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Occurrence of hydrocarbon-utilising bacteria in oil-polluted arid soils in Sudan

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ABSTRACT

Aims: This study aimed to isolate and identify hydrocarbon-utilising bacteria from oil- polluted sites and to develop a microbial consortium for use in pilot trial and commercial scale bioremediation treatment systems in the future.
Methodology and results: Ten hydrocarbon-utilising bacterial strains were isolated using enrichment culture technique from oil-polluted sites using crude oil as sole carbon source. The strains were tentatively identified on the basis of colony morphology, microscopic examination and biochemical characteristics. The growth of each strain was assessed by growing the bacteria in mineral salt medium amended with diesel oil as sole carbon source. The isolates exhibited differences in growth with the order of biomass production being *Enterobacter* sp. (OD_{620}=1.283)> *Bacillus subtilis* subsp. *subtilis* (OD_{620}=1.245) > *Aerococcus* sp. (OD_{620}=1.100) > *Bacillus firmus* (OD_{620}=0.970) > *Corynebacterium* sp. (OD_{620}=0.886) > *Bacillus lentus* (OD_{620}=0.743) > *Micrococcus luteus* (OD_{620}=0.656) > *Bacillus subtilis* (OD_{620}=0.367) > *Bacillus cereus* (OD_{620}=0.110) > *Kocuria flavus* (OD_{620}=0.065).
Conclusion, significance and impact of study: This study is a prerequisite for the design of future full-scale bioremediation treatment of oil-polluted sites using hydrocarbon-utilising bacteria. An efficient consortium was developed comprising the best three hydrocarbon-utilising strains, which include *Enterobacter* sp., *Bacillus subtilis* subsp. *subtilis* and *Aerococcus* sp. This efficient microbial consortium is suggested to be used in future to rehabilitate oil-polluted sites in Sudan.

Keywords: Crude oil, refinery oil sludge, hydrocarbon-utilising consortium, Sudan

INTRODUCTION

Petroleum oil has many benefits to human society. However, in line with oil development, particularly onshore exploration, it carries potential risks for plants, animals, and humans (Atlas, 1981). During the Gulf War in 1991, the release of eight million barrels of crude oil into the surrounding marine environment and Kuwaiti desert was an ecological disaster that led to serious environmental damage to the region (Al-Daher *et al*., 1998). Malallah *et al*. (1998), for example, studied damage caused by oil pollution to Kuwaiti desert flora and found that four flowering plants suffered from mutagenic modifications.

Accidental introduction of hydrocarbons from leaking pipeline or oil processing and storage facilities have been and will continue to be significant sources of environmental pollution (Morgan and Watkinson, 1989). Likewise, the release of crude oil and petroleum products through negligent disposal practices of wastes such as refinery oil sludge, have been shown to cause serious damage to natural ecosystems when improperly managed (Van Hamme *et al*., 2003).

A diverse number of microorganisms have the ability to degrade and utilise petroleum hydrocarbons as their sole carbon and energy sources (Leahy and Colwell, 1990; Atlas, 1991; Atlas and Bartha, 1992; Wang *et al*., 2011; Ahirwar and Dehariya, 2013; Panda *et al*., 2013). Such microorganisms have been isolated from various natural habitats severely contaminated with oil and over 200 different species of hydrocarbon-degrading microorganisms have been identified (Rahman *et al*., 2002; Survery *et al.*, 2004; Nwaogu *et al*., 2008; Roy *et al.*, 2014; Koshlaf and Ball, 2017).

For the past two decades, the discovery and development of petroleum oil in Sudan has resulted in large increase in the use of petroleum products. Improper handling represents a high risk of polluting the environment by petroleum hydrocarbons especially during crude-oil extraction, transportation, storage, refining of petroleum products and careless disposals of petroleum wastes (Xu *et al*., 2018). Unavoidable daily contamination has been the result of a rise of gasoline/diesel pumping station due to an increase in the number of vehicles in Khartoum and other cities. Spills of oil during tanker transfer, and leakage from underground storage tanks are

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the cause of groundwater contamination (Rahman *et al.*, 2002; Limaa *et al.*, 2019).

