Bioprocess and Biosystems Engineering
https://doi.org/10.1007/s00449-021-02534-6

RESEARCH PAPER

# Reduction of lag in crude oil degradation by *Aspergillus* when it is in synergy with *Bacillus* in biofilm mode

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Received: 26 September 2020 / Accepted: 9 February 2021


## Abstract

A major hindrance to the effective use of fungi in bioremediation is their inherent slow growth. Despite this, *Aspergillus* spp. may be used effectively. Our experiments demonstrate that bacteria, although inefficient in hydrocarbon degradation, may be effectively used in a consortium to overcome the lag in fungal utilization of petroleum hydrocarbons. Crude petroleum oil (160 mg; at 8 g/L) in minimal medium was inoculated with a previously isolated biofilm-forming consortium (*Aspergillus* sp. MM1 and *Bacillus* sp. MM1) as well as monocultures of each organism and incubated at 30 ℃ under static conditions. Residual oil was analyzed by GC–MS. Crude oil utilization of *Aspergillus–Bacillus* biofilm was 24 ± 1.4% in 3 days, increased to 66 ± 7% by day 5 and reached 99 ± 0.2% in 7 days. *Aspergillus* sp. MM1 monoculture degraded only 14 ± 6% in 5 days. However, at the end of 7 days, it was able to utilize 98 ± 2%. *Bacillus* sp. MM1 monoculture utilized 20 ± 4% in 7 days. This study indicates that there is a reduction of the fungal lag in bioremediation when it is in association with the bacterium. Although in monoculture, *Bacillus* sp. MM1 is inefficient in crude oil degradation, it synergistically enhances the initial rate of crude petroleum oil degradation of the fungus in the consortium. The rapid initial removal of as much crude oil as possible from contaminated sites is vital to minimize detrimental impacts on biodiversity.

**Keywords** *Aspergillus–Bacillus* biofilm · Lag time · Crude oil biodegradation · Synergism · GC–MS

## Introduction

Crude petroleum oil contamination is a global concern. The volume of crude petroleum oil released to the environment in 2018 was approximately 116,000 metric tons (www.itopf.org), following oil spills, oil seeps, and transportation accidents. This affects the quality of soil and water, and every form of life in both aquatic and terrestrial habitats [1]. Crude petroleum oil is a complex mixture of hydrocarbons of which the major fraction is saturated hydrocarbons. Remaining fractions are naphthenes, aromatics, resins, least soluble asphaltenes [2, 3], and non-hydrocarbon compounds such as nickel, vanadium, nitrogen, and sulfur [4].

Depending on their chemical and physical characteristics, various hydrocarbon fractions (typically 30–50%) may evaporate, while the remainder will spread over the surface and dissolve into water or absorb into the soil. This oil may subsequently accumulate as persistent residues or be slowly degraded by microorganisms and solar ultraviolet radiation [5]. Therefore, rapid removal of spilled oil

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Published online: 05 March 2021

from shorelines and wetlands is necessary to minimize potential environmental damage to these sensitive habitats.

Microbial bioremediation is the method that makes the most "eco-sense"; causing the least threat to the ecosystem while being economical. However, the process is relatively slow [6]. Physical and chemical methods, such as thermal desorption, soil washing, air sparging, chemical oxidation–reduction, incineration, etc. [7], are costly, yet more efficient in remediation of crude petroleum oil-contaminated sites. However, physicochemical methods produce hazardous chemical compounds such as carcinogens and immunotoxicants as a result of the underlying physical or chemical process [8]. Bioremediation has the advantage of producing $H_2O$ and $CO_2$ as the end products.

Numerous hydrocarbon-utilizing bacterial and fungal species have been identified, having varying rates of degradation and selectivity in carbon chain length. *Acinetobacter venetianus* strain RAG-1 was reported to degrade (1 g/L) diesel fuel in 7 days [9]. Individual strains of *Bacillus algicola*, *Rhodococcus soli*, *Isoptericola chiayiensis*, and *Pseudoalteromonas agarivorans* have been shown to degrade > 85% of crude oil (1% v/v) individually at 28 °C in 14 days at 180 rpm [10].

