
Pyrene	4	0.14	2.0×10^5	3
Benzo[a]anthracene	4	0.01	4.0×10^5	2A
Benzo[b]fluoranthene	5	Not reported	4.0×10^6	2B
Benzo[k]fluoranthene	5	Not reported	7.0×10^6	2B
Benzo[a]pyrene	5	0.0038	1.0×10^6	2A
Dibenz[a, h] anthracene	5	0.0005	1.0×10^6	2A
Benzo [g, h, i]perlene	6	0.0003	1.0×10^6	3
Indeno[1,2,3-c,d]pyrene	6	0.062	5.0×10^7	2B

[a] Octanol/water partition coefficient is defined as the ratio of a compound in all its forms between an aqueous phase (with buffer) and an oil phase, and the coefficient is used to represent the lipophilic character of a compound, which is a very useful physical-chemical parameter reflecting the transfer properties of a compound across biological membranes

[b] Pollutants are classified by the International Agency for Research on Cancer (IARC) as to their carcinogenic risk to humans. Group 1: carcinogenic; Group 2A: probably carcinogenic; Group 2B: possibly carcinogenic; and Group 3: not reported

Table 2. Summary of conventional treatment methods for PAH contaminated soil [14]

Technology	Examples	Factor Consider	Benefits	Limitation
In situ	1. In situ bioreactors 2. Biosparging 3. Bioventing	- Same as solid phase - Chemical solubility - Geological factors - Regulatory aspects for groundwater	- Soil and water treated - natural attenuation processes - simultaneously - Least cost - Complements - Noninvasive	- Physiochemical control - Monitoring progress and effectiveness - Extended treatment time
Bioreactor	1. Aqueous reactors 2. Soil slurry reactors	- Toxicity of amendments - Toxic concentrations of contaminants - Same as solid phase	- Use of surfactants and inoculants - Most rapid degradation - Enhanced mass transfer - Controlled conditions -	- Material input requires physical removal - Relatively high capital costs
Solid Phase	1. Soil Treatment 2. Land Farming 3. Engineered soil cell 4. Composting	- Presence of metals and other organics - Biodegradability - pH, temperature, moisture control - Catabolic capabilities of indigenous microflora	- Low O & M cost - Perform onsite Modestly effective for HMW PAHs -	- Bioavaibility - Space requirement - Extended - treatment tie - Control abiotic less

Earthworm is another alternative solution for PAHs degrader due to their ability to stimulate the accessibility of oxygen in the soil. In addition, earthworms have been extensively reported as soil engineer because of the prospective of earthworms ability to adapt in contaminated soil with high concentrations of PAHs and also to digest PAHs into simpler molecule [27-30]. Dabke reported [31], to eliminate pollutant such as phenolic compound from soil the earthworms were added to promote and enhance microbial activities added into contaminated soil by forming good conditions for bacteria [32-24]. Moreover, in the soil contaminated with petroleum hydrocarbons, *Eisenia fetida* is able to survive without constraints and accelerate degradation of crude oil in the soil [35, 36]. Hence, It can be considered that earthworms not only degrade pollutants however also stimulate and collaborate with other microorganisms in removal of pollutant in the soil. Analytical results reported by Asgharnia et al. [37] further showed that earthworms enhance potential soil quality, increased microbial activity and cause an increased in phenanthrene bioavailability, allowing more efficient in microbial degradation. Nonetheless, there is a essential to select a sustainable organism or biomaterial for PAHs degradation to avoid fatal by-products and production of unwanted toxic during degradation process.

In this study, earthworms (*Eisenia fetida*) were selected for pyrene degradation. In recent years, the use of earthworm have been extensively studied, consistent and stable degradation strategy have not been well developed. Therefore, to develop a suitable

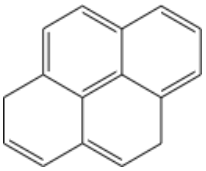
biodegradation approaches become our immediate research significance. Pyrene degradation pathway was developed in the study to investigate the role earthworms in the mechanism of ring cleavage and degradation of pyrene, which eventually led to pyrene breakdown and its end product.