Different physical and chemical techniques have been applied worldwide to remove hydrocarbons from contaminated aquatic and terrestrial habitats. Physical remediation techniques including the use of different kinds of booms (Schrader, 1991) and inorganic absorbent (Adebajo *et al.*, 2003) and oleophilic and hydrophobic sorbents have been employed to remove oils from aquatic environments (Deschamps *et al*., 2003). Chemical techniques include the use of dispersants (Lessard and Demarco, 2000), encapsulation (Khan *et al*., 2004) and chemical oxidation (Asghar *et al*., 2016). However, various physical and chemical methods have limited effectiveness with concomitant high operational costs and cause negative impacts on the environment (Singh and Chandra, 2014). Biological treatment (bioremediation), the use of hydrocarbon degrading microorganisms to treat the hydrocarbon contamination, is considered an alternative and less expensive method by oil companies to chemical and physical methods. Bioremediation is also a publicly acceptable method for the degradation of hydrocarbon pollutants to innocuous inorganic or mineral elements (Admon *et al*., 2001; Lynch and Moffat, 2005; Mikesková *et al*., 2012; Omotayo *et al*., 2012; Singh and Chandra, 2014; Sarkar *et al*., 2017).

In spite of the increasing information available on biodegradation of crude oil, the study of microbial degradation of petroleum hydrocarbons under tropical and sub-tropical conditions has received less attention than in temperate and polar regions. This is in part due to the paucity of information on the prevalence and geographical distribution of the hydrocarbon-degrading bacterial populations in soils under arid and semi-arid conditions (Chaillan *et al*., 2004; Adebusoye *et al*., 2007; Nwaogu *et al*., 2008; Boboye *et al*., 2010). Unlike temperate and polar regions, the constant warm temperatures in countries such as Sudan are expected to favour microbial activity throughout the year, thus offering favourable conditions for the enhancement of bioremediation. There is no field study of microbial degradation of petroleum hydrocarbons conducted in Sudan since the start of oil production in 1999. Additionally, no serious attempt has been made to tackle the problem of the highly contaminated brine (produced formation water) that is delivered to the surface along with petroleum oil during extraction. After separation from crude oil, the hydrocarbon-polluted produced water is stored in storage ponds until it can be decontaminated. Oily petroleum refinery sludges that are generated in Sudan by, for example, the Khartoum Refinery Company, require similar attention.

The present study aimed to screen oil-polluted sites in Sudan and examine the diversity of hydrocarbon-utilising bacteria in a preliminary study. The study explored the possibility of selected single bacterial species and mixed consortia of indigenous isolates to utilise diesel oil under controlled laboratory conditions. The prevalent hydrocarbon-utilising bacteria were identified, characterized and preserved for further use. The work will ultimately identify an efficient microbial consortium to be recommended for use in future decontamination trials of oil-polluted sites in Sudan.

MATERIALS AND METHODS

Source of oil utilising bacteria

Soil samples were randomly collected to 10 cm depths from: (i) crude-oil contaminated soil in Heglig Central Processing Facility, Heglig oil field (9° 53′ 00″ N 29° 50′ 00″ E), (ii) Shendi local petrol filling station in Khartoum North (15°37′ 44″ N 32° 37′ 33″ E) and (iii) dumping site of the oily sludge of the Khartoum Oil Refinery at Algaili, 45 km north of Khartoum (16° 7′ 44″ N 32° 41′ 18″ E). The samples were collected in pre-sterilised glass bottles and transported to the laboratory within 5 h of sampling and stored at 4 °C until further analyses.

Medium composition

Mineral salt medium (MSM) was used as the enrichment medium supplemented with 1% (v/v) Heglig Nile Blend (HNB) crude oil as sole carbon source for isolation of hydrocarbon-utilising bacteria. The composition of MSM is that of Zajic and Supplisson (1972) and was used throughout the study. HNB crude oil was obtained from Khartoum Oil Refinery at Algaili. The isolated bacteria were grown on the MSM. The pH of the medium was adjusted to 7.4 using 6 N hydrochloric acid or 1 N sodium hydroxide.

