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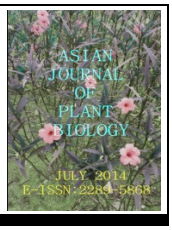

Bioremediation of Crude Oil by Different Fungal Genera

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One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Bioremediation is the promising technology for treatment of these contaminated sites as it is cost effective and lead to complete mineralization. This research attempts to study the potential of different fungal genera in bioremediation of hydrocarbon. *Aspergillus flavus*, *Aspergillus versicolor*, *Bionectria ochroleuca*, *Penicillium chermisinum* and *Trichoderma virens* were selected for the bioremediation purpose. Screening of fungi species sensitivity towards hydrocarbons was first conducted. To enhance the growth of fungi on hydrocarbon contaminated soil, suitable bulking agent was selected prior to addition into the soil. Hydrocarbon degradation trial was conducted followed by post-treatment tests for a period of six weeks. All fungal species show high tolerance towards hydrocarbon. Sago waste (*sago hampas*) was the most suitable bulking agent as all fungal species were capable to grow on it. Significant differences were found in the ability of *Bionectria ochroleuca* to degrade hydrocarbon. *Bionectria ochroleuca* was able to degrade more than 70 % of the C_{12} to C_{28} hydrocarbons, and with 100 % degradation of C_{12} and C_{28} hydrocarbons.

INTRODUCTION

Demand for petroleum as a source of energy and as primary raw material for the chemical industry has increased each year. A typical oil refinery in Malaysia which capable of producing 10 500 barrels per day will produce roughly 50 tons of sludge per [1]. Oil sludge need to handled carefully since its constituents are carcinogenic, immunotoxicants and mutagenic. Toxicity profiles of petroleum hydrocarbons to microorganisms, plants, animals and humans are well established. For example, low concentration (5-100 mg/l) of crude oil or petroleum fractions is sufficient to kill or inhibit the growth of microalgae and juvenile forms of marine animals [2]. Proficient technology in term of cost and efficiency is highly demanded in order to remove or degrade the hazardous constituents of oil sludge.

Bioremediation appear to suit the characteristics of the demanded technology. Bioremediation is a process of using microorganisms to convert hazardous pollutants into less toxic compounds. Over the past twenty years, fungal bioremediation or mycoremediation become the main desirability for all researchers who involve in bioremediation field. Due the chemical resemblance of lignin and PAHs and some other environmental pollutants, ligninolytic fungi have been regard as the most promising candidates to degrade PAHs [3]. White rot fungi produce three types of enzymes which involves in the degradation of lignin. The enzymes are lignin peroxidase (LiP), Manganese Peroxidase (MnP), and Laccase (Lac) [4,5].

The main objective of this research was to discover the full potential of fungi in bioremediation of hydrocarbon contaminated soil. To reveal fungi full potential, this research began with choosing suitable bulking agent and to determine whether fungi able to grow and penetrate into the contaminated soil with or without bulking agent. This study also reports on the results from bioremediation trial after proceed the selection of the best hydrocarbon degrading fungi on hydrocarbon contaminated soil.

MATERIALS AND METHODOLOGY

Microbial preparation

Five species of indigenous fungi, known with the potential of hydrocarbon degradation, were obtained from UNIMAS Molecular Biology Microbial Collection. The five species are *Aspergillus flavus*(UMAS-HDF8), *Aspergillus versicolor* (UMAS-HDF6), *Bionectria ochroleuca* (UMAS-BHDF7), *Penicillium chermesinum* (UMAS-HDF2), and *Trichoderma*

virens (UMAS-HDF7). Fungi were grown on potato dextrose agar (PDA, Merck) plates at 28 °C for 7 days before being stored at 4 °C and were subcultured every 3 months [6].

