International Journal of Environment and Climate Change

Volume 13, Issue 10, Page 3476-3487, 2023; Article no.IJECC.106072
ISSN: 2581-8627
(Past name: British Journal of Environment & Climate Change, Past ISSN: 2231–4784)

Isolation and Identification of Microfungi from Diesel Oil Contaminated Soils and Their Physico-Chemical Analysis

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJECC/2023/v13i103017

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/106072

Original Research Article

Received: 10/07/2023
Accepted: 13/09/2023
Published: 18/09/2023

ABSTRACT

Diesel oil contaminated soils were collected and analyzed for various Physico-chemical properties that included moisture content, total carbon, total nitrogen and phosphorus using standard protocol and the data compared from normal uncontaminated soils. Analysis revealed that all 5 samples of diesel oil contaminated soils possessed higher percent of moisture content, greater amounts of total carbon, nitrogen and phosphorus in comparison to non-contaminated control soils. The diesel oil contaminated soils are not suitable for cultivation of agricultural crops. Though, several species of fungi were isolated from diesel contaminated soils but only 6 species of *Aspergillus* utilized diesel oil as sole carbon and nitrogen source as these grew well and sporulated normally on 2% agar that contained 10% of diesel oil only as nutrient. These included *Aspergillus luchuensis, Aspergillus*

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violaceofuscus, Aspergillus niveus, Aspergillus terreus, Aspergillus japonicus, Aspergillus ustus. which also grew well in broth containing 0.5% jaggery (for initial growth) supplemented with 10% diesel oil only which suggest that these species could utilize diesel oil as sole carbon and nitrogen source because no other nutrient other than diesel oil was added into the medium. The role of carbon, nitrogen and phosphorus is soils has been discussed in this paper.

Keywords: Microfungi; diesel soil; soil analysis; carbon; nitrogen; phosphorus.

1. INTRODUCTION

Soil, water and air are the essential parts of any ecosystem. Soil harbour approximately 59% of the total species in the biosphere and is the home to 99% of *Enchytraeidae* worms, 90% of fungi, 86% of plants and more than 50% of bacteria but only 3% of mammals live in it [1]. The soil manage the recycling of nutrients, energy, and water to promote the plant growth in the ecosystem. Additionally, the soils play significant role in transfer of carbon between the atmosphere, plant, land, and ocean [2]. Soil microbiology and its impact on ecosystem is required to be studied in detail to understand the functional linkages between different life forms that live in there, as opposed to above ground systems [3]. Soil fungi play significant functional role in the cycling of carbon (C) and other nutrients. They can also exhibit a significant impact on plant growth, either as pathogens that kill plants or as commensal (mycorrhizal associations) that improve nutrient uptake [4]. In addition, prokaryotic functional groups involved in the nitrogen (N) cycling are also directly associated with soil fungi [5].The most significant chemical components of soil are carbon (C), nitrogen (N) and phosphorus (P), and their relative amounts greatly influence the growth and development of various life forms, nutrient cycling and energy flow [6]. Plant life depends on mineral elements, which are required for growth, development, and metabolism [7]. Exploring their intrinsic links can help reveal the mechanisms governing the accumulation and transformation of soil C, N, and P fractions because mineral elements and microorganisms have a significant impact on the transformation and transport of these fractions as well [8]. Enzymatic C: N: P stoichiometry has been investigated in detail to understand and evaluate the impact of nutritional limitations of soil microbes [9-11]. Soil microbes secrete extracellular enzyme that break down the complex molecules in the soil, and as a result, the ratios of enzymes reflect relative microbial nutritional constraint [12]. Nutrients can be immobilized and mineralized by soil microbial biomass [13]. Liu *et al.,* [14] opined that soil enzyme activities are connected to "plant-soil enzymes-soil nutrients" and are involved in the biochemical processes of the soil system.

