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Bioremediation of Polluted Soil with Hydrocarbons by Some of *Trichoderma* Spp in Al-Najaf-Iraq

Nihad Habeeb Mutlag[1*], Sara A. Hamoudi[1], Wafaa N. Radhi[2]
[1]Department of Ecology-Faculty of Science -University of Kufa
[2]Jaber Iben Hayyan Medical University, College of Pharmacy
Corresponding author: Nihad Habeeb Mutlag, email: nuhadh.alazerjawi@uokufa.edu.iq , Mobile: +9647706578230

ABSTRACT

Background: There are many types of microorganisms such as bacteria and fungi, which have the potential for biochemical analysis of hydrocarbons each organism has a specific role in the analysis process.

Material and methods: This study was conducted by addition some of biocontrol fungi *Trichoderma harzianum* (isolate1), *Trichoderma harzianum* (isolate2), *Trichoderma longibrachiatum* (isolate 3) in the treatment of contaminated soil with oil derivatives including the addition of 40 ml of white oil (WO) into the experimental pot containers containing 400 gm of soils and mixed well to achieve artificial pollution. Then, three studied isolates of *Trichoderma spp*. were added and mixed with all soil samples that artificially prepared in addition to controlling pots (without the addition of WO) three replicates for each treatment. The biodegradation of Total Petroleum Hydrocarbons (TPH) % and Biostimulation Efficiency (B.E) *%* were calculated for 30,60,90 days after the treatment with bioagent fungi. Spectrophotometer technique was used to estimate the total petroleum hydrocarbons) that showed *T. longibrachiatum* isolate gave the highest Total Petroleum Hydrocarbons (TPH) % reduction in site 1 reaching (93.2%) after 90 days.

Result: *Trichoderma* (isolate 2) has high rate of biostimulation efficiency% that reach (94.2%). Scanning Electronic Microscope (SEM) was used to compare the vegetative growth of fungi before and after the addition hydrocarbon with PDA media, which showed the clear and normal growth with short period to the growth in petri dish after addition 1ml of oil in comparison with the control petri dish in the three magnification forces values (1000x, 2000x, 5000x, 6000x, 10000x, 14000x) for all three studied bioagent fungi before and after the addition of oil.

Conclusion: *Trichoderma* has high rate of biostimulation efficiency, before and after the addition hydrocarbon with PDA media by using of SEM showed the clear and normal growth with short period to the growth in petri dish after addition 1ml of oil in comparison with the control petri dish in the three magnification forces values.

Keywords: *Bioremediation, Trichoderma,Total Hydrocarbons ,SEM, Biocontrol Fungi.*

INTRODUCTION

The problem of soil pollution with hydrocarbons is a common problem around the world. These compounds affect the texture of the soil and its apparent density and permeability [(1)]. This was noticed in the soils near industrial facilities and centers dealing with petroleum products from refineries, factories, storage centers, distribution centers and public and private fuel stations [(2)]. Environmental pollution is the most serious human disaster. Polycyclic aromatic hydrocarbon (PAHs) are organics components whose basic structure is H, C and O. They are found in nature on two types, aliphatic hydrocarbons and aromatic hydrocarbons. These substances are resistant to degradation and have more than 75 compounds classified as hydrocarbons [(3)].

These materials are found in the environment from the extraction of oil, industrial waste and other sources, soil treatment of sewage waste as fertilizer [(4)], which showed clear concentrations of PAHs. The residues contained concentrations of these compounds ranging from 1-10 mg / kg). In Iraq, petroleum fields distributed widely in many regions and linked together by a big net of transferring pipes to carry petroleum to all refineries and exporting ports in north and south of Iraq, but the accidents usually destroy these pipelines so, a huge quantities of oil may leakage through the soil and cause pollution of soil [(5)]. In addition to the flow of hydrocarbons into groundwater and which is considered a dangerous contaminant of the environment, this is done by the reduction of the microbial diversity that affected the elective phenomenon of these organisms [(6).]

There are many types of microorganisms such as bacteria and fungi, which have the potential for biochemical analysis of hydrocarbons each organism has a specific role in the analysis process [(7)]. Bacteria are activated in aquatic environments either fungi and their role is proved in terrestrial environments most often the series of decomposition and degrading processes of these materials have been called into simpler materials by biodegradation, mineralization, biotransformation, bioremediation and bioaccumulation [(8)]. The definition of biodegradation is biological treatment by cracking chemicals which are generally a series of biochemical reactions that occur to hydrocarbons when the cracking process is completed the mineralization process is called such as the products water, CO_2 gas and other inorganic materials the life transition refers to the path of chemical reactions that induce the molecule to turn into different materials. Microbes use chemical pollutants in the soil as a source of energy during oxidation and reduction reactions, to metabolize the target contaminants into an energy that can be used for microbes the (metabolic) outputs are again released into the environment typically in a less toxic form than the original pollutants for example, petroleum hydrocarbons can be destroyed by microorganisms in the presence of oxygen during respiration [(7)].

MATERIAL AND METHOD

The Study area: Al-Najaf oil refinery was chosen as a study area it is located between Al-Najaf governorate and Karbala governorate at the left side of the road.