2. Materials and Methods

2.1. Materials

The pyrene was purchased from TCI (Tokyo, Japan). The properties of the pyrene are presented in Table 3. In addition, N,N-dimethylmethanamide, Dichloromethane, toluene were supplied by Macherey-Nagel (German), meanwhile, Thin Layer Chromatography (TLC) silica gel 60 F254 aluminum sheets, Silica 60 (0.2-0.5mm) were supplied by Merck (German).

Table 3. Physical properties, application and harmful effect of pyrene

Name	**Pyrene**
Empirical Formula	$C_{16}H_{10}$
	
Melting Point (℃)	152
Boiling Point (℃)	378
Molecular weight (g mol^{-1})	202.3
Sources	Coal tar
Harmful effect	No carcinogenic effect (identified by IARC) Toxic to kidneys and liver
Application	For indicator for PAHs-contaminated wastes monitoring, to determine solvent environments, and dye production and dye precursors.

2.2. Microorganism and culture medium

The earthworms were collected in shady areas besides sewage ponds, in the vicinity of Kolej Rahman Putra, Universiti Teknologi Malaysia (1°34’13.1”N 103°38’40.6”E) (Malaysia). The collection activity is easier during cloudy day especially after rainfalls, earthworms will move to surface side of soil for respiration. Earthworms were kept in moist and shady moist condition. Meanwhile, for culture medium a total of 5 g of earthworms (4-5 earthworms) were added and weighed for 200 g of soil. Another alternative to soil was using cow dung that collected from UTM farm. Afterward, for 200 g of cow dung was added 5 g of earthworms. The moisture content was maintained at 60 % and the earthworms were fed with kitchen leftovers. After 7- and 14-days earthworms’ growth in cow dung and soil was recorded. 100 mg L^{-1} of pyrene was prepared, added and mixed into the cow dung and soil, respectively. The degradation rate of pyrene was calculated using Equation (1). The difference between initial pyrene concentration and final concentration was divided by initial concentration and multiply with 100 %.

$$Biodegradation\ Coefficient = \frac{Co-Ce}{Co} x\ 100 \qquad (1)$$

Where C_e is the final concentration of while C_o represents the initial concentration of pyrene.

2.3. Morphology and macroscopic characterization

Earthworms that degrade pyrene were characterized based on their morphology and macroscopic. The external morphology of the earthworms were further studied and recorded.

2.4. Soil moisture content

The soil was collected from origin habitat of earthworms and oven dried to measured soil moisture content. Constant value of soil weight was recorded. Based on Equation (2) moisture content in soil was calculated. Three soil samples with the weight of 20 g, 100 g and 200 g were subjected to soil moisture content analysis.

$$Soil\ Moisture\ Content = \frac{Initial\ soil\ weight - Final\ soil\ weight}{Initial\ soil\ weight} x\ 100\% \quad (2)$$

2.5. Soil to earthworm's ratio

Earthworms (4-5 earthworms) were weighed for 5 g and added into 20 g, 100 g and 200 g of soil, respectively. The moisture content was maintained by measuring the soil initial moisture content and adjusted with water according to need. The earthworms were fed with 5 g of kitchen leftovers (eaten apple waste) and the moisture content was maintained at 60 % $\pm$ 5 %. The growth rate of the earthworms was recorded after 7 and 14 days of incubation.

2.6. Effect of soil sterilization

The effect of sterilized soil on pyrene degradation by earthworms were evaluated in 200 g of soil sterilized at 120°C for 60 minutes. At the same time, 200 g of soil was prepared without autoclave. 100 mg L^{-1} of pyrene were formulated for both of soil samples. Thereafter, both of soil sample was added approximately 5 g of earthworms and left it for 7 and 14 days. The extraction was carried out and pyrene degradation rate was analyzed. Sterilized and unsterilized soil without the addition of wild worms were used as control in this study.

2.7. Effect of initial pyrene concentration

To evaluate effect of initial concentration of pyrene two initial concentrations of pyrene were used at 100 mg L^{-1} and 200 mg L^{-1}, formulated into 200 g of soil. The pyrene was dissolved in DMF before added into soil. The earthworms were added into contaminated soil after solvent vaporized (maximum 3 days left in open air) and incubated for 7 and 14 days. All samples were extracted and purified before analyzed.

