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RESEARCH ARTICLE

Bioremediation

An approach to develop bioremediation by isolation and characterization of microorganisms from soil contaminated with used lubricating oil

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Abstract: Soil contamination with used lubricating oil (ULO) is an emerging environmental threat in many parts of the world. Bioremediation is a cost-effective and green, technological approach with the potential to remediate soil contaminated with ULO. Therefore, as an attempt to develop a bioremediation technique, the present study aimed to isolate and characterize microorganisms from soil contaminated with ULO. Bacteria and fungi were isolated from soil exposed to ULO for a long period, by enrichment culture in a mineral salt medium (MSM). Biodegradation assays and total microbial activity (TMA) studies were carried out for the microbial isolates in laboratory-scale microcosms with 1–4% w/w contamination levels of ULO. The initial screening experiment confirmed that two novel strains, RUH-K01 and RUH-F07 were efficient ULO degraders. They were identified as *Pseudomonas aeruginosa* RUH-K01 and *Aspergillus fumigatus* RUH-F07 with respect to their morphological characteristics and 16S rRNA gene sequences. Microcosm experiments revealed the biodegradation percentage in soil with 1% w/w ULO was 82.27% and 70.09% for *P. aeruginosa* and *A. fumigatus*, respectively. A slight reduction in ULO degradation was observed by both strains when the ULO contamination level increased. At 4% w/w ULO contamination level, the degradation was 56.91% for RUH-K01 and 50.25% for RUH-F07. The potential to produce bio-surfactants and other species-specific metabolic activities may be reasons for the efficient biodegradation of ULO by *P. aeruginosa*. The resulting significant negative correlation ($p < 0.05$) between the residual total petroleum hydrocarbon (TPH) content and TMA of both strains confirmed the applicability of the FDA assay as an appropriate method to characterize the ULO degradation efficiency of microorganisms. In conclusion, the overall results indicate the potential of *P. aeruginosa* RUH-K01 and *A. fumigatus* RUH-F07 to be used in bioremediation of ULO contaminated soils.

Keywords: *Aspergillus fumigatus*, biodegradation, *Pseudomonas aeruginosa*, species-specific, total microbial activity, used lubricating oil.

INTRODUCTION

Soil is a complex natural ecosystem that is vitally important for the survival of life on earth. Soil contamination with used lubricating oil (ULO) has become a global environmental issue (Glibovytska *et al.*, 2019). Parallel to the increasing demand for lubricating oil (LO) from automobiles and industry, a large amount of ULO is generated as LO needs to be replaced from time to time due to the alteration of its chemical and physical properties during utilization (Yash, 2015). Accidental spills, careless handling, and illegal dumping that occur during routing oil replacement operations at mechanical workshops and automobile service stations have been identified as significant sources of environmental contamination by ULO (Ogunbayo *et al.*, 2012). ULO is a complex mixture of different types of hydrocarbon compounds including aliphatic (73–80%), mono-aromatic (11–15%), poly-aromatic (4–8%), di-aromatic (2–5%), and heavy metals (Vazquez-Duhalt, 1989). Further, degradation of ULO in the environment is very

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slow (Tang *et al.*, 2012) and the aliphatic and aromatic hydrocarbons, chlorodibenzofurans, additives, heavy metals, and decomposition products in ULO are known to be toxic, carcinogenic, and mutagenic (ATSDR, 1997; Wu *et al.*, 2008). Therefore, soil contamination with ULO may adversely affect not only soil quality (Abioye *et al.*, 2012; Tiwari & Saraf, 2017) but also environmental and human wellbeing. Hence, the application of sustainable remediation methods is vitally important to reclaim ULO contaminated soils.

At present, bioremediation is considered to be a promising, cost-effective, and green technological approach compared to conventional physical and chemical soil remediation strategies (Agarry & Ogunleye, 2012; Shahsavari *et al.*, 2017). Microorganisms such as bacteria, filamentous fungi, algae, and yeast (Margesin & Schinner, 2001), and higher plant species can be used as biological agents in the biodegradation of contaminants (Sardrood *et al.*, 2013). Microorganisms with the potential to degrade contaminants are widely applied to environments contaminated with a variety of organic compounds (Chang *et al.*, 2011) and heavy metals (Alisi *et al.*, 2009).

The application of microorganisms in the bioremediation of contaminated sites is considered as an efficient strategy due to their adaptability to a wide range of environmental conditions, rapid population growth, highly efficient metabolism, and production of biosurfactants (Tyagi *et al.*, 2011). Since screening and identification of efficient strains of microorganisms are crucial steps in the development of bioremediation techniques, many researchers have focused on the isolation and characterization of microorganisms capable of degrading hydrocarbons (Batista *et al.*, 2006; Mandri & Lin, 2007; Zhao *et al.*, 2008; Ling *et al.*, 2011; Masakorala *et al.*, 2013; Yuan *et al.*, 2014; Tiwari & Saraf, 2017). Although the biodegradation potential of petroleum pollutants by bacteria and fungi has been extensively studied (Tyagi *et al.*, 2011; Ghoreishi *et al.*, 2017), only a few studies indicate the degradation of ULO by bacteria and fungi. Umar *et al.* (2013) have identified that bacterial species *Pseudomonas fluorescens, Bacillus mycoides*, and *Acinetobacter* sp. and fungal genera such as *Aspergillus* and *Penicillium* (Jesubunmi, 2014) are capable of degrading ULO. The success of a bioremediation strategy is largely determined by the ability of inoculants to adapt to new environmental conditions, degradation efficiency, microbial activity, and their diversity (Wu *et al.*, 2016). Therefore, isolation and identification of effective and efficient ULO degraders from the environment are challenging.