The fungi isolated from petroleum-polluted soil were screened and applied as mono or co-cultures in the treatment of soil-contaminated microcosms in aerobic conditions [15] and evaluated the possibilities of native fungal bio augmentation as a tactic to encourage the bio remediation of an old, industrially damaged soil that was enhanced with heavy hydrocarbon fractions. Fungal bio augmentation's bio-degradation efficiency was compared to that of soil bio stimulation (the addition of water and nutrients) and untreated soil as a control. Total petroleum hydrocarbons (TPH) and HMW-PAHs were biodegraded more rapidly by fungi bio augmentation than by bio stimulation [16]. Oil sludge is produced in vast quantities during the drilling and refinement of petroleum. Utilizing certain microbes for bio remediation is one of the efficient approaches utilized in the waste degradation process [17]. Ten fungal species isolated from polluted soils were assessed for their capacity to break down crude oil samples in solid media based on their rates of growth [18]. According to Sun et al. [19], soil's biological and chemical conversion processes directly involves the soil's active organic carbon. Plants and microbes can readily have a considerable impact on the soil's active organic carbon [20]. However, nothing is known about how the amount of soil active organic carbon varies among various vegetation kinds. According to Lino et al. [21], soil enzyme activities are connected to "plant-soil enzymes-soil nutrients" and are involved in the biochemical processes of the soil system.

Particularly, the carbon cycle of the soil and the activity of certain enzymes, such as amylases, catalases, ureases, and sucrases, are significant markers of soil fertility [22]. The corner stone of modern soils and agricultural management approaches is inorganic chemical-based fertilizers, which are industrially produced compounds that are primarily water-soluble and have high accessible nutrient concentrations [23]. In many terrestrial and aquatic ecosystems, including forests, grasslands, crops, rivers, and wetlands, soil moisture and pH are extensively monitored and assessed [24,25]. According to Neina [26], soil biogeochemical processes like nutrient availability, soil microbial activity, and crop growth and development are all significantly influenced by soil moisture and pH. Given their beneficial impacts on soil C and N nutrient cycles and the growth of the soil biological community underneath the plant species, soil physiochemical characteristics, as well as their spatial pattern and distribution, might generally vary greatly with different plant species [27]. High potential fungal diversity levels in soil can also limit our capacity to draw broad conclusions about how environmental conditions such as global warming will affect various fungal species. To make biological reactions more predictable, phylogenetic methods that assess whether taxa react similarly to environmental change are being used more and more. According to Martiny *et al.,*[28], a finding of phylogenetic conservatism among taxa—close relatives behaving similarly to environmental factors—could improve predictability in response to climate change. Fungal species have great capabilities to degrade diesel oil by producing different amounts and varieties of extracellular secretory enzymes that can break down the complex compounds in nature including hydrocarbons and diesel oil. In fact, the hydrocarbons contaminated soils are not suitable for the production of agricultural crops but if these are rec-lamed using microbial strains, they may provide better production of crops.

2. MATERIALS AND METHODS

2.1 Collection of Samples

The samples were collected from diesel oil-contaminated soils like diesel oil-petrol pumps, workshops having spill of hydrocarbon oils, and parking areas with visible signs of petrol-diesel leakages. Those soils were collected which were exposed to diesel oil spills for a long time. The soil samples were collected 2-3 cm depth with sterile spatula in sterile glass containers, brought to the laboratory immediately and stored at 4℃for further examination.

2.2 Isolation of Diesel Oil Utilizing Microfungi

The enrichment culture techniques was used for the isolation of microfungi for which the soil samples were per-incubated in moist chamber rich with diesel oil at 37℃ for 7 days followed by inoculation of 1 mL suspension (1 g soil in 100 mL sterile distilled water) of per-incubated soil onto Petri plate containing sterile 2% (w/v)agar + 10% diesel oil only. The plates were incubated at 37 and 45℃ temperature for 3 - 7 days. The fungal colonies showing profuse mycelial growth and/or sporulation were sub-cultured on modified Czapeck's Dox Agar medium (agar 15 g/L; di-potassium hydrogen phosphate 1 g/L, Iron sulfate heptahydrate 0.01 g/L, magnesium sulfate hepthydrate 0.5 g/L, sodium nitrate 3 g /L and the Sucrose was replaced with 10g/L jaggery + 10mL diesel oil), identified using standard reference books for identification of fungi. These were further screened by culturing on broth with 0.5% jaggery supplemented with 10% diesel oil only. The species that grew well and sporulated on diesl oil broth were selected and identified as *Aspergillus luchuensis, Aspergillus violaceofuscus, Aspergillus niveus, Aspergillus terreus, Aspergillus japonicus, Aspergillus ustus.* The genus, *Aspergillus* is a large genus containing more than 300 species that are divided into 7 sub-genera and several sections. Its sexual stage lies in Ascomycotina and conidial stage is classified in class Hyphomycetes. The color of Aspergillus colonies Czapack's Dox Agar medium varies from white, gray, greenish, yellow, black, brown, smoky, and microscopically the mycelium is septate, hyaline or dark bearing globose, spherical, club shaped, oval vesicle of varying size. The vesicle bears uniseriate or biseriate phialides that bears conidia. The identification of species is based on size and shape of vesicles, uniseriate or biseriate phialides, size, shape and colour of conidia as described by Raper and Fennel [29] and Gams et al [30]. After identification the mycelia were stored at -20℃ temperature in 15% (w/v) glycerol until further used.