Liquid growth, buffer solution and soil preparation

The fungi were grown in glucose–malt extract–yeast extract (GYM) liquid growth media with slight modification before being applied to the bulking agents [7]. The liquid growth was composed of glucose 10 g l^{-1}, malt extract 3.5 g l^{-1}, yeast extract 2.5 g l^{-1}, KH_2PO_4 2.0 g l^{-1}, and $MgSO_4.7H_2O$ 0.5 g l^{-1}. The media was prepared in 500 mL batches in 1 L flasks and then were sterilized at 121–124 °C for 15 minutes before being stored in the refrigerator. Sixty millimolar (mM) sodium phosphate (Na_3PO_4) buffer solution at pH 6 wasprepared for the adjustment of soil moisture content and to keep soil pH slightly acidic. The uncontaminated soil samples were bought from a nursery in Kuching, Sarawak. After transportation to the laboratory, the soil was air dried. The drying process was conducted at room temperature to preserve soil indigenous microorganisms. Drying was performed to facilitate subsequent grinding and to increase contact between soil and the organic solvent used for extraction [8]. All large particles and extraneous materials were removed by hand and the soil was pulverized in an electric blender. The powdered soil was sieve using a sieve with 300 μm opening. The prepared soil was kept in airtight containers. Artificially contaminated soil is prepared by adding crude oil to the soil at a concentration from 1% of up to 5% (v/w). To make a homogenous mixture, the oil was first dissolved in hexane and the solution was then sprayed over the soil. The soil is then kept under fume hood for 24 h for the solvent to evaporate.

Screening of fungi strains sensitivity towards hydrocarbon

The sensitivity assay was used to determine the toxicity threshold of the five hydrocarbon degrading fungal isolates to crude oil contamination in the soil. The assay was conducted by comparing the differences between the fungal growth rates on the agar plates amended with different concentration of crude oil and control agar plates made of agar without the addition of crude oil. Potato Dextrose Agar (PDA) and Minimal Salt Agar (MSA) were used. Test plates were prepared by adding the crude oil to warm agar solution. In order to have a uniform concentration of crude oil in all plates, the solution was thoroughly mixed using a magnetic stirrer, right before pouring onto the plates. Five concentrations of crude oil–agar mixture [1%, - 5% (v/w)] was prepared. Control plates were prepared from pure Potato Dextrose Agar (PDA) and Minimal Salt Agar (MSA). All of the plates were inoculated with 1 mm diameter plugs of fungal mycelia taken from agar inoculum plates. The plates are incubated at 28 °C. Fungal mycelia extension on the plates was measured using a ruler on a daily basis and compared against the control plates in order to determine any possible toxicity**.**

Bulking agent selection

Sawdust, oil palm empty fruit bunch (OPEFB), rice husk, sago waste and mixture of bulking agents are used for fungal growth assay on different bulking agents. Fungi species were grown for 7 days in 50 mL of GYM media in a 250-ml flask, at 28 °C [7]. The cultures will be homogenized for 20 s using a homogenizer and 15 mL of each fungus homogenate were used to inoculate the bulking agents. In order to minimize the nutrients carry over from the growth media, the fungal homogenate was centrifuged and the supernatant was discarded. Then the remaining mycelia and Minimal Salt Media (MSM) were mixed and added to the bulking agents. During the experiments, the growth and penetration of the fungal test strains on different bulking agents was assessed qualitatively. The performance of the best bulking agent or a mixture of them was then further assessed in contaminated soil tests**.**

Fungal growth study in artificially contaminated soil

The main purpose of this growth study was to assess the ability of fungi to grow and penetrate into the contaminated soil with or without bulking agent. Growth studies in the artificially contaminated soil were performed using a bulking agent in artificially contaminated soil at concentrations of 0% to 5% (v/w). The inocula were prepared by homogenizing 1 cm^2 of mycelium (with part of PDA plug matter) from a colony on a PDA plate in 50 mL of GYM medium for 20 s. After 7 days of growth in a 250-mL flask at 28 °C, the fungus was homogenized and 15 mL of the fungal homogenate then centrifuged. Next, the supernatant was decanted and the remaining mycelia were used to inoculate the soil. Separation of the supernatant minimizes the nutrient carry over from the liquid media into the soil. Approximately 100 g of artificially contaminated soil was used for each test. The soil was placed into a 250-mL conical flask. The bulking agent was added on top of the soil. The fungi mycelia is then mixed with approximately 40 mL of Minimal Salt Media (MSM) and added to the flasks. The flasks were then kept in room temperature until the fungal mycelia had grown and covered the bulking agent surface completely. The flask's contents were then completely mixed and kept at room temperature for another 14 days. The growth and penetration of fungi in the soil were assessed qualitatively with the aid of a magnifier.