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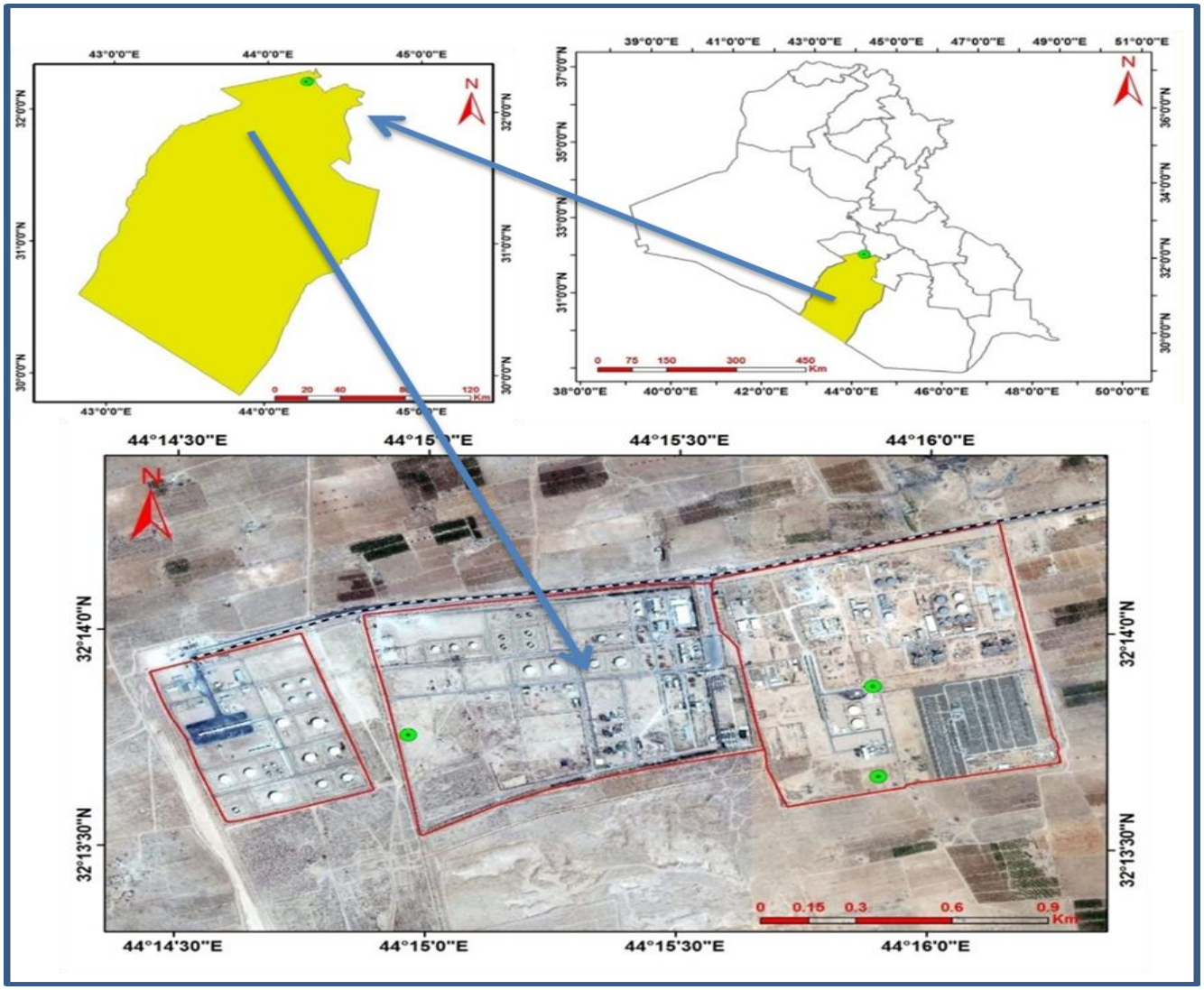

Image (1): GIS image for study area, Al-Najaf oil refinery.

The soil samples were collected from Najaf Oil Refinery from three sites inside the refinery, oil polluted soils samples (crude oil) and unpolluted soils samples were taken from the upper layer (5-10 cm in depth) of the soil placed inside poly ethylene bags, then transported to the ecology department laboratory and dried in the laboratory and passed through a sieve with a diameter of 2mm (2mm particles size) [(1).]

Soil Samples Preparation, Artificial Pollution and Treatment:

400 g of each soil samples were measured and put into clean dry experimental pots and moistened with distilled water to ensure proper mixing with the crude oil. Artificial pollution of the soil samples was done by measuring 40 ml of white oil (WO) into the experimental pot containers containing 400 g of soils and mixed well to achieve artificial pollution. Then, three studied isolates of *Trichoderma spp*. (1,2,3) were added and mixed with all soil samples that artificially prepared in addition to controlling pots (without the addition of WO) three replicates for each treatment.

The experimental pots contained soil, crude oil polluted soil (COPS) and *Trichoderma* spp (3 isolates). Amended soil for 30,60 and 90 days, Total Petroleum Hydrocarbon (TPH) and Biostimulation Efficiency (B. E) % were calculated.