Although contamination of soil by ULO is an emerging environmental issue in Sri Lanka, enough attention has not been paid on the management of contaminated soils. Though a few studies have been conducted to develop phytoremediation techniques for ULO contaminated soil (Gamage *et al.*, 2020), studies on the microorganism-mediated bioremediation have not been carried out up to date. Therefore, conducting a pilot-scale study is a prerequisite for establishing effective and efficient bioremediation using native microorganisms. Moreover, it would be beneficial for planning management strategies to restore ULO contaminated sites surrounding industrial areas and automobile service stations. Therefore, the present study aimed to isolate, identify, and characterize bacteria and fungi from ULO contaminated sites, with a capacity to degrade ULO and to determine their biodegradation efficiency at different levels of ULO contamination.

MATERIALS AND METHODS

Soil and ULO sample collection and preparation

Soil samples were collected from the Service Station of Sri Lanka Transportation Board (SLTB) bus depot in Matara, Sri Lanka. This has a long history as a service station and is known to be contaminated with ULO. After carefully scraping off the surface soil layer, soil samples were obtained at a depth of 5–10 cm. The collected samples was homogenized and sieved through a 2 × 2 mm mesh to remove large particles and other debris. Samples were then stored in polythene bags at 4°C to be used for further analyses. Total petroleum hydrocarbon (TPH) content was determined according to the method followed by Tang *et al.* (2012) with slight modifications as described in section 'Determination of biodegradation of ULO'. Uncontaminated soil was collected from the surface layer of an undisturbed site of the University of Ruhuna, Sri Lanka, at a depth of 5–10 cm, to carry out microcosm experiments as mentioned in section 'Biodegradation of ULO in microcosms'. Homogenized soil samples were obtained following air drying and sieving. An ULO sample, collected from an automobile service station at Kekanadura, Matara, Sri Lanka was filter-sterilized using Whatman® membrane filter (pore size 0.45 µm) before being used in the experiments.

Reagents and culture media

Several media were used for the isolation and maintenance of potential ULO degrading microorganisms. The mineral salt agar (MSA) medium used in the experiment was composed of (L^{-1}): NaCl 10 g; $MgSO_4.7H_2O$ 0.42 g;

KCl 0.29 g; KH_2PO_4 0.85 g; Na_2HPO_4 1.25 g; $NaNO_3$ 0.42 g; and 20 g of bacteriological agar. The nutrient agar (NA) medium used in the experiment was composed of (L^{-1}): NaCl 8 g; peptone 5 g; beef extract 3 g and 14 g of bacteriological agar. The nutrient broth (NB) was composed of (L^{-1}): pepton 5 g; beef extract 1 g; yeast extract 2 g; NaCl 5 g. The potato dextrose agar (PDA) medium was prepared by dissolving 35 g of PDA powder in 1 L of distilled water. The pH of the media was adjusted to 7.4 ± 0.2. Prepared culture media were sterilized by autoclaving at 121°C for 30 min.

Isolation of ULO degrading microorganisms

An enrichment isolation technique was used to isolate potential ULO degrading microorganisms (Zhao *et al.*, 2008; Masakorala *et al.*, 2013; Thenmozhi *et al.*, 2013) from ULO contaminated soil. Briefly, 1 g of ULO contaminated soil was suspended in 100 mL of MS broth in a 250 mL Erlenmeyer flask. As an enrichment substrate, 1 mL of filter-sterilized ULO (1% v/v) was added as the sole carbon source and incubated at 30°C in a horizontal orbital shaker (Lab Companion SK 300) at 180 rpm. Following 14 days of incubation, a 1 mL aliquot from the enriched culture was transferred weekly for a period of 4 weeks to 100 mL MS broth supplemented with 1% v/v ULO, and incubated under the same conditions. Bacterial cultures were obtained by uniform spreading of 100 µL aliquots of cultures at seven days of incubation on MSA medium coated with 100 µL 1% w/v ULO in dichloromethane. For the isolation of ULO degrading fungi, MSA supplemented with tetracycline (10 µg/mL) was used. Morphologically distinct bacterial and fungal colonies, which appeared following seven days of incubation at 30°C, were further purified. Pure cultures were obtained after three consecutive sub-cultures on NA and PDA. Stock cultures of bacteria were stored in 20% v/v glycerol at -20°C and fungi were stored in sterilized distilled water and PDA at 4°C (Zhao *et al.*, 2008; Masakorala *et al.*, 2013; Thenmozhi *et al.*, 2013).

Screening bacterial and fungal isolates for ULO degradability

The ULO degradability of pure isolates of bacteria and fungi were further screened on MSA medium coated with 100 µL 1% w/v ULO. Streak lines of similar lengths were drawn for all three bacterial isolates. Fungal isolates were inoculated by actively growing a mycelium plug (5 × 5 mm) in the middle of the Petri dish. Inoculated culture plates were incubated for 10 days at 30°C (Ibrahim *et al.*, 2018). The capacity of bacteria for biodegradability was estimated by measuring the area of clear zone surrounding the bacterial growth, while that of fungi was determined by visual observation of mycelial growth, sporulation, and distinct clear zone (Thenmozhi *et al.*, 2013). Un-inoculated ULO coated MSA plates were used as controls. The experiments were carried out in triplicate and repeated three times to confirm the reproducibility of the results.