Screening of fungi for bioremediation of hydrocarbon contaminated soil

The main purpose of this growth study was to assess the ability of fungi to grow, penetrate into the contaminated soil with bulking agent and degrade the hydrocarbon. Fungal inocula of all species that were used were prepared as described in previous section. Approximately, 100 g of hydrocarbon contaminated sludge was prepared by mixing the soil thoroughly with 10 ml of crude oil and placed in a 250 mL beaker. Bulking agent was first added on top of the soil. Approximately, 10 mL of fungal inocula resuspended in Minimal Salt Media (MSM) was added to the beakers. The beakers were then kept at room temperature until the fungi mycelia had grown and covered the bulking agent surface completely. The flask's contents were then completely mixed and kept at room temperature for 6 weeks. As for control, 100 g of hydrocarbon contaminated sludge was placed in a glass beaker without any addition of the fungal mycelia. Two types of control were used in the experiment: unsterile soil without the addition of fungal inoculum in order to evaluate the degradative capacity of indigenous microflora present in the soil (indigenous culture) (C1) and sterile soil without addition of inoculum used as an abiotic control to account for loss by evaporation (C2).

The experiment was conducted in duplicates. Residual crude oil was measured based on method described by [9], which is using cold toluene method. Before residual crude oil analysis conducted,

each sample was weighed to 10 g and mixed with sodium sulphate (Na_2SO_4) to bind water. The residual crude oil was then extracted using 20 mL toluene and measured using a spectrophotometer at 420 nm. This step was repeated three times to obtain mean value and standard deviation.

Bioremediation of hydrocarbon contaminated soil

Approximately, 200 g of hydrocarbon contaminated sludge was prepared by mixing the soil thoroughly with 20 mL of crude oil. Then it was transferred into a 1 L glass beaker. Bulking agent was first added on top of the soil. Approximately, 20 mL of the fungal inocula resuspended in Minimal Salt Media (MSM) was added into the flasks. The beakers were kept in water bath with the temperature set at 29 °C until the fungal mycelia had grown and covered the bulking agent surface completely. The flasks contents were then completely mixed and kept in water bath with the temperature set at 29 °C for a period of 6 weeks. As for the control, 200 g of hydrocarbon contaminated sludge was placed in a 1 L glass beaker without any addition of the fungal mycelia. Two types of control were used as described in the previous section. The experiment was conducted in triplicates.

Hydrocarbon analysis

Soxhlet extraction

Soxhlet extraction was performed with some modification [10]. Approximately, 10 g portions of the dry contaminated soil were transferred into a cellulose extraction thimble (Schleicher & Schuell, Germany) and inserted into the soxhlet assembly. This apparatus was fitted with a 250 ml flask containing 100 ml of toluene. The assembly was heated and refluxed for a period of 6 hours. The resulting extract was evaporated to partial dryness using a rotary evaporator.

Cold toluene

Hydrocarbon extracted by Soxhlet extraction method was analyzed using cold toluene method [9]. Approximately 20 mL of toluene was added to the extracted hydrocarbon and mixed well by shaking thoroughly. Absorbance of the mixture was analyzed at 420 nm and recorded. This step was repeated three times.

Gas Chromatography Flame Ionization Detector (GC/FID)

The analysis of all the extracts, (f_1) and (f_2) was carried out on gas chromatography apparatus which was equipped with flame ionization detector (FID)[11]. A HP-5 fused silica capillary column (50 mm x 0.32 mm x 0.25 µm). Nitrogen was used as the carrier gas. The GC oven temperature was held isothermally for 5 min at 70 °C, programmed to sequentially step from 70 to 290 °C at 3 °C min^{-1}, and then held isothermally for 40 min at 290 °C. Quantitation was achieved by integration of the peak areas.

Bioremediated soil toxicity test

Microbiological analysis

Microbiological analysis was conducted by quantifying the number of bacteria colonies and fungal growth from soil [12]. For bacterial colonies quantification, 1 g of bioremediated soil was added into 9 mL of sterile distilled water and serial dilution was conducted from the dilution power 10^1 to 10^5. Spread plate method was employed using Nutrient Agar (NA). For fungal growth, 1 g of bioremediated soil was added into 9 mL of sterile distilled water and serial dilution was conducted from the dilution power 10^1 to 10^5. Spread plate method was employed using Potato Dextrose Agar (PDA). The tests were conducted in triplicate for each of the dilution power. Colonies were counted for the enumeration of the microbes.