Scanning Electronic Microscope (SEM):

The fungi used in experiment is *Trichoderm spp.* isolates (1,2,3) P.D. A media were prepared by adding 39 gm of potato dextrose agar (PDA) to 1000 ml of distilled water with 25 mg of chloramphenicol (antibiotic against bacteria) which sterilized with an autoclave (15 bar,121 ^{o}C for 15 minutes), prepared P.D.A then added to petri dish that containing white oil with concentrations of (0.5, 1) ml and control without addition WO (three replicates for each treatment),then one disc of *Trichoderma spp*. (three studied isolates) translocate in the center of each petri dish, which incubated at 27 ^{o}C for two weeks.

All petri dish was taken from incubator after two weeks to scanning electronic microscope (SEM) to

compare the growth of vegetative spores with the control treatments [9].

Determination of Total Petroleum Hydrocarbon (TPH):

After 30, 60, 90 days of treatment take 1g each of the naturally and artificially polluted soil samples and dissolved in 10ml of hexane and shaken for ten minutes' using mechanical shaker the solution was filtered using a Whatman filter paper and filtrate diluted by taking 1 ml of the extract into 50 ml of hexane [10].

Procedural blanks and standard solutions were prepared and included to ensure analytical quality control so as to ensure the accuracy and reproducibility of the results. The absorbance of this filtrate solution was read at 460 nm with HACH DR/2010 Spectrophotometer using *n*-hexane as blank. Replicate analyses were carried out on the determination of TPH to yield a statistical mean which will be used to determine trueness and also. Total petroleum hydrocarbon (TPH) was determined at 15 days' interval for 30 days. Percentage of TPH degradation was calculated using the following equation:

$$\% \text{ of } TPH \text{ degradation} = \frac{TPHpo - TPHA}{\text{TPHpo}} * 100$$

Where TPHpo, is the hydrocarbon (white oil) in the untreated soils at zero (0) time and
TPH A is the degradations of hydrocarbon (white oil) in Treated soils at different times.

Biostimulation Efficiency %:

Evaluation of hydrocarbon (white oil) polluted soils and the fungus *Trichoderma spp*. Amended soils Biostimulation Efficiency (B.E) was calculate at 30day interval to 90-day bioremediation period utilize the following equation

$$B.E\% \frac{\text{TPH A\%} - \text{TPHP \%}}{\text{TPH A}} * 100$$

Where TPHA is the degradations of crude oil in fungus *Trichoderma spp*. amended soils at different times and TPHP the degradation of oil in white oil, polluted soils at different times on specimen tube [11].

Ethical approval:

The study was approved by the Ethics Board of University of Baghdad.

Statistical Analysis

Statistical analyses were performed by using SPSS software version 25.0 (SPSS, Chicago). Continuous data were presented as mean and standard deviation, and analysed with Student t-test. Categorical variables were expressed as number and percentage and analysed with Chi-square test. Receiver operating characteristic curve (ROC) was used to evaluate the predictive value of different markers in prediction of different complications and outcome. A p- value less than 0.05 was considered to indicate a statistically significant difference.

RESULT AND DISCUSSION

Scanning Electronic Microscope (SEM):

Image (2) represents a microscopic image of the *Trichoderma* isolate 1 before adding the Oil (control). It was at a magnification power (mag) of (9440x) working Distance (WD) was (9.5 mm) and high accelerating voltage (hv) (12.50 kv). And the image (3) represents a microscopic image of the *Trichoderma* isolate 1 after adding the Oil on the power of magnification (9249x), WD (10.20 mm), and hv (10.00 kv). Also from the images diameter of the spores was measured.

The diameter of the spores before adding the Oil (control) was (16 mm) and the diameter of the spore after adding the Oil (18 mm). This indicates that the fungus after adding the Oil continued to grow and there was no reduction in growth This may be due to the ability of fungi to grow in Oil-containing medium. Image (4) represents a microscopic image of *Trichoderma* isolate 2 before adding Oil (control) to a magnification force of (10087x), WD (10,6mm), and hv (12.50kv) while image (5) represents a microscopic image of Trichoderma isolate 2 after adding Oil to a magnifying force of (10889x), DW (10.2), and hv (10.00kv). From the above images we were able to measure the diameter of the spores, where it was in the control (14 mm) and the diameter of the spores in the image (5) was (16 mm), meaning that the growth of the fungus did not decrease or be affected by the presence of White Oil (WO). Image (6) represents a microscopic image of *Trichoderma* isolate 3. Before adding the Oil on a magnification force (6000x), WD (9.4mm) and HV (12.50kv), the image (7) represents a microscopic image of the fungus *Trichoderma* isolate 3 After adding the Oil on a magnification force (6038x) and WD (9.9mm), HV (12.50kv) where we note that the growth of Spores (Conidia) continues and has not stopped after adding the Oil may be due to the fungi using the oil as nutrients for their survivals, growth [13].