Characterization of ULO degrading bacteria and fungi

Morphological and biochemical characterization

The bacterial isolate RUH-K01, which showed the highest degradation potential, was identified and characterized based on colony morphology and pigmentation on NA medium and Gram staining. The biochemical properties of the isolate were determined by conducting a series of biochemical tests, such as triple sugar fermentation, methyl red, citrate, nitrate reduction, oxidation and fermentation, urease, indole, starch hydrolysis, catalase, and Voges-Proskauer. The results were analyzed following Bergey's Manual of Determinative Bacteriology (Breed & Bergey, 1948). The fungal isolate RUH-F07, with the highest degradation potential, was identified based on macroscopic and microscopic colony characters on the PDA medium. Macroscopic colony characters such as pigmentation, visible surface texture, and growth rate (time taken to cover a 90 mm Petri plate) were recorded. In addition, features of hyphae, arrangement of conidiophores, and conidia were examined on slide cultures (Riddell, 1950) under high power (× 400) of the phase-contrast microscope (OLYMPUS DP 21). High-resolution images were captured using an in-built camera to the microscope.

Molecular characterization

Genomic DNA was extracted from RUH-K01 and RUH-F07 isolates using commercial extraction kits (Qiagen). Bacterial genomic DNA from RUH-K01 was amplified by Polymerase Chain Reaction (PCR) with universal primers for the 16S rRNA gene; 785F (5'GGATTAGATACCCTGGTA3') and 907R (5'CCGTCAATTCMTTTGAGTTT3'). Fungal genomic DNA from RUH-F07 was amplified by universal primers for the ITS1, 5.8S rRNA, and ITS2 regions of the fungal genome, ITS1 (forward primer, 5'TCCGTAGGTGAACCTGCGG3') and ITS4 (reverse primer, 5'TCCTCCGCTTATTGATATGC3'). Amplicons were sequenced (Macrogen, Korea). Sequences were assembled using the software, Geneious R11 (Kearse *et al*., 2012), and searched for nucleotide identities in NCBI/GenBank (*https://blast.ncbi.nlm.nih.gov/Blast.*

cgi) using the non-redundant database. Sequences of RUH-K01 and RUH-F07 were deposited in the NCBI/ GenBank database under the accession numbers MH828325 and MK949124, respectively.

Biosurfactant production

Oil displacement assay was conducted to determine the ability of bacteria to produce biosurfactants as described by Rodrigues *et al.* (2006). In this assay, a thin smear of ULO was formed by spreading 20 µL of ULO (1% w/v) on 25 mL of sterilized distilled water in a Petriplate. Subsequently, 15 µL of cell-free supernatant obtained from the saturated culture of RUH-K01 was placed on the center of the oil-water surface and the formation of a distinct clear zone was observed. Sodium dodecyl sulphate (SDS) was used as the positive control. The experiment was carried out in triplicate and repeated three times to confirm the reproducibility of the results.

Biodegradation of ULO in microcosms

Experimental setup

Soil samples obtained from an undisturbed site of the University of Ruhuna, sterilized by autoclaving at 121°C for 30 min, were mixed with ULO at 1–4% w/w levels in laboratory-scale microcosms prepared in 250 mL Erlenmeyer flasks. The water holding capacity was adjusted to 70%. Three replicates were used for the treatments and control at each contamination level. All replicates of the treatments were allowed to stand for three days before adding 5 mL of RUH-K01 bacteria suspension (at OD_{600nm} ~ 0.4) and mycelia-free spore suspension of RUH-F07 (at OD_{600nm} ~ 0.4) separately. The control setup was not inoculated, while 5 mL of sterilized distilled water was added. The microcosms were incubated at 30°C. After every 10, 20, and 30 days, total petroleum hydrocarbon (TPH) content and total microbial activity were measured using standard procedures as follows.

Determination of biodegradation of ULO

TPH content in ULO contaminated soil was estimated gravimetrically according to the method described by Tang *et al.* (2012) with slight modifications. Five grams of ULO contaminated soil from the microcosms were extracted into 10 mL of dichloromethane. The mixture of soil and dichloromethane was sonicated for 15 min and the supernatant was collected by repeating the extraction procedure three times. The collected supernatants were centrifuged at 996 × g for 10 min, and the dichloromethane was evaporated in a water bath at 40°C (Tang *et al.*, 2012). Finally, the residual TPH content was determined according to equation number 1.

$$TPH\ Content = \begin{pmatrix} Final\ weight\ of\ the\ crucible \\ containing\ residual\ oil \end{pmatrix} - \begin{pmatrix} Weight\ of\ the\ empty \\ dried\ crucible \end{pmatrix} \quad ...(1)$$

The percentage biodegradation of ULO was determined according to the weight loss method using equation 2 (Bartha & Bossert, 1984).

$$Biodegradation = \frac{Average\ weight\ of\ oil(Control) - Average\ weigh\ \ of\ oil\ (Treatment)}{Average\ wei\quad of\ oil(Control)} \times 100 \quad ...(2)$$

Total Microbial Activity

Changes in the inoculated microbial population or their enzymatic activity during the process of biodegradation of ULO were determined by the fluorescein di-acetate (FDA) hydrolysis method described by Adam & Duncan (2001). The amount of fluorescein released after 10, 20, and 30 days of incubation of microcosms containing 1–4% w/w ULO contaminations at 30°C was quantified at a wavelength of 490 nm using UV/Visible spectrophotometer (Evolution 260 Bio, Thermo Fisher Scientific Inc. Germany). A standard concentration series of 1–5 µg/mL was prepared by diluting 20 µg/ mL of fluorescein stock solution in 60 mM potassium phosphate buffer at pH 7.6 (Adam & Duncan, 2001).