Root length analysis

Root length test of mung bean (*Vigna radiata*) was carried out on water extracts by resuspending the bioremediated soil samples with distilled water at soil to distilled water ratio of 1:10 (w/v) ratio for one hour [13]. The samples were then filtered. The extract from non-inoculated soils and untreated soils were used as control and conducted in duplicates while the extract from inoculated bioremediated soils was conducted in duplicate (Zeng *et al*., 2009).

RESULTS AND DISCUSSION

The sensitivity assay was used to determine the sensitivity threshold of five hydrocarbon degrading fungi isolates to crude oil contamination of the soil. The assay was conducted by comparing the differences between the fungal growth rates on the Petri dishes test and control Petri dishes. The results are as shown in Table 1. As for the process of screening fungi species sensitivity towards hydrocarbons, all of fungal species was found able to grow onto Minimal Salt Agar (MSA) and Potato Dextrose Agar (PDA) amended with crude oil within the range of 0% to 5% (v/w) of crude oil.

Table 1: Results for visual observation of mycelia extension on contaminated PDA plate

Concentration of crude oil (v/w)	1%		2%		3%		4%		5%	
Time passed (days)	7	14	7	14	7	14	7	14	7	14
P. chremesinum	**	**	**	**	**	**	**	**	**	**
A. versicolor	**	**	**	**	**	**	**	**	**	**
T. virens	**	**	**	**	**	**	**	**	**	**
A. flavus	**	**	**	**	**	**	**	**	**	**
B. ochroleuca	**	**	**	**	**	**	**	**	**	**

A scale of '--' to '**' was used to indicate rate of fungal growth inhibition in the presence of crude oil. '--' represents the complete inhibition and '**' no fungal inhibition.

However, only *A. versicolor* did not show full plate growth as compared to the other species. *A. flavus*and *T. virens* showed the highest tolerance towards crude oil with the range of 0% to 5% (v/w) of crude oil on Minimal Salt Agar (MSA) and Potato Dextrose Agar (PDA) as they showed complete growth on day 7. Meanwhile, *B. ochroleuca* and *P. chermesinum* showed complete growth on day 9. Time taken by each species to grow fully on a plate differs based on the growth rate of each species respectively. Although *A.flavus* and *T. virens* have faster growth rate compared to the others, there is no difference in time needed for the fungi to grow fully on the plate compared to the control plate. Therefore, high concentration of crude oil amended into the agar did not give any effect on the growth rate of the fungi [14].

Bulking agent is vital in bioremediation because some fungi are not capable for using hydrocarbon from petroleum or crude oil as their sole carbon source.Throughout the two weeks incubation period, sago waste turn out to be the most suitable bulking agent. Although the growth of each species emerged on different number of days, all of the species are capable of growing on sago waste with *T. virens* and *A. flavus* growth emerged on day 4, *A. versicolor* and *B. ochroleuca* growth emerged on day 5 and finally, *P. chermesinum* on day 7. Respectively the growth of *B. ochroleuca, P. chermesinum, A. versicolor, T. virens* and *A. flavus* are presented in Figure 1.

Sago waste (*sago hampas*) is a starchy lignocellulosic by-product generated from pith of *Metroxlon sagu* (sago palm) after starch extraction. Sago waste (*sago hampas*) contains the pith from sago trunk with some straw like substance which is significantly contributed to the void space in the soil. Therefore, it can be used alone as the bulking agent. Sago waste (*sago hampas*) is largely made of cellulose and lignin which have some potential to be a biosorbent [15]. Sago waste (*sago hampas*) itself acted as another carbon source for the fungal growth in the hydrocarbon contaminated environment. Bulking agent played a role in reducing soil bulk density as well as serving as an additional organic material during the bioremediation process in addition to increasing soil porosity and oxygen diffusion this stimulates microbial activity [16,17]. Apart from the co-metabolism strategy of some fungal species, bulking agent that is used in bioremediation provides acclimatization before the fungal species can start to degrade the contaminants. Acclimatization is a very important step as it will help the fungi to adapt to the environment [18].