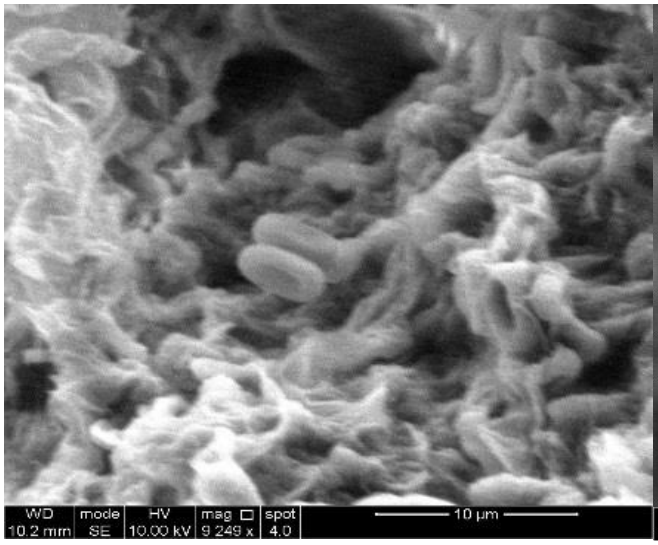

Image (2): *Trichoderma* isolate 1 before adding hydrocarbons.

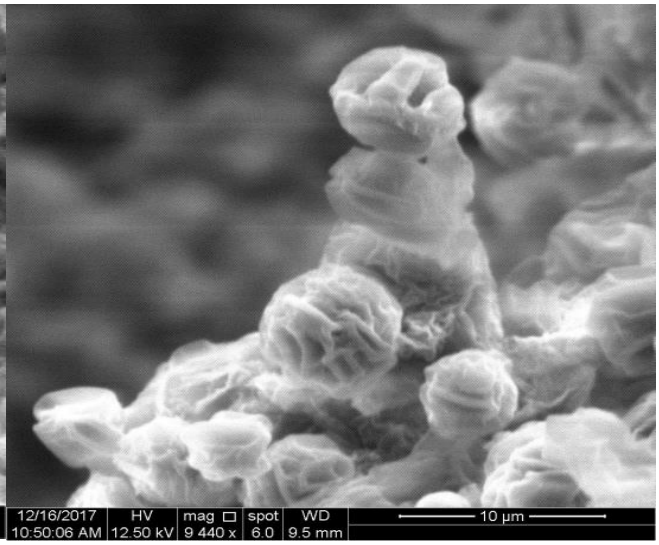

Image (3): *Trichoderma* isolate 1 After adding hydrocarbons

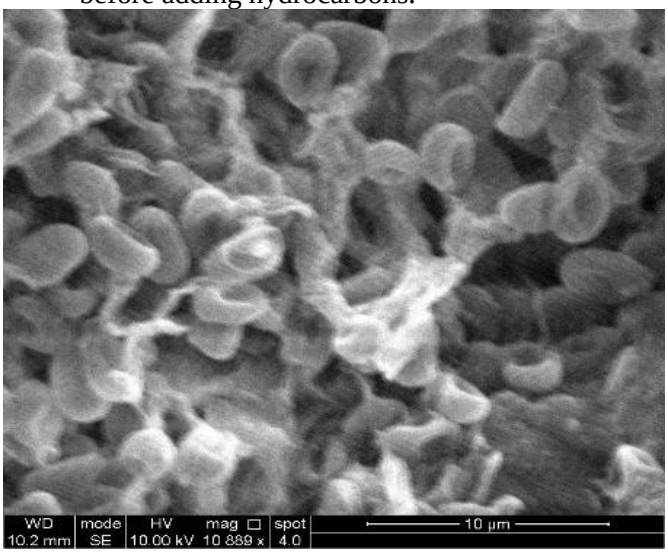

Image (4): *Trichoderma* isolate hydrocarbon.

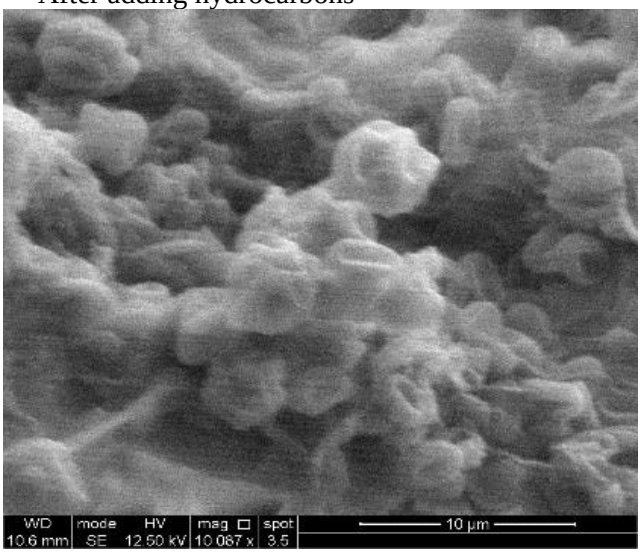

Image (5): *Trichoderma* isolate 2- 2-before adding after adding hydrocarbon.

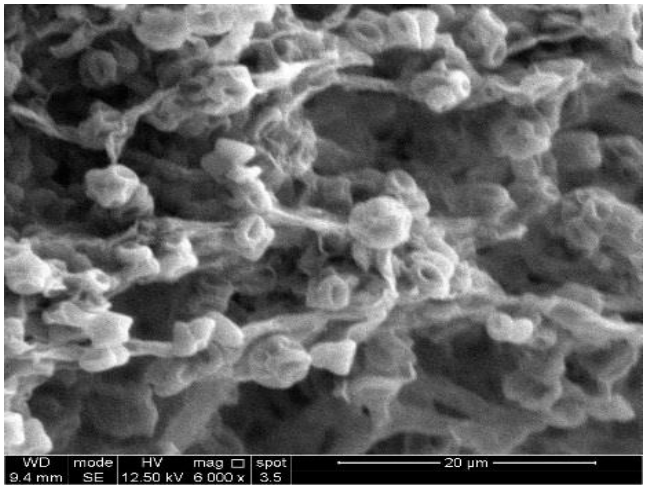

Image (6): *Trichoderma* isolate hydrocarbon.