2.8. Pyrene extraction and purification

Wet filter paper was prepared for earthworm after removed from soil and kept for 24 hours. Earthworms were dried with liquid nitrogen and allowed to freeze at 0°C. 5 g of sodium sulfate (Na_2SO_4) were added and mixed to the 5 g of earthworms [38]. Meanwhile for earthworm extraction, 20 mL of ethyl acetate was added and kept for overnight. The same procedure was conducted twice. In the meanwhile, soxhlet extraction method was used for soil extraction of pyrene. Before thimble being placed in soxhlet extractor total of 50 g of soil were filled into soxhlet thimble. 150 mL dichloromethane was used as solvent extraction and filled into distillation flask. Anti-bumping granules were added into round flask. The extraction was heated for 24 hours with dichloromethane to boiling point (40°C). The compounds were

concentrated in the distillation flask after many cycles of extraction. Then, 1-2 mL of concentrated extracts were collected after concentrated with rotary evaporator. The column chromatography was used for purified concentrated crude extracts. In the column hexane were diluted into 5 g packed of silica gel. The crude extract was concentrated using rotary evaporator up to 1-2 mL by 150 mL of hexane eluted through the column. Then, toluene was added into concentrated purified extract up to 10 mL to make final volume before injected into Gas Chromatography.

2.9. Comparison of pyrene degradation using different experimental approach

2.9.1. Soil incubation degradation

Prepared 200 g of soil was formulated with pyrene. DMF and polyethylene glycol sorbitol monooleate was used as solvent to dissolved 100 mg L^{-1} of pyrene before added into soil. 5 g of earthworms (4-5 earthworms) was added into the soil after the solvent allowed to dry. The soil moisture content was maintained at 60%. kitchen leftovers were added into soil to fed earthworm. Analyzed was investigated after 3, 7, 10 and 14 days. Meanwhile, 200 g of soil with pyrene formulated without any addition of earthworm were prepared as control in experiment. 200 mg L^{-1} of pyrene were used following the method used above. Sterilized and unsterilized soil was used to compare the effectiveness of earthworms in pyrene degradation. All experiments were conducted in duplicate.

2.9.2. Crude enzyme and earthworm powder

In this method, ethanol was added and blended into 5 g crushed earthworms [39]. After the earthworms were pureed evenly in a blender, filtration was used to collect supernatant (ethanol and crude enzyme). 200 g of soil containing 100 mg L^{-1} of pyrene was added by crude enzyme. After 3, 7, 10 and 14 days degradation rate was analyzed. Meanwhile, powder form of earthworm was collected after earthworms bodies dried in the oven. 10 mg L^{-1} of pyrene was added into 20 mL distilled water followed by earthworm powder. After 3, 7, 10 and 14 days of incubation degradation rate was analyzed.

3. Results and Discussion

3.1. Morphology and macroscopic characterization

Earthworms used in this research were collected from shady areas under the leaf litter in UTM Johor. It was dark brown in color. The worms has a mean length of 6-12 cm, diameter of 0.3-0.6 cm and 80 to 120 segment number depends on the size of individual worm. A worm will excrete mucus as it moves through soil or floor. The worms can easily survive and can live up to 4 weeks without food. They can survive and tolerance in polluted environment and have high tolerance towards PAHs.

3.2. Soil moisture content

Soil collected from wild worms were collected. The results shown in Table 4. The subject for analyzes was selected from three different weight of soil. Based on the results, the soil moisture content for 20, 100 and 200 g was 58, 59 and 62 %, respectively. In this study, that moisture content 60 $\pm$ 5 % in soil is suitable for earthworms' growth. Several studies showed that the optimum growth function of earthworm in the soil was at 60-75 % of moisture content [40-42].

It is important to maintain a correct moisture content to ensure optimum growth rate of earthworms achieved. Low moisture conditions could also delay sexual development and it was found that earthworms developed clitella at different times under different moisture conditions [43, 44]. Therefore, at 60 ± 5 % of soil moisture content was maintained when the experiments involved earthworms' culture.