Enrichment and isolation of pure cultures of hydrocarbon-utilising bacteria

Hydrocarbon-utilising bacteria were isolated and purified using the enrichment procedure described by Ganesh and Lin (2009) with some modification. In brief, 1 g soil sample was added into 100 mL of MSM that contains 1% (v/v) HNB crude oil as sole carbon source in 250 mL Erlenmeyer flasks. The flasks were then shaken for seven days at 170 rpm at 30 °C ± 2 °C. Serial dilutions were carried out for all samples and 1 mL of each dilution was streaked on MSM agar containing 1% (v/v) HNB crude oil as sole carbon source. The plates were incubated at 30 °C ± 2 °C for 3-7 days. Colonies of hydrocarbon-utilising bacteria were randomly picked and further purified by streaking onto nutrient agar plates for several times until pure colonies were obtained. The purified isolates were preserved on slants of nutrient agar and kept in a refrigerator at 4 °C and subcultured every month until ready to use. For long term preservation, the isolates were stored in 50% glycerol at –20 ºC.

Identification and characterisation of bacterial isolates

Ten purified hydrocarbon-utilising bacterial strains which were isolated from the contaminated soil were given the letters: A (HOF 1.4), B (HOF 1.14), C (SPS 3.10), D (HOF

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1.23), E (HOF 1.26,), F (HOF 2.17), G (ERS 1), H (SPS 3.1), I (SPS 3.14), and J (SPS 3.30). The isolates were characterised with tentative identification to species/genus according to standard laboratory and taxonomic manuals on the basis of morphological characteristics of the cells and colonies as well as different types of biochemical tests such as Gram staining, endospore staining, oxidase test, catalase test, oxidation fermentation, hydrogen sulphide production test, nitrate reduction test, indole test, methyl-red test (MR), Voges-Proskauer test (VP), citrate utilisation test, triple sugar iron agar test, starch hydrolysis test and urease test (Holt *et al*., 1994; Cappuccino and Sherman, 2014). In this study, no genetic analyses were done.

Capabilities of the ten purified bacterial strains to grow in diesel oil by measuring turbidity of the culture broth

The capabilities of the 10 isolates to grow in refined diesel oil were tested and compared. The diesel oil was obtained from a local petrol filling station and stored in dark bottle at 30 °C ± 2 °C throughout the study and the diesel oil was filter sterilized using cellulose membrane filter, pore size 0.2 µm, before it is used. In this study, the growth of the bacterial isolates in MSM containing 1% of diesel oil was considered having the ability to utilise the diesel oil, which was assessed by measuring the optical density of the liquid medium at 620 nm at the end of incubation period using spectrophotometer (Apel model PD-303). Testing involved adding 1 mL of culture broth from each bacterial isolate (OD equivalent to 1.0 at 620 nm) to 250 mL Erlenmeyer flask containing 100 mL sterile MSM supplemented with 1% diesel oil as the sole carbon source. The flasks were stoppered with cotton wool stoppers and kept on a shaker at 170 rpm at 30°C ± 2°C for seven days**.** All media and glassware were sterilized by autoclaving for 15 min at 121°C. Non-inoculated flasks and flasks without diesel oil served as controls. All treatments were conducted in duplicate. The optical density was recorded at the end of the incubation period at 620 nm to estimate bacterial growth and biomass. The growth was categorized as: (i) excellent growth of bacteria (OD_{620} = 0.81->1.0) denoted by ++++ indicating very high hydrocarbon-utilising capability (ii) high growth of bacteria (OD_{620} = 0.61-0.80) denoted by +++ indicating high hydrocarbon-utilising capability (iii) moderate growth of bacteria (OD_{620} = 0.41-0.60) denoted by ++ indicating moderate hydrocarbon-utilising capability (iv) low growth of bacteria (OD_{620} = <0.40) denoted by + indicating low hydrocarbon-utilising capability (Rahman *et al*., 2002).

Preparation of the best bacterial consortium for utilisation of diesel oil

One mL from each bacterial broth was transferred into a separate 250 mL Erlenmeyer flask containing 100 mL sterile MSM medium (Zajic and Supplisson, 1972) supplemented with 1% diesel oil. The flasks were closed with cotton wool stoppers and kept on a shaker at 170 rpm at 30 °C ± 2 °C until the optical density was approximately 1.0 at 620 nm. In this study seven bacterial consortia were prepared. Each of the seven consortia was prepared by mixing together 1 mL of the pure culture broth from each of three or four bacterial isolates. Six consortia were prepared at random and labelled as HBI, EGC, HBEF, IEFG, HGCI, and HEIG. One system (HBE) contained a consortium representing a mixture of the best three hydrocarbon-utilising bacteria isolated from this study. The overall growth of the bacterial strains comprising each consortium to survive in 1% diesel oil was assessed as outlined above to choose the best consortium for further studies.