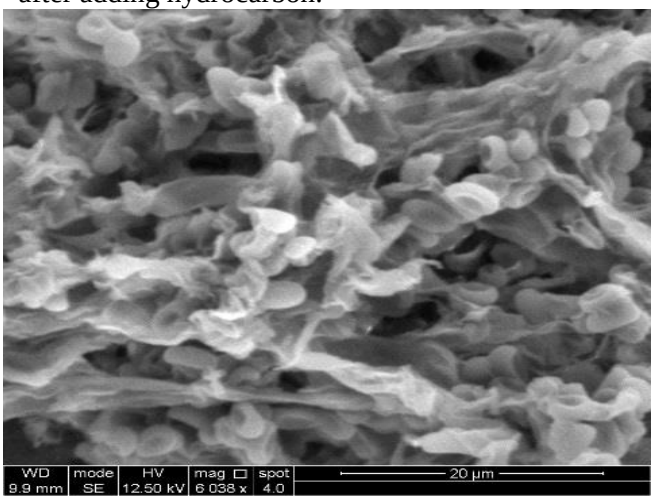

Image (7): *Trichoderma* isolate 3- 3-before adding after adding hydrocarbon

Total Petroleum Hydrocarbon Degradation:

The Result showed that the studied, fungi *Trichoderma spp*. (1,2,3) after addition to Oil polluted soil for different incubation period (30, 60, 90) days were able to remove White Oil from The soils containing with Oil after estimating the percentage of hydrocarbon.

After 30 days, the total petroleum hydrocarbon degradation rate by *Trichoderma spp*. (2) for the three sites (S1, S2, S3) respectively were (36.1 %, 37.9%, 35.8%), fig. (2). the TPH degradation rate by *Trichoderma spp*. Isolate (1) for the three sites respectively were (33.2%, 52.7%, 32.8%), as in fig. (2). the TPH degradation rates by *Trichoderma spp*. Isolate (3) were (34.3%, 34.2%, 31.6%) for the three sites respectively as in fig. (2).

After 60 days the rate of TPH degradation by *Trichoderma spp*. Isolate (2) were (58.6%, 60.5%, 56.8%) for the three sites respectively (S1, S2, S3) as in fig. (3). The rate of the total petroleum hydrocarbon TPH degradation by *Trichoderma spp*. Isolate (1) were (54.8%, 55.7%, 53.2%) for the three sites respectively fig. (3), and the rates of TPH degradation by *Trichoderma* isolate (3) were (56.4%, 57.3%, 54.3%) for the three sites respectively, as in fig (3).

After 90 day of the study, the highest values of total petroleum hydrocarbon degradation by *Trichoderma spp*. Isolates (2) were (93.2%, 91.6%, 89.7%) for three sites (S1, S2, S3) respectively, as in fig. (4), the rate of TPH degradation by *Trichoderma spp*. Isolate (1) were (91.7%, 90.5%, 88.2%) for the three sites respectively, as in fig. (4) and the rate of TPH degradation by *trichoderma spp*. Isolate (3) were (90.9%, 89.2% 87.3%) for the three sites respectively, as in fig. (4).

The results here clearly demonstrated that the bioremediations of soils which are polluted with white Oil can be enhanced by treating the polluted soil with *T. longibrachiatum* which is in line with some previous studies [14].

Our results showed the isolated *Trichoderma longibrachiatum* have high removal efficiency to the degradation of hydrocarbon for all periods (30, 60, 90) days The reduction rates were increased with the increasing in the incubation rate the highest TPH reductions observed in 90 day (93.2%), This might be due to the *Trichoderma longibrachiatum* increasing the bioavailability of hydrocarbons components leading to a larger population of hydrocarbon degraders by using carbon source for the nutrition [15].

The reduction may be due to the *Trichoderma longibrachiatum* presences in crude Oil polluted soil that utilized carbon and energy in the white Oil for their metabolism White Oil is used as sources of nutrients and energy into microbial growth. At the same time microorganism degradation hydrocarbon to numerous substances includes naphthenic acids and phenols, alcohol, hydro peroxide, esters and carbonyl compound", finally to carbon dioxide and waters [16]. This is achieved by the role of *Trichoderma* produce enzymes capable of breakdown hazardous organic pollutants [16].