2.3 Physico-chemical Properties of Soil Samples

The amounts of soil organic matter, readily accessible phosphorus, were measured in accordance with Das *et al.*, [31] explanation.

2.4 Soil Moisture Content

The moisture contents of the control and diesel contaminated soil were measured by weighing a fresh sample of soil (15g) and dried in the oven maintain at 80 ℃overnight till constant weight, cooled down in the desiccators and weighted. Moisture was calculated as per the formula given below.

$$\text{Moisture Content } \% = 100\text{X}\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}$$

2.5 pH

Soil pH was determinedby suspending10g of air-dry soil with 100 mL of deionizedin 250 mL Erlenmeyer flask, stirred well, allowed to settle for 10 minutes. pH of the suspension was determined using glass electrode with digital pH meter (SYSTRONICS model µ pH System 362).

2.6 Organic Carbon

Organic Carbon For determination of organic carbon, soil sample was air dried, sieved through (0.5 mm sieve size), 1 g sample was taken in 250 mL Erlenmeyer flask, to which 10 mL of 1N solution of potassium di-chromate was added and spread over the soil sample by swirling, 20 ml of concentratedSulphuric acid was added. The flask was immediately gently stirred until the soil and reagent were combined, then aggressively stirred for one minute. After being spun once again, the flask was left to stand on an asbestos sheet for around 30 minutes. Then 100mL of distilled water was added and allowed to cool once more and the suspension was filtered, 5 mL O- phosphoric acid + three to four drops of 0.5N ferrous sulphate solution were added. The solution initially showed greenish hue as the tip drew closer before turning dark green. At this point, ferrous sulphate was gradually added until maroon color was reflected in light on a white background abruptly changed from blue to red. However, there was no soil used to standardize the dichromate for the blank determination. The following formula was used to calculate % organic carbon:

% organic C in soil = ($K_2Cr_2O_7$ – $FeSO_4$ X 0.003 X100X(F) / % of air in dry soil

2.7 Total Nitrogen

Total nitrogen was estimated using Kjeldahl method as described by Anurag and Garg [32] where the soil sample was first digested with concentrated H_2SO_4 in presence of catalyst selinium, condensed and then allowed to cool to which boric acid (H_3BO_3) was added, combined with 40% NaOH. The released NH_3 was titrated with regular HCl and terminal point's color changed from green to pink.

2.8 Phosphorus

Phosphorus 1g of air-dried soil was taken into a 15mL centrifuge tube, 7mL of the extraction solution was added, mixed well for 1 minute, and centrifuged at 2,000 rpm for 15 minutes. The supernatant was evaluated Colorimetrically using ascorbic acid molybdate blue as described by Ibrahim et al. [33].