RESULTS AND DISCUSSION

The hydrocarbon-utilising bacteria were obtained by culture enrichment technique with Heglig Nile Blend (HNB) crude oil as sole carbon and energy source. Pure cultures of 10 bacteria capable of utilising crude oil hydrocarbons as sole carbon source were successfully isolated under aerobic conditions from three different habitats contaminated with petroleum hydrocarbons. Table 1 shows that the growth performance of the 10 identified strains in MSM with diesel oil arranged in ascending order, exhibited noticeable differences varying from low to very high growth.

Tables 2 and 3 show the morphological characteristics of the 10 isolates as well as the results of biochemical tests. It is worth noting that the genera and species presented have been reported in the literature as typical heterotrophic bacteria present in the oil contaminated soils and capable of degrading hydrocarbons (Rahman *et al*., 2002; Survery *et al*., 2004; Hunter *et al.*, 2005; Toledo *et al*., 2006; Nwaogu *et al*., 2008; Sathishkumer *et al*., 2008; Al-Saleh *et al*., 2009; Boboye *et al*., 2010; Roy *et al.*, 2014; Koshlaf and Ball, 2017). Table 2 shows that 9 out of 10 isolates were Gram positive bacteria belonging to the five genera identified as *Bacillus, Micrococcus, Corynebacterium*, *Kocuria* and *Aerococcus*. The one Gram negative isolate belongs to the genus *Enterobacter*. The genus *Bacillus* dominated with 5 isolates; several other studies have shown dominance of *Bacillus* spp. from crude oil contaminated soils and soils from petrol stations. Ijah and Antai (2003) attributed the prevalence and survival of *Bacillus* in soil samples with high concentrations of hydrocarbons up to 40% to the possession of resistant endospores.

The seven isolates with the highest growth on MSM (Table 1) were chosen to construct 7 consortia to test growth in diesel oil under controlled laboratory conditions (Table 4). The growth performance of these 7 isolates was in the order *Enterobacter* sp.> *Bacillus subtilis* subsp*. subtilis*> *Aerococcus* sp.> *Bacillus firmus*> *Corynebacterium* sp.> *Bacillus lentus*> *Micrococcus luteus* (Table 1). The top 3 isolates with highest growth were chosen to formulate the consortium HBE (number 7, Table 4). In addition, another 6 consortia (consortia number 1-6, Table 4) of 3 - 4 isolates were constructed from these

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Table 1: The growth performance of the ten identified strains in mineral salt medium (MSM) with 1% diesel oil at optical density 620 nm (OD_{620}).

Represented letter	Bacterial strains	Source of isolate	OD_{620}[a]	Bacterial growth[b]
A	*Kocuria flavus*	Heglig field	0.065 ± 0.047	+
D	*Bacillus cereus*	Heglig field	0.110 ± 0.040	+
J	*Bacillus subtilis*	S. P. station	0.367 ± 0.001	++
I	*Micrococcus luteus*	S. P. station	0.656 ± 0.017	+++
C	*Bacillus lentus*	Heglig field	0.743 ± 0.013	+++
G	*Corynebacterium* sp.	R. sludge	0.886 ± 0.016	++++
F	*Bacillus firmus*	Heglig field	0.970 ± 0.047	++++
E	*Aerococcus* sp.	Heglig field	1.100 ± 0.014	++++
B	*Bacillus subtilis* subsp. *subtilis*	Heglig field	1.245 ± 0.001	++++
H	*Enterobacter* sp.	S. P. station	1.283 ± 0.017	++++

Heglig field = Heglig oil field; R. sludge = Refinery sludge; S. P. station = Shendi petrol station
[a] Data represents averages ± standard deviation (SD) of duplicate samples after incubation for seven days.
[b] excellent growth of bacteria (OD_{620}= 0.81->1.0) denoted by ++++ indicating very high hydrocarbon-utilising capability; high growth of bacteria (OD_{620}= 0.61-0.80) denoted by +++ indicating high hydrocarbon-utilising capability; moderate growth of bacteria (OD_{620}= 0.41-0.60) denoted by ++ indicating moderate hydrocarbon-utilising capability; low growth of bacteria (OD_{620}= <0.40) denoted by + indicating low hydrocarbon-utilising capability

seven isolates to test and compare growth in diesel oil. Table 4 shows that consortia 5, 6 and 7 achieved the highest optical densities of 0.880, 0.886, and 0.940 respectively. However, the optical densities of these best three consortia (Table 4) were slightly lower compared to the top 3 individual strains that were tested singly in diesel oil (Table 1).