Statistical Analysis

Data were analyzed statistically using the MINITAB (version 17) statistical software package. Results of the measured parameters were given as arithmetic means of three independent replicates. Two-way ANOVA was conducted to determine the significant differences between percentage level of contamination, time, and the effect of both contamination level and time on reduction of residual TPH content and total microbial activity. The correlation between the residual TPH content and total microbial activity was determined using the Pearson product correlation coefficient (r). The statistical significance in all analyses was defined at $p < 0.05$.

RESULTS AND DISCUSSION

The amount of petroleum hydrocarbon content in the ULO contaminated soil of the composite sample was determined before the isolation of the microorganism. Results showed the presence of 100,500 mg/kg ± 200 mg/kg or 10.5% (w/w) of TPH in the analyzed soil implying a high level of ULO contamination.

Isolation of potential ULO degrading microorganisms

ULO degrading bacteria

Through the enrichment isolation of potential ULO degrading bacteria, three morphologically distinct bacteria (RUH-K01, RUH-J01, and RUH-I01) were isolated in MSA medium supplemented with 1% (w/v) ULO as the sole source of carbon. In the literature, it has been reported that the formation of a clear zone and considerable growth of bacteria along the streaked path are indications of the biodegradation potential of bacteria (Vinothini *et al.*, 2015). Therefore, isolates were further screened on ULO coated MSA medium to confirm and differentiate their ULO degradation potentials. At the end of 10 days of incubation, a clear zone and bacterial growth were visible along the streaked path of each bacterial isolate on MSA medium coated with ULO. The mean of the measured area of the clear zones from the isolates RUH-K01, RUH-J01, and RUH-I01 in three trials were 403 mm^2, 229 mm^2, and 394 mm^2, respectively. Therefore, the results infer the difference in the potential of the isolates to degrade ULO, and the RUH-K01 (Figure 1) was identified as the isolate with the highest potential.

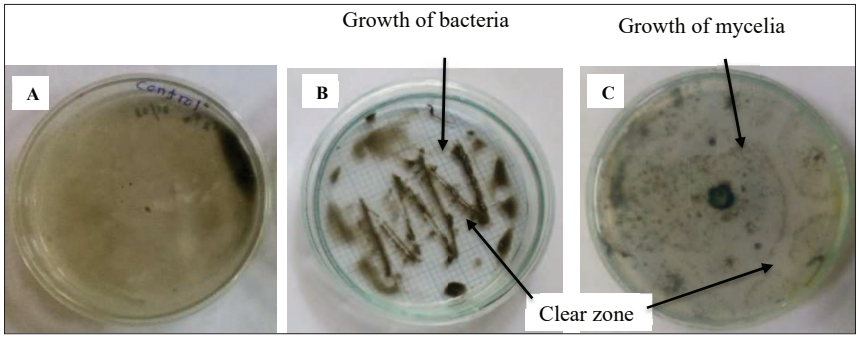

Figure 1: ULO degradation by bacteria and fungi. (A) Un-inoculated control; (B) growth and clear zone formation of bacterial isolate RUH-K01 and (C) growth and spore formation of fungal isolate RUH-F07 on MSA coated with 100 µL 1% w/v ULO after 10 days of incubation at 30°C

ULO degrading fungi

Two morphologically distinct fungal colonies (RUH-F06 and RUH-F07) were isolated in MSA medium supplemented with 1% w/v ULO as the sole source of carbon. The mycelium of the fungal isolate RUH-F07 proliferated and sporulated rapidly on MSA coated with ULO and formed a clear zone surrounding colonies (Figure 1). Thenmozhi *et al.* (2013) have reported that the large colony diameter, abundant mycelial growth, and heavy sporulation of fungal isolate are characteristic morphological features of efficient hydrocarbon degraders. Thus, the reported visual observations of the present study infer that the isolate RUH-F07 is likely to have the capability of utilizing ULO as the sole source of carbon for its growth and reproduction, and the potential to degrade ULO.

Morphological and biochemical characterization of ULO degrading bacteria and fungi

Morphological characterization and Gram's staining indicated that the RUH-K01 is a rod-shaped Gram-negative bacterium. The colonies were pale brown, circular-shaped with an entire margin, and had a convex elevation on NA medium (Figure 2). Biochemically, the RUH-K01 isolate gave positive results for citrate, catalase, nitrate reduction, and oxidase tests, produced acids by the fermentation of D-glucose and was negative for sucrose, lactose, starch hydrolysis, Indole, Voges-Proskauer (VP), methyl red, and urease tests. Based on the morphological and biochemical characteristics, the novel isolate RUH-K01 was provisionally identified as a member belonging to genus *Pseudomonas* (Breed & Bergey, 1948), and was confirmed by the sequencing

of 16s rRNA. The analysis of the partial sequence (1486 nucleotides) of the 16s rRNA showed that the strain RUH-K01 was 99% identical to *Pseudomonas aeruginosa*. Therefore, the novel strain RUH-K01 with the GeneBank accession number MH828325 was tentatively nominated as a *Pseudomonas aeruginosa* RUH-K01.