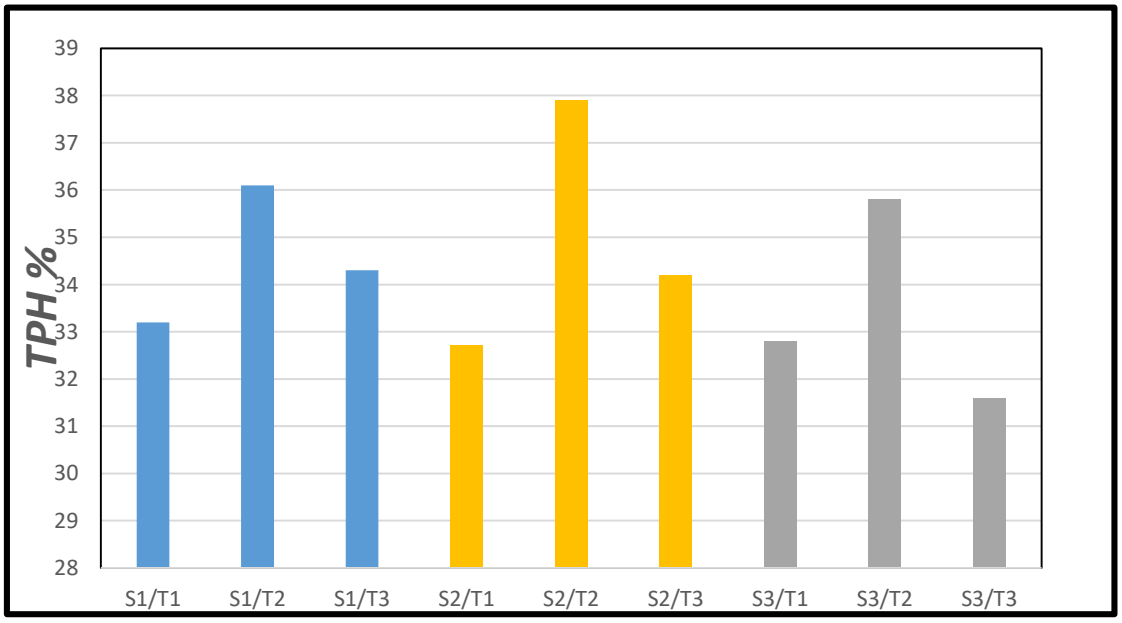

Fig. (2): (TPH%) total petroleum hydrocarbons by *T. longibrachiatum* amended soils after 30 days.

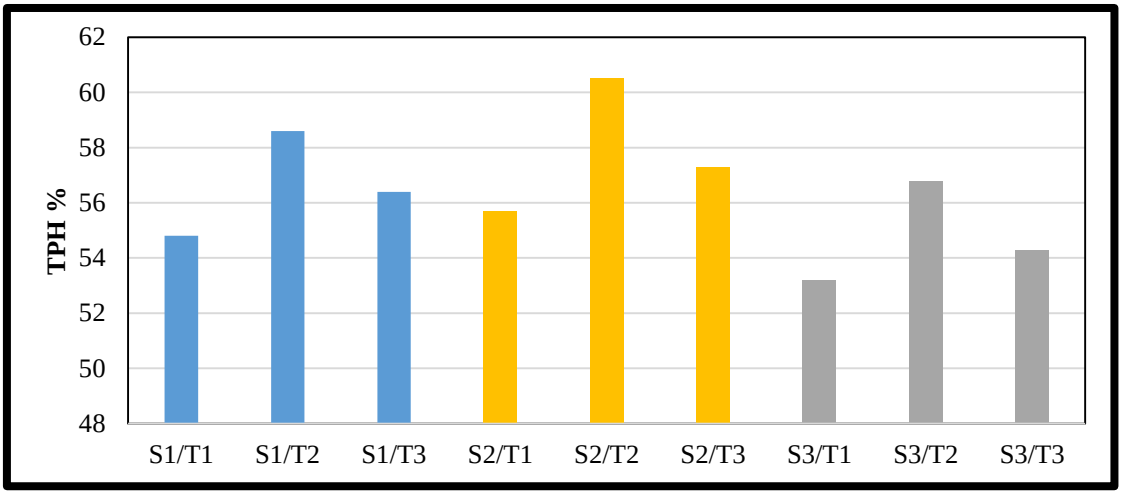

Fig. (3): (TPH) total petroleum hydrocarbons degradation by *T. longibrachiatum* amended soils after 60 days.