Table 4. Soil moisture content collected from UTM

Moisture content (%)	Weight (g)
58	20
59	100
62	200

3.3. Soil to worm ratio

The earthworms (4-5 worms) were weighed for 5 g and placed in container containing 20, 100 and 200 g of soil, respectively. The constant moisture content 60 ± 5 % was ajusted in containers with worms and placed in shady area. The survival rate and weight of wild worms were recorded in Table 5. According to the data shown in Table 5, 3 out of 5 worms placed in 20 of soil (worm to soil ratio of 1:4) was dead after 14 days. The weight of worms reduced from 5.14 to 1.87 g. The worm struggled to adapt to the new living environment and try to crawl out from the flask. Meanwhile, there were no death worms found in soil ratio of 1:20 and 1:40. There was only 1.9 % of weight lost in 1:40. This happen due to the lack of congestion, lack of restriction in term of moving and the worms feel comfortable in a larger and wide soil ecosystem.

3.4. Earthworms culture medium

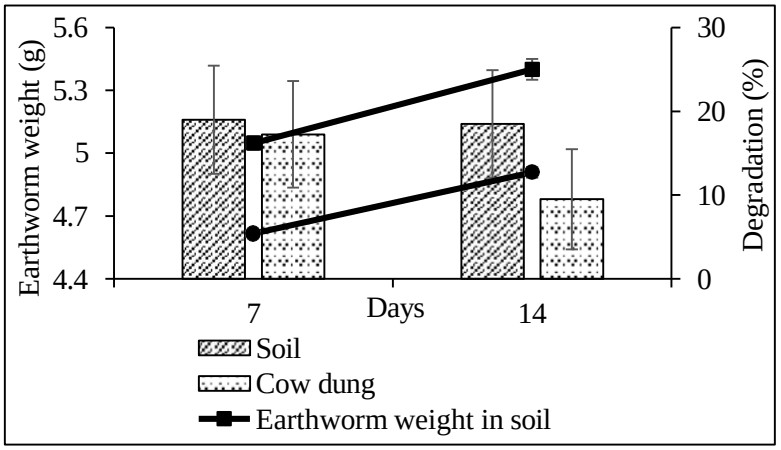


Figure 1. Pyrene degradation using earthworms in cow dung and soil

Two types of culture media were used to cultivate the worms, which are cow dung and soil. Each medium was formulated by 100 mg L^{-1} of pyrene. Degradation of pyrene was investigated in both media in order to confirm the survival rate of earthworm. Figure 1 shows that the wild earthworms exhibited better pyrene degradation efficiency in the soil where it was collected. A total of 16.2 % and 25 % of pyrene was degraded by wild worms in soil after 7 and 14 days, respectively. In cow dung, wild worms successfully removed 5.4 % and 12.7 % of pyrene after

7 and 14 days. Besides that, the weight of worms was maintained after 14 days in soil, with a loss of only 1.2 % in total worms' biomass. In contrast, worms in cow dung lost 0.39 g or 7.5 % of biomass after 14 days. Earthworms can accelerate PAHs degradation rate by itself or stimulate bacteria or microorganisms present in soil by improving the aeration in the contaminated soil. Similar studies had investigated the effect of the addition earthworm in PAHs removal process. In this regard, the degradation rate has vastly improved compared to the treatment using only indigenous microorganisms [28, 45]. Earthworms improved the soil structure when they burrow and crawl in the soil by contracting their bodies. This introduces the oxygen needed by microorganisms to breakdown the pollutants.

3.5. Effect of initial pyrene concentration

Pyrene with the concentration of 100 mg L^{-1} and 200 mg L^{-1} were mixed into 2 separate containers with 200 g of soil. As a control, 5 g of earthworms were added into uncontaminated. Meanwhile, contaminated soil was added a total 5 g of earthworm and incubated for 7 and 14 days, respectively. Figure 2 shows the effect of initial pyrene concentration on pyrene degradation by earthworms. Figure 2 shows the capability of earthworms in pyrene degradation when 2 pyrene concentrations were used, 100 mg L^{-1} and 200 mg L^{-1}. After 7 days incubation 16,2% of pyrene was removed from initial concentration 100 mg L^{-1}, while after 14 days the value increased to 25 %. There was no growth after 7 days incubation (weight reduce by 0.4 g compared to first day) while after 14 days incubation the weight increased by 0.9 g. This happen because of earthworm has adapted with harmful condition or environment (the presence of pyrene) at the beginning of the study. Meanwhile, the degradation rate was slower when the initial concentration increased to 200 mg L^{-1}. As the result shown that, after 7- and 14-days incubation was achieved 6.4 and 11.7 %, respectively. Based on the result observation, 200 mg L^{-1} of pyrene in contaminated soil were found earthworm dead, however not in lower concentration. Thus, the higher initial pyrene concentration in soil proved to be harmful to earthworms.