The features of colonies and microscopic examination of the fungal isolate RUH-F07 showed conidiophore and conidia characters similar to that of genus *Aspergillus* (Figure 2). Conidiophores were hyaline, short and simple. Conidia were unicellular, smooth-walled, green, spherical in shape and formed as chains. A partial sequence (593 nucleotides) of the ITS region confirmed that the strain RUH-F07 was 99% identical to *Aspergillus fumigatus*. Therefore, the novel strain RUH –F07 with the GeneBank accession MK949124 was tentatively named as *Aspergillus fumigatus* RUH –F07.

The phylogenetic trees constructed using neighbour-joining method to illustrate the relationships of RUH-K01 and RUH-F07 with nearest phylogenetic relatives are given in Figure 3 (A) and (B) respectively. All the accession numbers of the reference sequences are given in Figure 3. According to the phylogenetic trees, strains RUH-K01 and RUH-F07 cluster with the largest clades

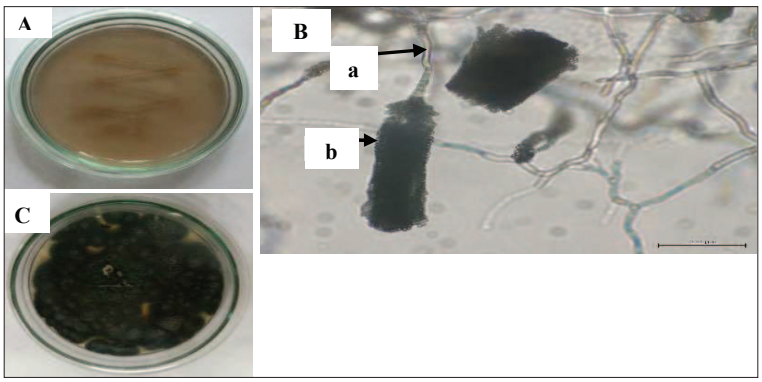

Figure 2: A pure culture of RUH-K01 on NA (A), RUH-F07 observed under the high power (×400) of the microscope (a-Conidiophore, b-Conidia) (B) and a pure culture of RUH-F07 on PDA (C).

Biosurfactant Production

Biosurfactants are amphiphilic molecules produced by microorganisms. Depending on the origin of biosurfactants, chemical composition may comprise different structural components such as glycolipids, phospholipids, peptides and glycopeptides (Gautam & Tyagi, 2006). Bioavailability of hydrocarbons for microbial degradation is less, due to their hydrophobic nature (Das *et al.*, 2014). Biosurfactants enhance the bioavailability of hydrocarbons for hydrocarbon-degrading bacteria by reducing surface tension and increasing contact surface and solubility in the aqueous phase (Gautam & Tyagi, 2006; Masakorala *et al.*, 2013). Therefore, much attention has been paid towards the potential of microorganisms to produce surfactants in planning bioremediation or bio-augmentation strategies for decontamination of hydrocarbon contaminated sites. Although the biosurfactant production potential of *Aspergillus* spp. is well documented (Sperb *et al.*, 2018), there is limited information on biosurfactant production by bacteria (Ghazala *et al.*, 2019). Therefore, the biosurfactant production ability of the isolated ULO degrading bacteria was studied in this research. Our results showed that *P. aeruginosa* RUH-K01 has a potential to synthesize biosurfactants (Figure 4). Since the biodegradability of ULO by *P. aeruginosa* RUH-K01 has been shown to be significantly efficient, this strain may have utilized alternative mechanisms such as microbial mobilization and microbial attachment (Ortega-Calvo, 2017) to enhance the bioavailability of ULO during the biodegradation process, in addition to the biosurfactant production.

Biodegradation of ULO

Previous studies have revealed the potential of microorganisms isolated from hydrocarbon-

contaminated sites to degrade hydrocarbon compounds (Husaini *et al.*, 2008; Wu *et al.*, 2008; Das & Chandran, 2011; Liu *et al.*, 2011). Further, genes responsible for the hydrocarbon degradation process have been identified in indigenous microorganisms isolated from hydrocarbon-contaminated soils (Zhang *et al.*, 2016). Furthermore, some studies have revealed that the monitoring of residual TPH content with time is a direct measurement