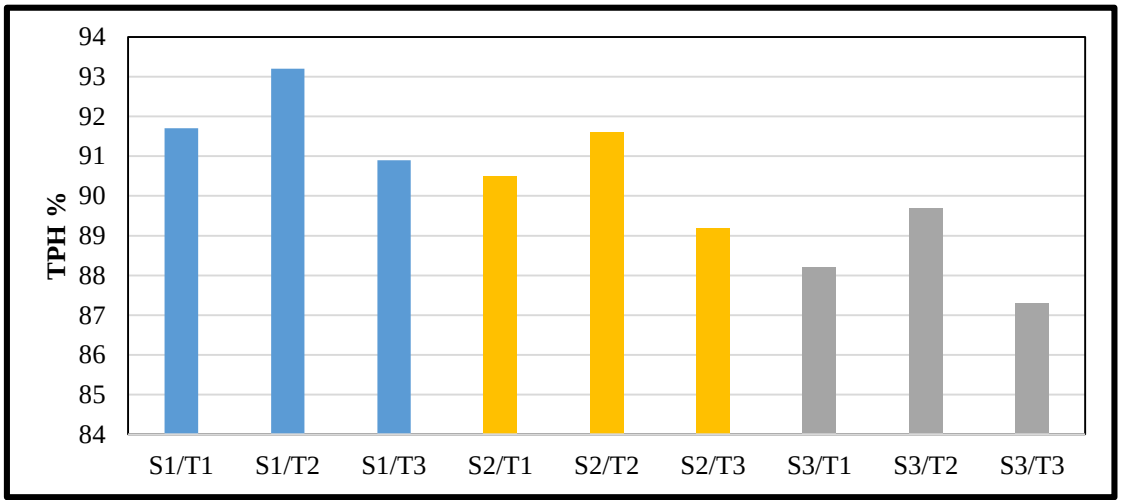

Fig. (4): (TPH) total petroleum hydrocarbons degradation by *T. longibrachiatum* amended soils after 90 days.

Biostimulation Efficiency:

Evaluation of white Oil polluted soil and *Trichoderma spp*. (1.2.3) amended soil Biostimulation Efficiency (B.E) % was calculated at 30 days' intervals for 90 days. Fig (5) showed the rate of biostimulation efficiency by *Trichoderma spp*. (2) at 30 days were (89.5%, 88.3%, 87.2%) for the three sites (S1, S2, S3) respectively, the rate of B.E by *Trichoderma spp*. (1) were (87.7%, 86.5%, 85.1%) for the three sites respectively as in fig (5) and the rates of B.E by T*richoderma spp*. (3) were (86.6%, 85.6%, 84.5%) for the three sites respectively as in fig (5).

At 60 days the biostimulation efficiency increased, the rates by *Trichoderma spp*. (2) were (92.6%, 91.25, 89.4%) for the three sites (S1, S2, S3) respectively as in fig. (6), the rates of B.E% by *Trichoderma spp*. (1) were (87.7%, 86.5%, 85.1%) for the three sites respectively as in fig (6), and the rates of by T*richoderma spp*. (3) were (86.6%, 85.6%, 84.5%) for the three sites respectively as in fig (6).

After 90 days, the rates of the bio stimulation efficiency by *Trichoderma spp*. (2) were (94.2%, 93.6%, 91.7%) for the three sites (S1, S2, S3) respectively as in fig. (7), the rates of B.E% by *Trichoderma spp*. (1) were (92.7%, 91.5%, 89.2%) for the three sites respectively, as in fig. (7), and the rate of the biostimulation efficiency by *Trichoderma spp*. (3) were (91.9%, 90.2%, 88.3%) for the three sites respectively, as in fig (7).

Our results showed that increases in TPH reduction (%) and biostimulation efficiency (B. E) with the increasing in the incubation rate with the presence of *Trichoderma spp*. (1,2,3) for all period (30.60.90). Biostimulation efficiency (B. E) increased when total petroleum hydrocarbon TPH reductions increase. The TPH reductions (%) and increased B. E results are in line with the finding of Ahmed et al. after estimating the ration of B.E % [17].

The results showed the *Trichoderma* (2) has high rate of biostimulation efficiency% for all periods (30, 60, 90) belonging to the same fungus *T. longibrachiatum* This might because nutrients stimulation of fungi to the degradation of petroleum hydrocarbon contaminated sites nutrient is potentially helpful as stimulation nutrients to bioremediation [17].

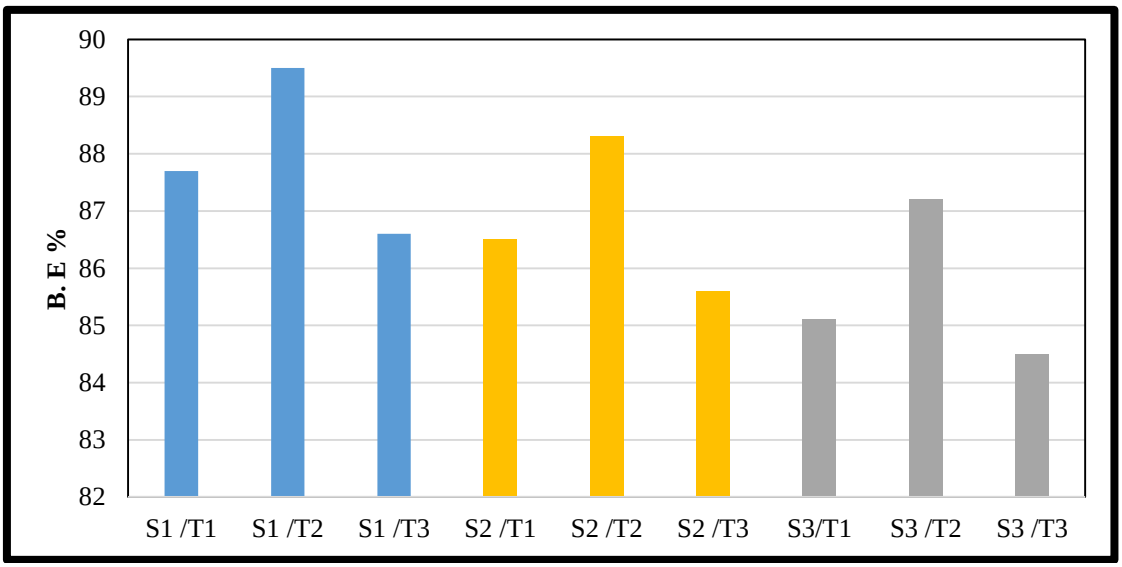

Fig. (5): Biostimulation Efficiency, (B. E) of *T. longibrachiatum* Amended Soil for 30 days.