3. RESULTS AND DISCUSSION

All 5 samples contaminated with diesel oil exhibited slightly acidic soils when compared with adjoining non-contaminated control soils (Table 1). The acidity of the soils may be due to the degraded products of hydrocarbon contaminated soils. Soil pH has a direct impact on nutrient availability [34, 65]. Schroder et al. [35] found that long-term N fertilizer application can lower soil pH, which may result in a fall in nutrient availability. Long-term inorganic fertilizer application facilitate the nitrification and acidification processes, which results in the drop in soil pH [36,37]. The kind of soil, the source of the manure, manner of its application, and numerous other soil dynamics all affect the soil's pH [38].The presence of carbonate and bicarbonate, as well as carboxyl and phenolic groups, typically results in the rise of soil pH [36]. Soil pH is a key condition with substantial influence on soil biology, chemistry and physical processes which have direct impacts on plant growth and their development. Soil pH and crop productivity are linked together. The United States Department of Agricultural National Resources Conservation Service has categorized soil pH as follows: ultra-acidic (<3.5), extremely acidic (3.5–4.4), very strongly acid (4.5–5.0), strongly acidic (5.1–5.5), moderately acidic (5.6–6.0), slightly acidic (6.1–6.5), neutral (6.6–7.3), slightly alkaline (7.4–7.8), moderately alkaline (7.9–8.4), strongly alkaline (8.5–9.0) and very strongly alkaline (>9.0) [39,40]. Agricultural crop production is generally conducted within the range of slightly acidic to slightly alkaline which facilitate optimal availability of soil nutrients. In all soils, solubility, mobility and bio availability of trace elements is strongly affected by pH. A full understanding of pH is necessary for optimizing nutrient cycling, soil remediation and plant nutrition, as it affects the entire interacting system.

Mean nitrogen, phosphorus, and carbon concentrations were found higher in hydrocarbon contaminated soil samples when compared with their corresponding non-contaminated normal control soils (Table 1). It shows that hydrocarbon contaminated soils provide additional nitrogen, carbon and phosphorous to the soils, however, the diesel oil contain various other components that are harmful to the biology of the soil which makes the soils unsuitable for cultivation of crops. If the biology of such soils is activated using microbial inoculations, these may be used better fertile soils for cultivation of crops. This paper suggests that infertile hydrocarbon soil can be recovered and soil nutrients may be restored using soil fungi. It has been reported that microfungi like nitrogen-fixers, phosphorus and carbon solubilizers, and arbuscular mycorrhizal fungi affected the growth of agricultural crops [41-43]. The soil that had been colonized with microfungi absorbed more carbon and phosphorus to varied degrees compared to the control. Higher nutrient concentrations were seen in pots contaminated with the concoction of arbuscular fungus, phosphorus and carbon solubilizers, and nitrogen-fixers.

Diesel contaminated soils contained higher amounts of carbon 0.41% (S-1), 20.40% (S-2), 0.38% (S-3), 0.37% (S-4), and 0.39% (S-5), at depths of 0 to 1 cm in comparison to uncontaminated normal control soils that exhibited only 0.3% of total organic carbon. The organic carbon is crucial for the growth and cultivation of crop plants and the increasing amounts of chemical fertilizers have fastly depleted soil organic carbon from 3-4% to 0.3% in Indian soils over the past 70 years and this depletion has been very fast during the last 20 years. At a soil depth of 2 cm, S-1 and S-2 had a greater soil organic carbon (SOC) amount than control soils. The SOC is crucial to soil processes that provide an array of ecosystem services. Abdelhafez et al. [44], found that the organic matter in manure has more stable organic components as it decomposes.SOC levels in normal soils were higher at the soil's top than they were at deeper soil layers in all treatments, indicating a slower translocation of SOC through the soil profile [45]. There is a significant association between soil aggregate stability, soil structure, and SOC content [46]. Present study revealed that diesel soil treatment resulted in greater soil organic matter (SOM).

The moisture content was also higher in diesel contaminated soils 13.1% (S-1), 13.5 % (S-2), 14.0 % (S-3), 13.9 % (S-4), 12.7 % (S-5) as compared to control soil. The moisture content is important physiological parameter of soil biology as all the minerals and nutrients are absorbed by the plants only in water soluble form. The additional supply of hydrocarbon rich diesel oil may increase the water holding capacity of the soils to retain more amount of water. The water holding capacity of the soil is an important factor of soil ecosystem that determine the rate of photosynthesis, carbon allocation, plant growth, nutrient cycling, microbial activities, soil biodiversity, soil physiology including enzymatic availability for breakdown of different complex compounds in the soil [47].