According to Varjani (2017), petroleum hydrocarbons utilizing microorganisms are widely distributed in nature and that they naturally biodegrade pollutants and thereby remove pollutants from the environment. Varjani *et al.* (2015) developed a hydrocarbon utilizing bacterial consortium consisting of four bacterial isolates that was found to be good at degrading crude oil. Many authors are in the opinion that mixed bacterial populations are superior to single cultures in degradation of petroleum hydrocarbons due to different degradative enzymes and pathways occurring in different members of the consortium (Leahy and Colwell, 1990; Bouchez *et al.*, 2000; Mishra *et al*., 2001; Vinas *et al.*, 2002; Gallego *et al*., 2007; Cerqueira *et al.*, 2011; Malik and Safia, 2012; Santisi *et al*., 2015; Patowary *et al*., 2016).

Jasmine and Mukherji (2014) pointed out that the use of mixed bacterial populations is more effective than single bacterial populations because of broad enzymatic capacities, synergistic effect and co-metabolism in the degradation of complex mixtures of hydrocarbons. Sarkar *et al*. (2013) and Patowary *et al.* (2016) indicated that for a microbial consortium to achieve higher degradation, the bacterial mixed populations must be compatible with each other without any antagonism among them. It is possible that one species outgrows the others in the system used and one would need to use genetic analyses such as DNA sequencing following extraction to identify and provide information on the composition of microbial communities.

In the present study, the growth of the three individual isolates: *Enterobacter* sp., *Bacillus subtilis* subsp. *subtilis* and *Aerococcus* sp. in diesel oil obtained OD620 1.100-1.283, which is slightly higher than consortia of three to four isolates (Tables 1 and 4). From Table 4, the best consortium to grow in MSM with 1% of diesel oil is made up from these three bacterial isolates attaining OD 0.940. It is interesting to note that this consortium of three bacteria showed higher growth (OD_{620} 0.940) than the consortium that consists of four isolates (OD_{620} 0.880) that included *Bacillus firmus* as an added member to the consortium of the three isolates (Table 4). An assumption may be made that by adding the fourth strain (*B. firmus)* to the selected consortium of three strains, an antagonistic effect is exerted. However, this assumption must be verified by carrying out antagonistic tests to determine if there is any growth inhibition from every single isolate toward other strains. We recommend to screen isolates of the consortium for the production of quorum sensing signal molecules in future work, in order to illustrate synergistic and antagonistic effect. In this study, the consortium which consists of *Enterobacter* sp., *Bacillus subtilis* subsp. *subtilis* and *Aerococcus* sp. possessing good growth in diesel oil is useful for crude oil degradation in laboratory and field studies in the future.

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Table 2: Morphological and cultural characteristics of the bacterial strains isolated from three different habitats in this study.

Represented letter	Bacteria	Colony shape	Colour	Surface	Elevation	Margin	Opacity	Size	Gram	Endospore	Cell shape
A	*Kocuria flavus*	circular	yellow	n/a	convex	entire	transparent	small	+	–	coccus
B	*Bacillus subtilis* subsp. *subtilis*	irregular	cream	n/a	flat	undulate	transparent	large	+	+	rod
C	*Bacillus lentus*	circular	cream	n/a	flat	entire	transparent	medium	+	+	rod
D	*Bacillus cereus*	circular	cream	n/a	flat	entire	transparent	medium	+	+	rod
E	*Aerococcus* sp.	circular	orange	n/a	flat	entire	transparent	small	+	–	coccus
F	*Bacillus firmus*	circular	cream	n/a	flat	entire	transparent	small	+	+	rod
G	*Corynebacterium* sp.	irregular	cream	granular	umbonate	serrate	transparent	–	+	-	rod
H	*Enterobacter* sp.	circular	white	smooth	convex	entire	transparent	–	–	-	short rod
I	*Micrococcus luteus*	circular	yellow	smooth	convex	entire	transparent	–	+	-	coccus
J	*Bacillus subtilis*	circular	cream	smooth	raised	serrate	transparent	–	+	+	rod

n/a= not available; (+) = positive; (–) = negative

Table 3: Biochemical characteristics of the bacterial strains isolated from hydrocarbon contaminated habitats.