The ability of various fungi to degrade hydrocarbon in chemically defined liquid media has been conducted in many laboratory experiments. However, introduction of fungi into the field for bioremediation purpose is relatively few. This is especially for bioremediation of hydrocarbon contaminated soils, sediments, and waters. One major setback to bioremediation is the inability of the mycelium to go through the soil [19,20]. Therefore, the fungal growth study of artificially hydrocarbon contaminated soil was conducted to understand the growth pattern of the fungi.In a period of two weeks and addition of sago waste as bulking agent into artificially hydrocarbon contaminated soil, all of fungal species did not show any difficulty to grow and penetrate the contaminated soil based on visual observation. *A. flavus* and *T. virens* were visibly able to grow and penetrate the soil starting from day 3. On day 5, *B. ochroleuca* and *P. chermesinum* were observed to do the same. As for *A. versicolor*, fungal growth and penetration was observed on day 6. The growth and penetration of each fungus are presented in Figure 2.

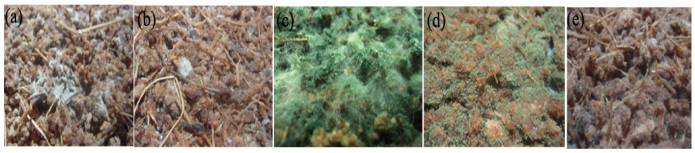

Figure 1: (a) *Penicillium chremesinum*UMAS-HDF2, (b) *Aspergillus versicolor*UMAS-HDF6, (c) *Trichoderma virens*UMAS-HDF7, (d) *Aspergillus flavus*UMAS-HDF8, and (e) *Bionectria ochroleuca*UMAS-BHDF7 on sago waste.

Figure 2: Fungi growth on soil with addition of sago wastes (a)*Bionectria ochroleuca*. (b)*Penicilin chermesinum*. (c) *Aspergillus versicolor*. (d) *Trichoderma virens*. (e) *Aspergillus flavus*.

The main purpose of growth study was to select for the most suitable fungi species that can be used in bioremediation trial based on the ability of fungi to grow, penetrate into the contaminated soil with bulking agent and degrade the hydrocarbon. Cold toluene method was employed for the quantification of degraded hydrocarbon [9]. Within the period of six weeks, two patterns of hydrocarbon degradation were observed. *P. chermesinum*, *A. flavus* and *T. virens* showed that they absorbed the hydrocarbon from the soil before releasing the hydrocarbon as the absorbance reading of toluene decreasing from week 2 and started to increase by week 5. The increasing absorbance observed might be due to the release of byproducts from biodegradation. This statement can be supported by studies conducted by [21] and [22]. Both studies suggested that contaminants are being absorbed before degradation occurs and the byproducts are released back to the surrounding. Meanwhile, for *B. ochroleuca*, no hydrocarbon was absorbed as the absorbance reading kept on decreasing until week 6.

Based on cold toluene absorbance reading, volume of crude oil (hydrocarbon) left in the soil was quantified using equation from prepared hydrocarbon standard curve (presented in Appendix B). Percentage of hydrocarbon degradation for week 6 is shown in Table 2. From the results, *B. ochroleuca* is shown to be able to degrade up to 89% of the 10 mL hydrocarbon (crude oil) that was added into the soil at the initial stage of the experiment. The four other strains showed just a slightly higher percentage of degradation as compared to sterile and unsterile

Table 2: Percentage of hydrocarbon degradation for week 6 for all species with sterile and unsterile control.

Fungus	Absorbance	Hydrocarbon residue (mL)	Hydrocarbon degraded (mL)	Degradation percentage (%)
P. chremesinum	1.291	4.26	5.74	57.4
A. versicolor	1.027	3.581	6.419	64.19
T. virens	1.141	3.874	6.126	61.26
A. flavus	1.517	4.841	5.159	51.59
B. ochroleuca	0.061	1.098	8.902	89.02
Sterile control	1.639	5.154	4.846	48.46
Unsterile control	1.639	5.154	4.846	48.46

control. Studies done by other researchers have shown that *Penicillium*sp.[23], *A. versicolor* [24], *Trichoderma* sp. [25], and *Aspergillus* sp. [26] were capable of degrading hydrocarbon efficiently. However, in this study, such efficiency was not found. The main reason that contributes to this is the chemical of composition in the hydrocarbon itself, in this case the crude oil. Chemical composition of a crude oil determines the type of fungi which grow on it and some fungi may grow on some type of crude oil and not others [27,28]. This is also supported by [29] and [30], which reported that degradation rate of different crude oil, differs despite being utilized by the same species of microorganism due to its geographical origin.