Some bacterial species have been shown to be effective when they are in association with several other species which have the similar degradation potential or when they are provided with additives. A consortium of two strains of *Geobacillus* and a *Bacillus* sp. (50 ℃; 120 rpm in 20 days) degraded *n*-alkanes C32 and C40 (0.1%) to 90% and 87%, respectively [11]. A consortium of five strains of bacteria (*Exiguobacterium* sp., *Pseudomonas aeruginosa*, *Alcaligenes* sp. ASW-3, *Alcaligenes* sp. ASS-1, and *Bacillus* sp.) immobilized in a calcium alginate activated carbon embedding carrier degraded 75.1% crude oil (1%, w/v) at 25 ℃ in 7 days at 150 r/min [12]. *B. subtilis* BL-27 has been reported to degrade 65% of crude oil (0.3%, w/v) within 5 days at 45 ℃, while the use of additives such as SDS (50–100 mg/L) and Tween 80 (200–500 mg/L) significantly increased the strain's biodegradation efficiency up to 75–80% [13].

Fungi are more resistant to harsh environmental conditions than bacteria [14] and therefore, they have acquired the ability to utilize a wider range of components of crude oil. *Aspergillus* and *Rhizopus* sp. have been shown to degrade crude oil (0.5%) by 29.10% and 26.32%, respectively; while their combination removed 48% in 28 days at 28 ℃ [15]. A mixed culture of *A. niger* and *A. fumigatus* degraded 90% of crude oil (2%, w/w) in 28 days at 25 °C, while a community of four strains (*A. niger*, *A. fumigatus*, *P. funiculosum,* and *Fusarium solani*) was able to remove only 70% in 28 days [16].

Both *Aspergillus* and *Bacillus* spp. have been identified as dominant hydrocarbon degraders from petroleum-polluted sites. Various *Aspergillus* and *Bacillus* strains have been tested for petroleum biodegradation [11, 13, 16–19].

Over the last decade, efficiency has been shown to be improved by the use of microbial biofilms in bioremediation of contaminants [20–22]. Biofilms can be formed either by a population of a single species or a mixed community of different species of the same or different genera. Fungal-bacterial biofilms are a special form of biofilms where bacterial cells are attached to the well-spread fungal hyphae by secreted extracellular polymeric substances (EPS) to cement the interaction, thus increasing resistance to stress conditions [23]. Due to their interaction and cellular communication, biofilm communities acquire increased metabolic activity which leads to their efficiency compared to planktonic cells / monocultures [24].

Further, the combined use of fungi and bacteria that are able to form biofilms has proven to be synergistic in removal of the underlying substrate. The use of *Aspergillus* and *Bacillus* strains in a combination has been successfully demonstrated in synergistic degradation of *n*-hexadecane [25]. Here, we tested the role / effect of bacilli in the *Aspergillus–Bacillus* consortium in utilization of crude oil.

## Materials and methods

### Microbial strains

Three fungal–bacterial consortia (C1, C2, and C3) were previously isolated from a municipal landfill in Colombo district, Sri Lanka based on their ability to degrade *n*-hexadecane. C1 and C2 consortia had a single bacterium associated with a single fungus; while, C3 comprised of three fungi associated with a single bacterium. C1 which showed the highest activity towards *n*-hexadecane degradation was found to be comprised of *Aspergillus* sp. MM1 (GenBank accession no. MH503926) and *Bacillus* sp. MM1 (GenBank accession no. MH503924) [25]. In the present study, crude petroleum oil biodegradation of C1, C2, and C3 consortia was investigated.

### Culture media

Nutrient broth (NB) or nutrient agar (NA) and sabouraud dextrose broth (SDB) or sabouraud dextrose agar (SDA) (Hardy Diagnostics, USA) were used to maintain bacterial and fungal cultures, respectively. Bushnell and Haas (BH) medium [26] was used in assays conducted to measure the ability of microbes to utilize the given source of carbon and energy. Filter sterilized (0.22 µm pore size) crude petroleum oil obtained from Ceylon Petroleum Corporation (CEYPETCO) refinery, Sri Lanka was used as the sole source of

carbon and energy in BH medium at a concentration of 1% (v/v). The crude petroleum oil used in this study corresponds to 8.25 g/L.