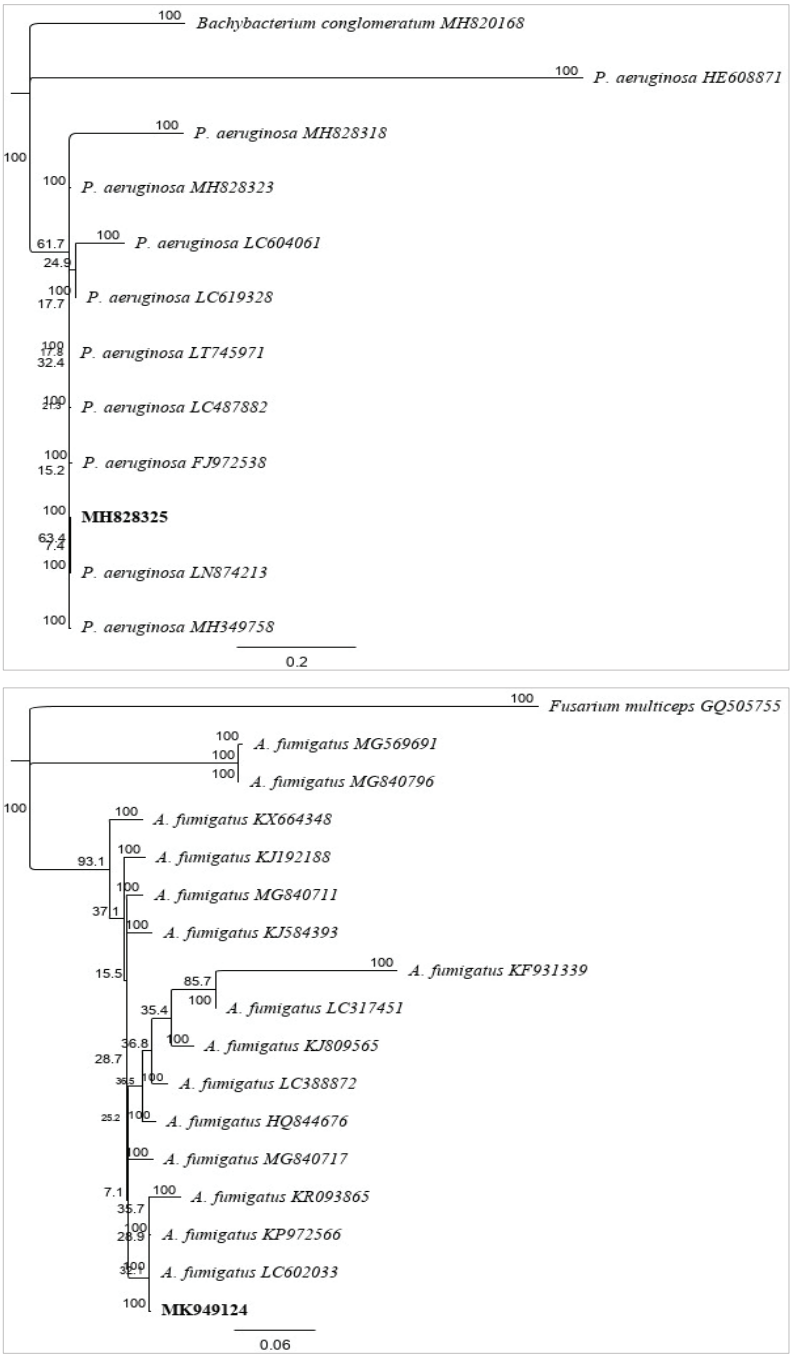

Figure 3: Phylogenetic trees of the novel strain RUH-K01 (A) and novel strain RUH-F07 (B). Bootstrap values, expressed as percentages of 1000 replications, are given at branch points. NCBI/GeneBank accession numbers are given after the scientific name.

of biodegradation which reflects the efficiency of bioremediation (Margesin & Schinner, 2001; Tang *et al.*, 2012; McIntosh *et al.*, 2017; Zeneli *et al.*, 2019). Hence the characterization of the degradation potential of microorganisms isolated from ULO contaminated soil is vitally important prior to implementing bioremediation. Therefore, the ULO degradation potential of isolated microorganisms was evaluated.

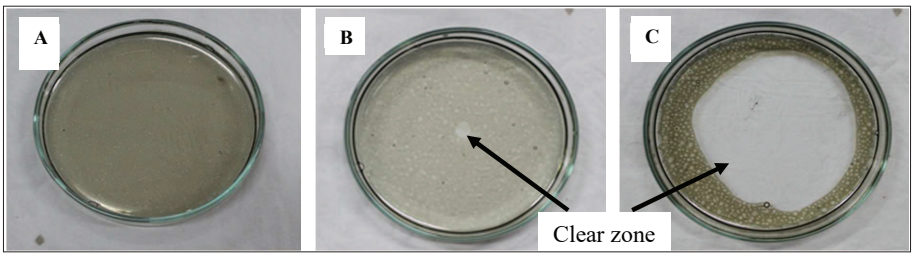

Figure 4: Biosurfactant production of *P. aeruginosa* RUH-K01. Negative control (A), oil spreading test for cell-free supernatant taken from the saturated culture of RUH-K01 (B), positive control SDS (C).

Results obtained from microcosm experiments showed an obvious degradation of ULO at all the tested contamination levels 1-4% (w/w) of ULO after inoculation with novel strains *P. aeruginosa* RUH-K01 and *A. fumigatus* RUH-F07 (Figure 5 (a)). However, *P. aeruginosa* RUH-K01 seems to be more efficient in degrading ULO compared to RUH- F07, at all the tested contamination levels. At the end of the first 10 days at 1% (w/w) ULO levels of contamination, RUH-K01 and RUH-F07 reached 70% and 45% degradation, respectively. The highest degradation was recorded in *P. aeruginosa* RUH-K01 inoculated soil at 1% (w/w) contamination level with a degradation efficiency of 82.27% after 30 days of incubation. At the end of 30 days, the recorded degradation efficiency of *A. fumigatus* RUH-F07 inoculated soil at 1% (w/w) ULO was 70.09%. It is stated that the activity of microorganisms is a result of the expression of specific genes responsible for a certain function (Yu *et al.*, 2013). Therefore, the recorded ULO degradation efficiencies by the novel bacterial isolate *P. aeruginosa* RUH-K01 and novel fungal isolate *A. fumigatus* RUH-F07 could be due to species-specific reasons.

Members of the genus *Pseudomonas* are well-known bacteria with the potential of utilizing different types of hydrocarbons as the sources of energy and carbon (Das & Chandran, 2011; Kadali *et al.*, 2012; Puškárová *et al.*, 2013; Pacwa-Płociniczak *et al.*, 2014). Safdari *et al.* (2017) have reported 62% diesel removal from contaminated water by *P. aeruginosa* during 20 days, highlighting a strong diesel degradation capacity.