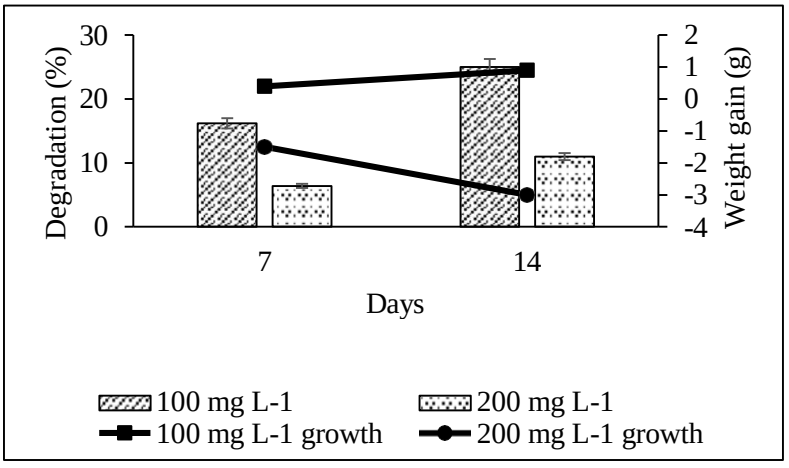


Figure 2. Effect of initial pyrene concentration on pyrene degradation rate using earthworm

3.6. Effect of soil sterilization

Figure 3 shows the results of pyrene removal in sterilized and unsterilized soil after 7 and 14 days by earthworms. In unsterilized soil, the removal of pyrene was achieved at 16.2 % and

25 % after 7- and 14-days incubation. In the meantime, degradation of pyrene became slower in sterilized soil and a total of 5.6 % and 9 % of pyrene were degraded after 7 and 14 days of incubation. Moreover, by sterilizing the soil directly eliminate all advantages microorganisms in the soil and the indigenous promoting bacteria. In the ecosystem, symbiotic between bacteria and enzyme produced from earthworm bodies could play role important for metabolism in degradation of pyrene [46], however, better degradation requires longer time. In this light, earthworms improved biological activity and conditioning substrate to act as a catalyst for the degradation process [47]. In unsterilized soil, earthworms enhance pyrene degradation by stimulating microbial activity and growth via excretion of readily degradable carbon [48]. The presence of earthworm in unsterilized soil increased the bioavailability of pyrene and degradation of contaminants due to re-arrange of soil particles and create aerobic conditions of the soil through continuous mixing [47, 49, 35].

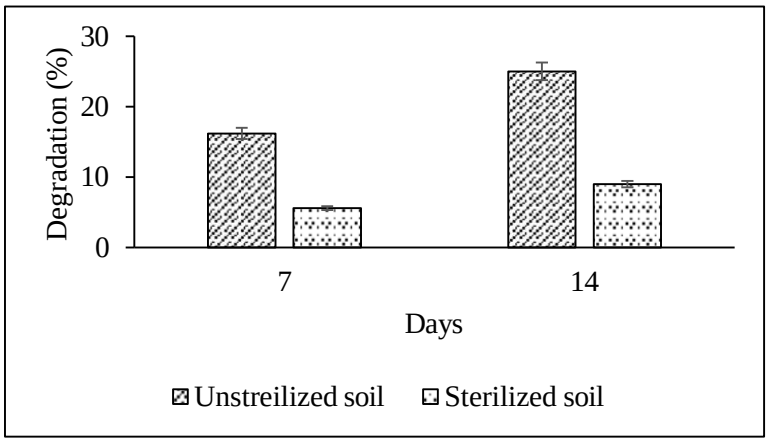


Figure 3. Pyrene degradation performance of wild worms in sterilized and unsterilized soil