The total nitrogen (TN) level and total phosphorus (TP) was 2.95% (S-1), 2.0% (S-2), 2.5% (S-3), 2.8% (S-4), 2.7% (S-5) than 1.5% (control soil) showing that total carbon, nitrogen and phosphorus all three were higher in hydrocarbon contaminated diesel oil rich soil. Such soils are dominant in oil rich Arabian counties and our studies reveal that using microbial inoculation of such soils, these may be converted into fertile soils rich in carbon, nitrogen and phosphorus that may be used for agricultural crop production. Both soil total nitrogen (STN) and soil total phosphorus (STP) responded similarly to rising SOC and soil pH, rising in tandem with SOC and falling in tandem with rising soil pH in both alpine meadows and wetlands. On the Qinghai-Tibetan Plateau, alpine shrub land has shown similar connections between STN and soil pH and SOC [48]. Fang et al. [49], demonstrated that soil organic matter decomposition is a prominent mechanism for soil N buildup, which causes a positive association between STN and SOC. The Three Rivers Source Region's alpine wetland alpine meadows show a positive correlation between STP and SOC because soil P can also be affected by organic matter supply and loss [50]. Both STN and STP demonstrated a notable negative connection with soil pH, in contrast to their favorable relationship with SOC. The facilitation of microbial activity and moderate alkalinity promotes the breakdown of soil organic matter, whereas acidic conditions often slow the turnover of soil organic matter. As a result, raising of soil pH caused a decline in soil organic matter [51].

The mineralization of organic phosphorus is a crucial soil process and only the inorganic phosphorus can be absorbed by the plants which is released into the soil solution only by microbial activity, particularly the microfungi found in soil and on plant roots [42,52]. Numerous soil fungi, mainly belonging to the genus *Aspergillus* have been demonstrated to have the capacity to

Table 1. Physico chemical properties of control and diesel contaminated soils

Physico-chemical properties	Control Soil	Diesel Contaminated Soils				
		S-1	S-2	S-3	S-4	S-5
pH	7.2	6.9	6.8	7	6.6	6.9
Organic Carbon	0.3 %	0.41%	0.40%	0.38%	0.37%	0.39%
Total Nitrogen	1.5%	2.9%	2.0%	2.5%	2.8%	2.7%
Phosphorus	0.5%	1.1%	1.0%	0.9%	1.1%	1.3%
Moisture Content	11.8%	13.1 %	13.5%	14.0%	13.9%	12.7%

convert insoluble phosphates into the soluble forms in soils by secreting organic acids [53]. These acids cause the breakdown of bound phosphate forms and lower pH. Several researchers have demonstrated that plants injected with P solubilizing fungi have improved phosphorus uptake and promote the growth [54].

The purpose of the present investigation was to isolate microfungi from diesel contaminated soils using substrate enrichment technique so that we may further evaluate the potential of these soil fungi to degrade the diesel oil in nature for reclamation of such soils using microbial ecosystem. Present study revealed that the species of *Aspergillus* were highly dominant in diesel contaminated soils and we have isolated total *Aspergillus luchuensis, Aspergillus violaceofuscus, Aspergillus niveus, Aspergillus terreus, Aspergillus japonicus, Aspergillus ustus* (Fig. 1) that were actively associated from hydrocarbon contaminated diesel oil soils. These species were isolated using nutrient enrichment technique and were found to grow fast and optimum on 2% agar containing diesel oil as sole carbon and nitrogen source. For identification, colony morphology, mycelial structure, conidiophore formation (size and color), vesicle orientation and morphology of conidia. The genus *Aspergillus,* family Trichomaceae, and phylum Ascomycota were used to categorize *Aspergillus sp*. A common species that is isolated from diesel soil is *Aspergillus spp.*that have been utilized to produce a variety of significant industrial enzymes, including cellulases, hemicellulases, and proteases, which are employed hydrolysis of lignocarbohydrates in nature and under laboratory conditions. *Aspergillus* is one of the most extensively studied biological control agents for the management of plant-parasitic nematodes [55]. Under *in vitro* conditions, Doilom et al., [56],assessed the phosphate-solubilizing capacity of several isolates of *Aspergillus spp.,* an insoluble tricalcium phosphate, were tested among the fungi. The capacity of *Aspergillus spp*. to solubilize phosphate and nitrogen in soil has been described in scientific literature [29]. It was found that *Aspergillus aculeatus* was able to dissolve rock phosphate. Doilom et al. [56], reported the mineralization of organic phosphates by *A. japonicus* and showed strong acid phosphatase activity in Pikovskaya's broth, indicating that a high percentage of phosphorus was liberated. *A. japonicus* has also been investigated in the past as a rock phosphate solubilizer. By increasing the quantity of soluble phosphorus, *Aspergillus sp.* successfully solubilized a large amount of insoluble rock phosphate [57].