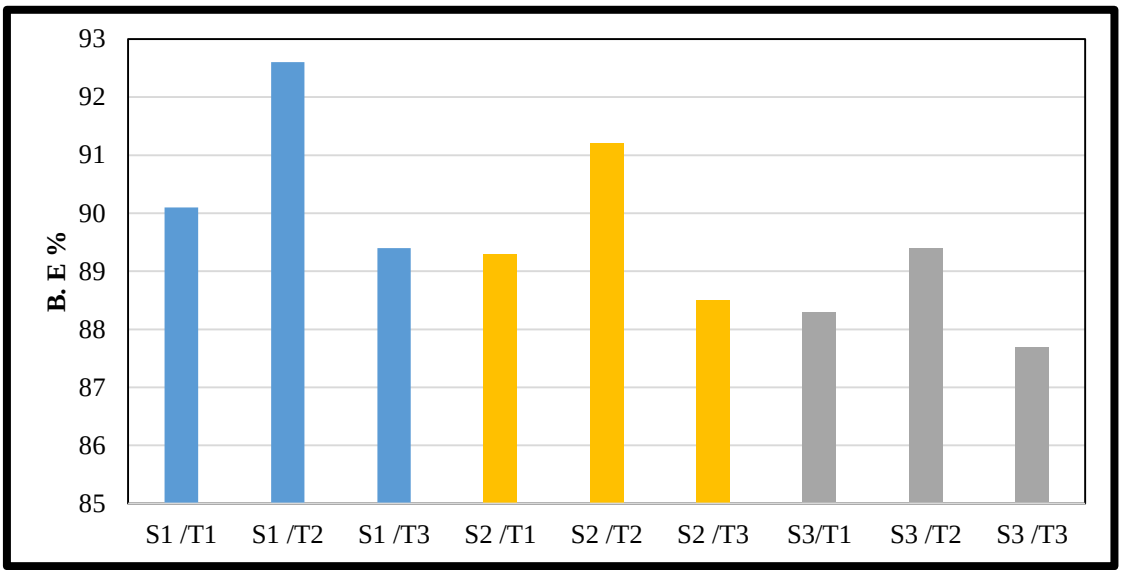

Fig. (6): Biostimulation Efficiency, (B. E) of *T. longibrachiatum* Amended Soil for 60 days.

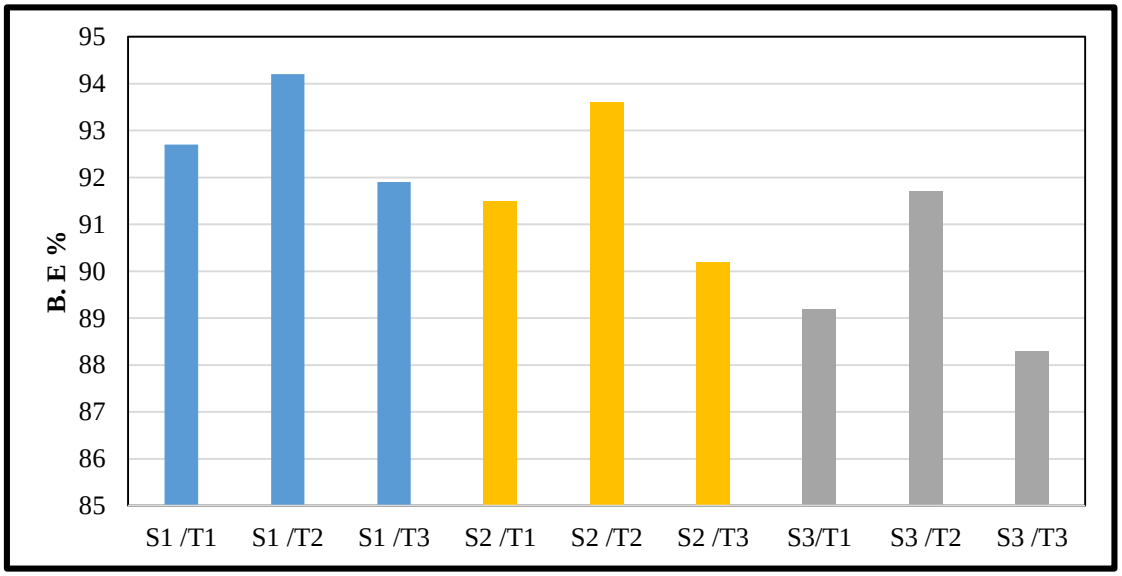

Fig. (7): Biostimulation Efficiency, (B. E) of *T. longibrachiatum* Amended Soil for 90 days

CONCLUSION

In this study concluded that *Trichoderma* has high rate of biostimulation efficiency, before and after the addition hydrocarbon with PDA media by using of SEM showed the clear and normal growth with short period to the growth in petri dish after addition 1ml of oil in comparison with the control petri dish in the three magnification forces values

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Conflict of interest: The authors declare no conflict of interest.

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