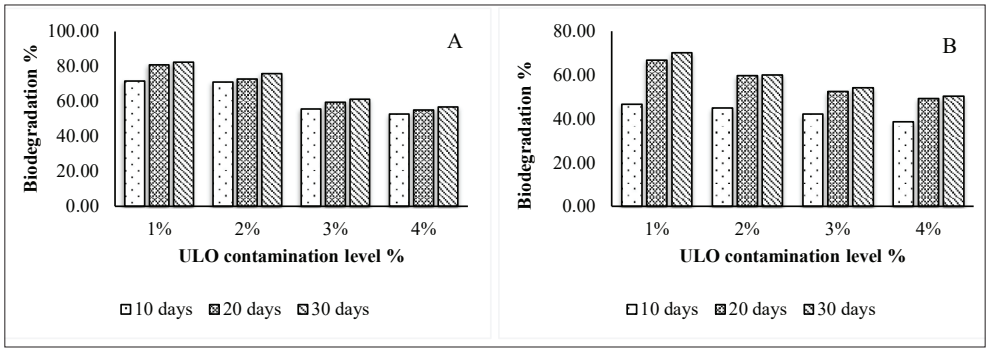

Figure 5 (a): Percentage biodegradation of ULO at 1–4 % (w/w) contamination levels over 30 days of incubation inoculated with *P. aeruginosa* RUH-K01 (A) and *A. fumigatus* RUH-F07 (B).

Similarly, it has been shown that *P. aeruginosa* was able to reduce TPH concentration in crude petroleum-contaminated soil from 84 to 21 g/kg within 60 days (Das & Mukherjee, 2007). Results obtained for *A. fumigatus* RUH-F07 in the present study and results of similar studies available in the literature indicates that *A. fumigatus* is known to be a frequent fungal species in soils polluted with petroleum, emphasizing its tolerance and ability to grow and survive in such soils (Al-Jawhari, 2014). Further, 78% degradation of crude oil by *A. fumigatus* at 2% (v/v) contamination level during 28 days has been reported, highlighting its suitability for remediation of hydrocarbon polluted soil at low contamination levels (Al-Jawhari, 2014). Moreover, Tiwari and Saraf (2017) have showed 71.1% of crude oil degradation at a 1% (v/v) contamination level within seven days by *A. fumigatus* isolated from ULO contaminated soil. Therefore, the results of the present study are in agreement with the previous findings. However, the observed decrease in the degradation efficiency at the ULO contamination levels higher than 1% (w/w) and the resulting degradation plateau after 20 days of incubation indicate dependency of ULO degradation rate on both ULO contamination levels and time. However, even at a higher contamination level such as 4% (w/w) ULO, the recorded 56.91%, and 50.25% biodegradation from the microcosm inoculated with RUH-K01 and RUH-F07, respectively, at the end of 30 days indicate the higher ULO degradation potential of both novel strains, highlighting their suitability to be used in bioaugmentaion or bioremediation.

The results of Two-way ANOVA revealed significantly higher ($p < 0.05$) residual TPH content (mg/g) with respect to the contamination level and time, in treatments inoculated by both strains. However, the interactive effect between ULO contamination level and incubation time on the residual TPH content (mg/g) in both strains were not significant ($p > 0.05$). Wu *et al.* (2017) have observed a similar pattern of degradation in soils contaminated with TPH and alkane following three weeks of incubation.

This type of degradation pattern may be due to the inhibition of the degradation as a result of the accumulation of toxic metabolites produced early in the degradation process (Chaineau *et al.*, 2003). In addition, accumulation of high molecular weight aromatic hydrocarbons that are resistant to microbial degradation in the soil matrix can also be a reason for limited biodegradation after 35-40 days of incubation (Lee *et al.*, 2007). Therefore, the observed limitations in the degradation following the initial phase in the microcosms inoculated with both RUH-K01 and RUH-F07 might be due to accumulation of toxic intermediate metabolites along with residual high molecular weight aromatic hydrocarbons, or the limitation of carbon source for microbes, or due to the combined effect of both.

Total microbial activity

Microbial activity is an important parameter to understand the capability of microbial degradation of xenobiotic compounds. Previous studies have reported the possible negative impacts of hydrocarbons on the metabolic pathways of soil microorganisms, through changes in the activities of enzymes such as dehydrogenase, lipase, urease, and catalase (Margesin *et al.*, 2000). Most such enzymes produced by microorganisms have the potential to hydrolyze colorless fluorescein di-acetate (FDA) into a colored end product called fluorescein (Patle *et al.*, 2018). Since the rate of fluorescein production indicates the total microbial activity (TMA), FDA assay was employed as a tool to assess the changes in TMA during the degradation of ULO in the microcosms inoculated by RUH-K01 and RUH-F07. FDA assay revealed the decreasing trend in the production of fluorescein in both *P. aeruginosa* and *A. fumigatus* inoculated soils when the ULO contamination level increased (1–4% w/w) (Figure 5 (b)). The highest fluorescein concentrations reached were 6.825 µg/mL and 6.124 µg/mL at 1% ULO contamination level following 10 days of incubation with *P. aeruginosa* and *A. fumigatus* respectively. Therefore, results indicate the contamination level and time-dependent decrease in TMA with increasing ULO contamination level and incubation time. Abioye *et al.* (2012) have reported that low levels of ULO in soil may not have a negative impact on the metabolic activities of the microbial population in microcosms. In a previous study, a concentration-dependent reduction has been reported in microbial biomass which was exposed to increasing crude oil concentration (Vanishree *et al.*, 2014). Thus the possible reasons for the observed trend in the current study might be due to the inhibition of the growth and proliferation of microorganisms as a result of direct toxic effects (Tshikantwa *et al.*, 2018) or indirect toxicity caused by the secondary metabolites generated during the biodegradation process. The resulting differences in the decreasing trend in the production of fluorescein by *P. aeruginosa* and *A. fumigatus* with respect to the ULO contamination level and incubation time reflect the species-specific differences of the microorganisms employed in the microcosms. The bioavailability of contaminants in the soil environment affects microbial activity (Antizar-Ladislao, 2010). Therefore, a larger reduction of the bioavailable fraction of ULO in the microcosm inoculated by *P. aeruginosa*