Primary productivity and nitrogen cycling are both constrained by the availability of nitrogen in practically all ecosystems, including those that are agricultural [58]. The rate of photosynthesis can be directly correlated with the availability of nitrogen because a plant requires a relatively significant amount of nitrogen for photosynthesis [59]. Inorganic nitrogenous substances are a kind of nitrogen that plants can absorb. To release an inorganic nitrogen compound that plants may utilise, fixed nitrogen from the atmosphere in terrestrial systems may be bound in organic matter and must first be mineralized [60]. Fungal decomposers in the soil play a significant role in mediating nitrogen conversion [61]. The energy contained in the redox potential of organic nitrogen can be used by a number of soil fungi [62], most fungi have the capacity to release exo-enzymes like proteases and peptidases that can degrade organic materials and then can capture molecules that contain nitrogen, creating a direct pathway from organically bound nitrogen in the soil to the plant [55]. Numerous Aspergillus species have been found to produce proteases with optimal activity expressed at pH 7. Aspergillus oryzae and A. fumigatus are two more Aspergillus species that have been identified as having the ability to make protease [63,64]. Aspergillus spp. are known to contain several extracellular secretory oil degrading enzymes that have multiple capabilities to utilize and degrade. The degraded products have high nutritive value for the agricultural crops. Hence, the objective of the present investigation was to isolate, screen and characterize oil degrading microfungi for their commercial exploitation for improvement of soil fertility. Soil microorganisms have various capabilities to degrade petroleum hydrocarbons at low concentrations of 1 to 5% The species of Fusarium and Mortierella have been reported to degrade high petroleum content [66]. Daasi and Almaghrabi [67] recently in the year 2023 have reported 6 fungal isolates that have capabilities to degrade total petroleum hydrocarbons (TPHS), these included Aspergillus niger, Sycephalastrum, Paecilomyces, Fusarium and Coniochaeta. Bioremediation of petroleum contaminated soils has been reviwed by Ravi et al [68] who have summarized that fungi provide sustainable solutions for degradation of petroleum pollutants.