Bioremediation of hydrocarbon contaminated soil was conducted to further prove that *B. ochroleuca* was the most superior fungal species in degrading hydrocarbon as compared to the others. Hydrocarbon residue was analyzed based on three different methods namely; cold toluene method, gravimetric analysis and quantification of peak areas based on gas chromatography equipped with flame ionization detector.

Based on the cold toluene absorbance reading on week 6, *B. ochroleuca* managed to degrade up to 87.5% of the spike crude oil as compared to the control. Gas chromatography analysis was then conducted to assess the degradation of the hydrocarbon individually based on carbon number. Figure 3 summarized the percentage of degradation based on the carbon number. At the end of the bioremediation trial, *B. ochroleuca* was found to be able to completely degrade C_{12} and C_{28}. For $C_{14} - C_{26}$, with at least up to 70% degradation was observed as presented in Figure 3. Shorter hydrocarbon chain (C_{10}) was not detected due to the fact that short hydrocarbon chains are easily evaporated and may be loss during the analysis process.

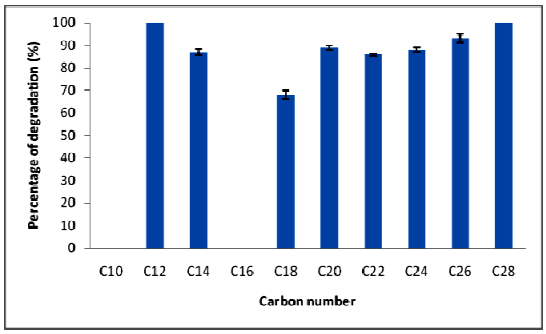

Figure 3: Percentage degradation of fraction 1 (f_1) from treatment against sterile control.

Typical crude oil, consist of 58.2% saturates, 28.6% of aromatics, and 14.2% of polar compounds [31]. Of all petroleum fractions, *n*-alkanes (saturates) of intermediate length (C_{10}-C_{20}) are preferred substrates as carbon source [32,33]. Several species of Ascomycetes were found to have the ability to degrade hydrocarbons. These species preferably degrade aliphatic fraction of crude oil, ranging from C_{12}-C_{26} [34]. Although there are no literatures on the abilities of *B. ochroleuca* to degrade hydrocarbon, findings from this experiment can be supported by study conducted by [34] as *B. ochroleuca* is a member of phylum Ascomycota. Efficiency of bioremediation for soil restoration often to be determined by assesment of soil chemical parameters such as the concentration of residual hydrocarbon after bioremediation.However, not much information is available concerning the efficiency of bioremediation and soil restoration as far as soil's function as habitat for fauna and flora. There is a need to develop biological criteria for post-treatment efficiency test as an alternative to arbitrary chemical criteria cleanup levels [35,36,37]. In order to fulfill those requirements, microbial analysis and root length analysis were conducted. The data for both analyses are presented in Table 3 and4.

There are more than 350 colonies of bacteria formed on each nutrient agar after 24 hours except for replicate B, dilution 10^{-3}. This is an indication that the soil is suitable for habitat of microorganisms. When foreign microorganisms are added into soils as inoculumm, the population activity rapidly decline [38,39]. This may be contributed by factors of biotic and abiotic. Biotic factors are predation by protozoa and competition from other soil indigenous microorganisms, while abiotic factors are moisture content, quality of organic carbon, available nutrients, pH, and temperature [40; 41,42].

However, [43] and [38] suggested that the introduced inoculumm will declined over time as the level of the substrate, in this case hydrocarbon, is reduced. When substrate levels become so low, the microorganisms are only able to fulfill basic requirements of maintenance, and are unable to grow and divide. This phenomenon is known as a threshold [44]. Depletion of the substrate will stop the growth and division of the inoculum. Thus, the microbial population will equilibrated through various forces which tend to revert to non-hydrocarbon sources of organic nutrition, and the population will likely stabilize through nutrient limitation. The results of root length analysis is shown in Table 5. Clearly showed the effect of the bioremediation with the decrease of toxicity of hydrocarbon contaminated soils for seed growth. The toxicity of both sterile and non-sterile control were much higher than that of the treated soil as the seeds root lengths are much shorter (p<0.05).