Represented letter	Bacteria	H_2S	Glucose	MR test	VP test	Indole test	Urease test	Citrate test	Catalase test	Oxidase test	Nitrate test	Oxidation lactose/starch
A	*Kocuria flavus*	–	–	+	–	–	–	+	+	–	n/a	–
B	*Bacillus subtilis* subsp. *subtilis*	–	+	–	+	–	–	+	+	+	n/a	–
C	*Bacillus lentus*	–	+	+	–	–	+	–	+	–	n/a	–
D	*Bacillus cereus*	–	+	–	+	–	–	+	+	–	n/a	–
E	*Aerococcus* sp.	–	+	+	–	–	–	+	+	–	n/a	+
F	*Bacillus firmus*	–	+	+	–	–	–	–	+	+	n/a	–
G	*Corynebacterium* sp.	–	+	+	-	–	+	+	+	+	+	-
H	*Enterobacter* sp.	–	–	-	+	–	+	+	+	–	+	+
I	*Micrococcus luteus*	–	–	+	–	–	–	–	+	–	+	-
J	*Bacillus subtilis*	–	–	–	+	–	+	+	+	–	+	+

n/a= not available; (+) = positive; (–) = negative

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Table 4: The growth performance of the ten identified strains in mineral salt medium (MSM) with 1% diesel oil at optical density 620 nm (OD_{620}).

Consortia numbers and represented letters of isolates	Mixtures of consortia	OD_{620}[a]	Bacterial growth[b]
consortium (1) EGC	*Aerococcus* sp. *Corynebacterium* sp. *Bacillus lentus*	0.561 ± 0.078	++
consortium (2) IEFG	*Micrococcus luteus* *Aerococcus* sp. *Bacillus firmus* *Corynebacterium* sp.	0.631 ± 0.009	+++
consortium (3) HEIG	*Enterobacter* sp. *Aerococcus* sp. *Micrococcus luteus* *Corynebacterium* sp.	0.662 ± 0.103	+++
consortium (4) HGCI	*Enterobacter* sp. Corynebacterium sp. *Bacillus lentus* *Micrococcus luteus*	0.717 ± 0.009	+++
consortium (5) HBEF	*Enterobacter* sp. *Bacillus subtilis* subsp. *subtilis* *Aerococcus* sp. *Bacillus firmus*	0.880 ± 0.053	++++
consortium (6) HBI	*Enterobacter* sp. *Bacillus subtilis* subsp. *subtilis* *Micrococcus luteus*	0.886 ± 0.032	++++
consortium (7) HBE	*Enterobacter* sp. *Bacillus subtilis* subsp. *subtilis* *Aerococcus* sp.	0.940 ± 0.041	++++

[a]Data represents averages ± standard deviation (SD) of duplicate samples after incubation for seven days.
[b]Excellent growth of bacteria (OD_{620}= 0.81->1.0) denoted by ++++ indicating very high hydrocarbon-utilising capability; high growth of bacteria (OD_{620}= 0.61-0.80) denoted by +++ indicating high hydrocarbon-utilising capability; moderate growth of bacteria (OD_{620}= 0.41-0.60) denoted by ++ indicating moderate hydrocarbon-utilising capability; low growth of bacteria (OD_{620}= <0.40) denoted by + indicating low hydrocarbon-utilising capability

CONCLUSION

Ten hydrocarbon-utilising bacterial strains were isolated by enrichment technique from oil-polluted sites using crude oil as sole carbon source. The consortium consisting of three bacterial strains showing the best growth in diesel is recommended to be tested in future studies before applying in field. *B. subtilis* subsp. *subtilis* and *Aerococcus* sp. were isolated from crude oil polluted soil and *Enterobacter* sp. was isolated from diesel oil contaminated soil of a petrol station. It is expected that these indigenous hydrocarbon-utilising bacteria are more adapted to arid conditions and it is recommended that this consortium can be utilised in *in situ* landfarming remediation of oil-polluted sites to reduce concentrations of the contaminants to safe levels.

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