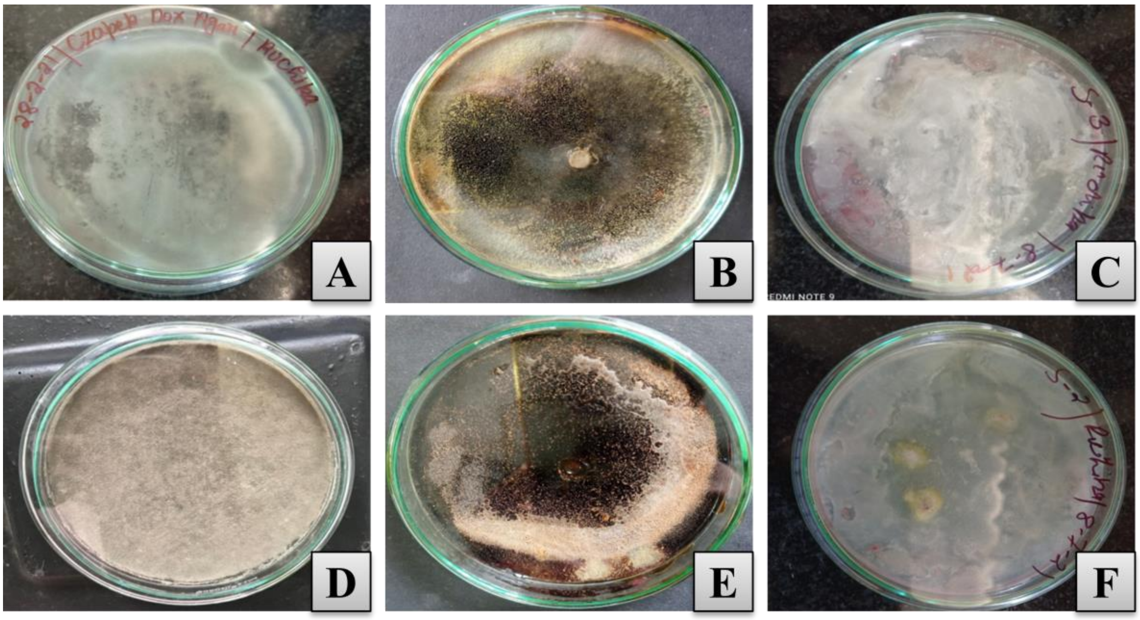

Fig. 1. Fungal growth on Czapeck's Dox Agar (CDA) medium with carbon and nitrogen source as 10% diesel oil incubated at 37℃ temperature A) *Aspergillus luchuensis* B) *Aspergillus violaceofuscus* C) *Aspergillus niveus* D) *Aspergillus terreus* E) *Aspergillus japonicus* F) *Aspergillus ustus.*

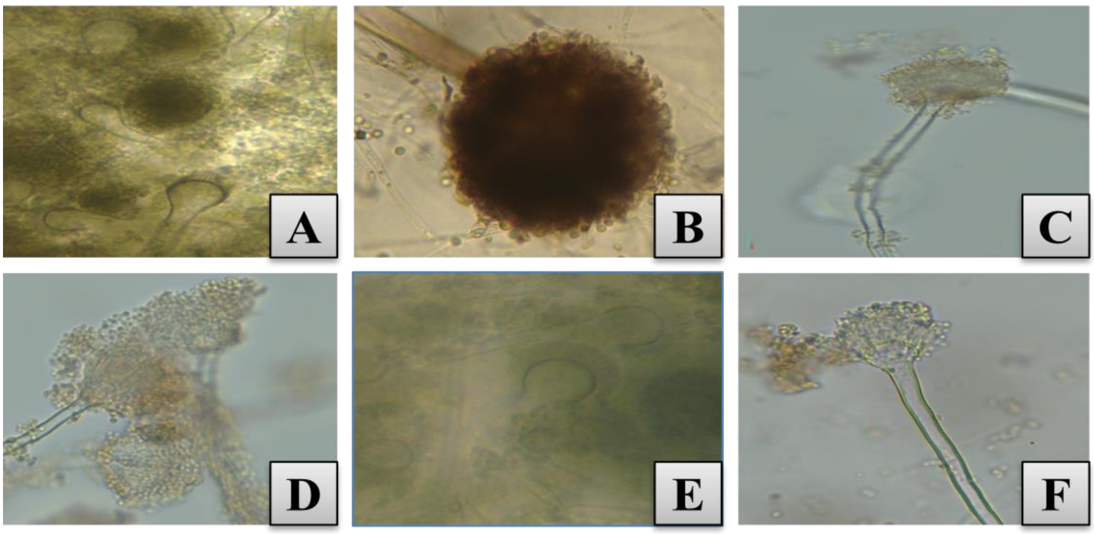

Fig. 2. Microscopic characters of species isolated: (A) *Aspergillus luchuensis*(B) *A. violaceofuscus*(C) *A. niveus*(D) *A. terreus*(E) *A. japonicus*(F) *A. ustus* (All under 10 X x 40X)

4. CONCLUSION

Though several species of fungi were isolated from diesel contaminated soils but only *Aspergillus luchuensis, A. violaceofuscus, A. niveus, A. terreus, A. japonicus, A. ustus* effectively utilized diesel when cultured on 2% agar with 10% diesel oil only and incubated at 37℃temperature. These species were also cultured in broth that contained 0.5% jaggery and 10% diesel oil only which suggest that these species utilized diesel oil as sole carbon and nitrogen source. The soils contaminated with diesel oil possessed higher amounts of moisture content, higher percent carbon, nitrogen and phosphorus in comparison to non-contaminated control soils. These soils are not suitable for agricultural crops mainly because of the known antimicrobial activities of diesel oil. The treatment of diesel oil contaminated soils with fungi can degraded the oil and enhance their nutrient quality for cultivation of agricultural crops.

ACKNOWLEDGEMENT

The authors are thankful to Shobhit Institute of Engineering & Technology, Deemed-to-be-University, Meerut for laboratory facilities.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/106072