Table 3: Number of bacteria colonies on Nutrient Agar after 24 hours

	Beaker	Dilution	Number
Nutrient Agar	AC	10^{-1}	TNTC
		10^{-2}	252±21
		10^{-3}	182±4
		10^{-4}	41±8
		10^{-5}	0
	C	10^{-1}	197±8
		10^{-2}	80±10
		10^{-3}	38±9
		10^{-4}	0
		10^{-5}	0
	10A	10^{-1}	TNTC
		10^{-2}	TNTC
		10^{-3}	TNTC
		10^{-4}	TNTC
		10^{-5}	95±6
	10B	10^{-1}	TNTC
		10^{-2}	TNTC
		10^{-3}	TNTC
		10^{-4}	TNTC
		10^{-5}	144±6

Table 4: Number of fungal growth on Potato Dextrose Agar

	Beaker	Dilution	Number
Potato Dextrose Agar	AC	10^{-1}	25± 2
		10^{-2}	11± 1
		10^{-3}	1± 1
		10^{-4}	0± 1
		10^{-5}	0± 0
	C	10^{-1}	43± 3
		10^{-2}	8± 2
		10^{-3}	2± 1
		10^{-4}	1± 1
		10^{-5}	0± 0
	10A	10^{-1}	TNTC
		10^{-2}	TNTC
		10^{-3}	194± 5
		10^{-4}	81± 4
		10^{-5}	28± 2
	10B	10^{-1}	TNTC
		10^{-2}	TNTC
		10^{-3}	233± 14
		10^{-4}	77± 5
		10^{-5}	30± 2

TNTC – Too numerous too count (>350 colonies)
AC – Sterile control; C – Unsterile control;
10A - replicate A of soil with *B. ochroleuca*;
10B - replicate B of soil with *B. ochroleuca*.

Table 5: Root length analysis (n=10)

Sample	AC		C		10A		10B	
	i	ii	i	ii	i	ii	i	ii
	3.5	2.0	2.0	3.5	5	7.5	5	3.5
	5.0	4.5	2.0	3.5	5	8	5	5.5
	5.5	4.2	2.5	1.5	8	2	4	4.5
	9.0	5.5	1.0	3.0	2	7	5.2	3.5
	5.0	7.0	3.5	4.0	3	2	6	4.5
	1.0	6.0	3.5	0.5	3.5	6	2	3.3
	2.0	7.0	2.7	7.0	9	6	5	6
	5.0	2.0	4.0	5.0	5	3.5	10	4
	1.0	3.0	2.5	3.5	7	7	6.5	8.5
	1.0	2.1	0.0	0.0	4	6	0	0
Mean	3.80± 2.60	4.33± 2.00	2.37± 1.20	3.15± 2.08	5.15± 2.23	5.50± 2.21	4.87± 2.65	4.33± 2.18

AC – Sterile control; C – Unsterile control; 10A - replicate A of soil with *Bionectria ochroleuca*;
10B - replicate B of soil with *Bionectria ochroleuca*.

CONCLUSION

The findings in this study have shown that *P. chermesinum, A. versicolor, T. virens, A. flavus*, and *B. ochroleuca* are capable of tolerating high concentration of crude oil. This is shown where each fungus is able to grow on agar media amended with 5% crude oil (v/v). Apart from that, addition of sago hampas as bulking agent into crude oil contaminated soil, proven to support in the penetration of fungal mycelia cords in the soil. In addition, sago hampas also act as other substrate in co-metabolism of crude oil in the soil. *B. ochroleuca* was the best isolate for bioremediation purposes. This is supported by the ability of the fungus to degrade up 87% of the total petroleum hydrocarbon in the bioremediation trial. From the 87% of the total petroleum hydrocarbon, b. ochroleucawas able tocompletely degrade C_{12} and C_{28}. For $C_{14} – C_{26}$, with at least up to 70% degradation was observed. The efficiency of *B. ochroleuca* as bioremediation agent was also proven in the improvement of soil's fertility as the treated soil hold more microorganisms. apart from that, significant differences were observed in root length analysis between water extract from treated and non-treated soil, where the root length of seeds are longer when planted with water extract from treated soil.

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