3.7. Comparison of pyrene degradation using different experimental approaches

To investigate the relationship between earthworm and degradation of pyrene several experimental approaches were conducted. In this section, the filtrate crude enzymes from earthworms' bodies and earthworm powder was investigated and applied in degradation of pyrene. The experiments were conducted in 3, 7, 10 and 14 days. The results shown in Figure 4, the most effective method for degradation of pyrene was used earthworm powder. After 3 days incubation, 100 mg L^{-1} of pyrene was removed up to 30.7 %. Nevertheless, after 14 days incubation removal rate was decreased to 16.8 %. The surface or pore of earthworm has covalent bond, that function was similar with activated carbon that allows the attachment of pyrene on the surface [50]. Meanwhile, the crude enzymes extracted from earthworms' bodies were investigated with similar incubation times. After 7 days incubation the highest degradation rate was achieved at 19.1 % and gradually decreased after 14 days incubation to 12.1 %. In this case, it was happened due to the enzymes extracted from earthworms' bodies not preserved well. Therefore, the enzymes extracted from earthworms' bodies was denatured. It could be concluded that crude enzyme from wild earthworms started to denature after 7 days. Capability of earthworms' enzyme in pyrene degradation was due to the broad substrate specificity of extracted enzymes. Protease is a common enzyme found in earthworms' bodies, shows several processes of activity in the present different of substrates and this could make

their existing in an unpleasant environment [51]. In conclusion, enzymatic reactions are performed effectively well under good conditions and processed without waste-generating protection and deprotection steps common in conventional organic syntheses [52]. During the soil incubation method, after 3 days incubation was found 14.1% of pyrene removed. The degradation rate was increased after 7 days incubation up to 22.7%. In this case, prolong the incubation time does not assist in the further degradation activities. Therefore, can be concluded it was reached capability of wild earthworms on the PAHs degradation. Pyrene degradation rate increased with time in soil incubation method. Hence, this method is deemed as the most suitable pyrene degradation method.

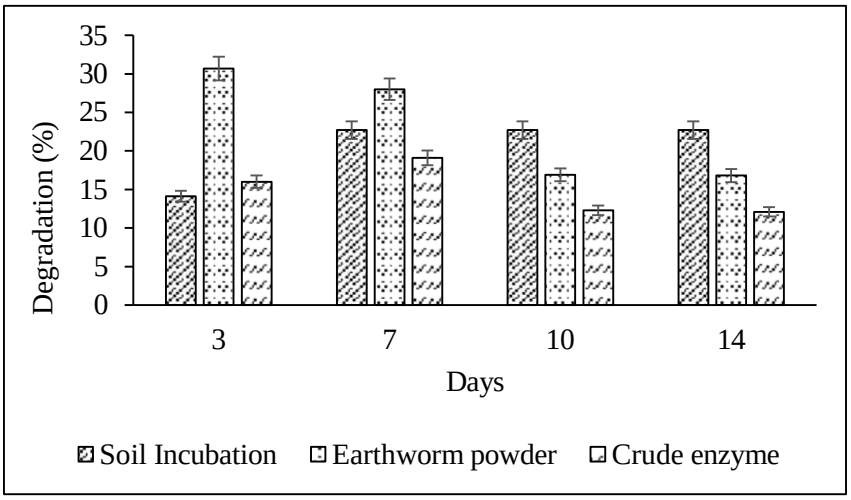


Figure 4. Pyrene degradation using different experimental approaches by using earthworm

4. Conclusions

In this study wild worms were collected from shady areas in UTM Johor and then cultured in the soil with the moisture content of 60 $\pm$ 5 %. Earthworms grows better in a wider environment, with ratio of earthworm to soil at 1:40. Earthworms exhibit better degradation efficiency in soil compared to cow dung, with 25 % of pyrene was degraded by worms in soil while only 12.7 % of pyrene was degraded in cow dung. In earthworm analysis, earthworm to soil ratio was fixed at 1:40 and 5 g of earthworm in 200g of soil containing 100 mg $L^{-1}$of pyrene are enhance degradation up to 25 % of pyrene. Meanwhile, by increased pyrene concentration cause increased death rate of earthworms. Sterilized the soil could eliminated the bacteria presence in the soil, thus reducing pyrene degradation rate. The most effective method for degradation of pyrene was used earthworm powder, with 30.7 % of pyrene was removed after 3 days. there is no metabolite produced during pyrene degradation pathway by wild earthworms. The pyrene might be fully degraded or accumulated in fatty tissue in earthworms. This research provides a framework on the exploration of earthworm as a PAHs degrader. Furthermore, this method can be used in petroleum spillage cleanup under extreme conditions. As whole this study present a potential environmental benefit and low cost operations.

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Competing Interest

There is no competing interest to declare.

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