compared to that of *A. fumigatus* inoculated soil might be another reason for the observed disparity in TMA reflected by FDA hydrolysis due to RUH-K01 and RUH-F07. Results of the Two-way ANOVA revealed that there was a significantly higher ($p < 0.05$) interactive effect between ULO contamination level and incubation time on the TMA of *P. aeruginosa* inoculated soil.

The observed decrease in TMA might be the reason for the reduction of the percentage of biodegradation at higher levels of ULO contamination. According to Ramos *et al.* (1991), the population density of microorganisms present in a hydrocarbon-contaminated medium after inoculation with a potential microbial degrader is one of the main criteria that determines the efficiency of biodegradation. Therefore, in the present study, the bioremediation process was monitored by estimating residual TPH content and total microbial activity. At the end of 30 days, a significant negative correlation ($p < 0.05$) of -0.636, and -0.929 between residual TPH content and TMA were observed in both the strains, RUH-K01 and RUH-F07, respectively. Margesin *et al.* (2000) reported that the effects caused to the microbial population by organic compounds can be positive, negative, or indifferent, and the degree of the effect on the microbial population may vary with the level of the contamination or the concentration, and with the period of exposure of microbes. Limited studies have been conducted so far to explore the relationship between degradation of hydrocarbons and microbial populations and their activity. According to the study of Tang *et al.* (2012), a significant negative correlation was observed between TPH content and bacterial population. Another study has shown a negative correlation for the biodegradation of polycyclic aromatic hydrocarbons (PAHs), due to the reduction of organic pollutants causing less bioavailability for microorganisms with time (Sverdrup *et al.*, 2002). Therefore, the results of the present study are consistent with the finding of similar studies available in the literature.

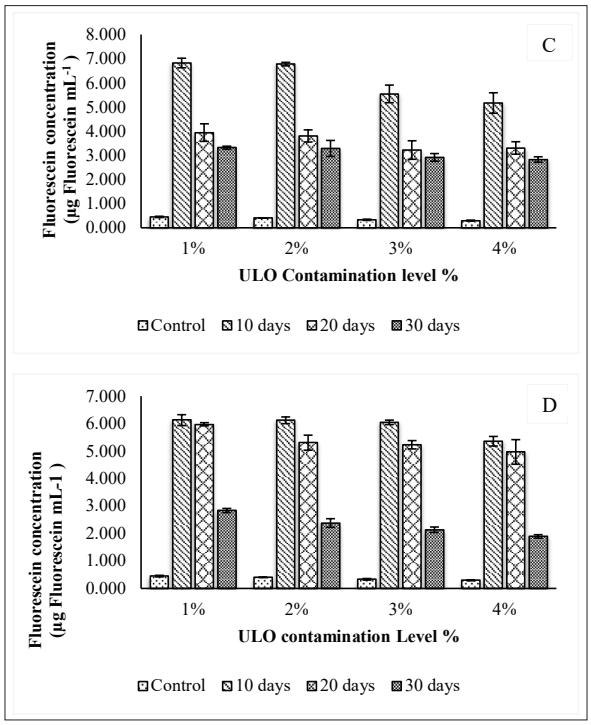

Figure 5 (b): Concentration of Fluorescein produced at 1–4 % (w/w) contamination levels over 30 days of incubation inoculated with *P. aeruginosa* RUH-K01 (C) and *A. fumigatus* RUH-F07 (D). Error bars represent the standard deviation of three independent measurements.

CONCLUSION

Two novel strains, bacterium RUH-K01 and fungi RUH-F07, with the potential to degrade ULO efficiently were isolated from soil with prolonged exposure to ULO contamination. Subsequent phylogenetic analysis following initial morphological characterization confirmed the novel strains as *P. aeruginosa* RUH-K01 and *A. fumigatus* RUH-F07. Therefore, the present study contributed to upgrading features of *Pseudomonas aeruginosa* and *Aspergillus fumigatus* with a potential to degrade ULO. While the increase in the level of contamination (1–4% w/w) showed a slight reduction in the degradation of ULO, *P. aeruginosa* RUH-K01 was found to be more efficient in ULO degradation than *A. fumigatus* RUH-F07. The FDA assay revealed the possibility of asssessing TMA as an appropriate method to characterize ULO degradation efficiency of microorganisms. The positive results for the oil spreading test confirmed the potential of *P. aeruginosa* RUH-K01 to produce biosurfactants. The metabolic activities of both strains would make them promising agents for bioremediation of ULO contaminated soil. Therefore, findings of this study provide important information for establishing bioremediation by *P. aeruginosa* and *A. fumigatus*. However, it is recommended to carry out small scale field trials using the two strains in order to further confirm the laboratory derived data.

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