### Culture conditions

Crude petroleum oil degradation experiments were carried out under aerobic conditions (without agitation, to enhance the formation of a microbial biofilm). GC–MS assays were conducted in duplicate and all others in triplicate, with negative control (under same conditions, without microbial inoculum) where necessary.

### Crude petroleum oil biodegradation assay

C1, C2, and C3 consortia were assayed for crude petroleum oil biodegradation. Co-cultures of C1, C2, and C3 were prepared by adding $5 \times 10^4$ bacterial cells/mL and $5 \times 10^3$ fungal spores/mL of each strain in the consortium. Erlenmeyer flasks (100 mL) containing 20 mL BH medium with 1% crude petroleum oil (8.25 g/L) was inoculated with each co-culture and incubated continuously for 7 days at 30 ℃. Control flasks were incubated under identical conditions but devoid of the microbial inoculum (negative control). Disintegration / removal of the oil layer was visually observed in comparison to the negative control. The most efficient consortium was selected based on visual observation and the composition of the consortium.

### Quantitative analysis of crude petroleum oil biodegradation by Gas Chromatography–Mass spectrometry (GC–MS)

Microbial strains in the selected consortium were cultured separately as well as in co-culture, maintaining the same inoculum size and the culture conditioned mentioned above. The residual oil in the growth medium of microbial cultures and negative control were extracted at day 0, day 3, day 5, and day 7 for quantitative analysis by GC–MS.

Residual crude petroleum oil was extracted four times with dichloromethane (DCM) (5 mL) and the combined solvent extract was dried using anhydrous sodium sulfate ($Na_2SO_4$). Crude petroleum oil in DCM was then analyzed chromatographically via GC–MS using Agilent 7890a GC system equipped with 5975c MS system, split injector, and a capillary column (Agilent 19091 s-433HP-5MS 5% Phenyl Methyl Silox; 30 m × 250 µm × 0.25 µm). The optimized temperature program was as follows: oven temperature 60 °C for 2 min, then increased to 290 ℃ at a rate of 20 ℃ / min and kept at 290 ℃ for 5 min where the total run time was 18.5 min. The injector temperature was maintained at 320 ℃. The carrier gas used in the column was helium at a flow rate of 1 mL/min [27].

The total ionic current (TIC) spectra obtained by GC–MS analysis displayed the relative abundance of the components of crude petroleum oil present in the DCM extract. The degradation percentages were calculated from the difference in the area under the respective peak in the TIC spectrum.

## Results

### Selection of an efficient fungal-bacterial consortium in crude petroleum oil biodegradation

Crude petroleum oil biodegradation by C1, C2, and C3 consortia were visually observed. Crude petroleum oil (1%, 20 mL BH medium in the Erlenmeyer flask) was visible by its brown coloration and formed a continuous oil layer on the colorless salt medium (Fig. 1). The bottom view of the control flask (Fig. 1a) clearly showed the brownish oil layer inside the flask. Biofilms formed by the consortia were brown in color due to the aggregation of oil around the growing biofilm. After 7 days of incubation, the brown coloration was absent in C1 and C3, indicating that the oil has been utilized; whereas in C2, some color was still observable. Therefore, it can be concluded that both C1 and C3 were equally capable of removing the crude petroleum oil in the liquid medium within 7 days at 30 °C according to the visual observation of the crude petroleum oil biodegradation assay.

According to the previous characterization of the community composition of C1, C2, and C3 [25], C1 comprised of a single bacterium (*Bacillus* sp. MM1) and a single fungus (*Aspergillus* sp. MM1) and C3 had at least three species of fungi interacting with a single bacterium. Therefore, based on the visual observation of the efficiency of crude oil degradation as well as the simplicity of the consortium, C1 (*Aspergillus–Bacillus* biofilm) was selected for further investigation.

### GC–MS analysis of crude petroleum oil degradation by the *Aspergillus–Bacillus* biofilm and the synergistic association of its community counterparts

#### Abiotic loss of crude petroleum oil in the growth medium

The aliphatic fraction of the crude petroleum oil used in this study comprised of straight chain alkanes ranging from C9 to C30 as well as pristine (Pr) and phytane (Py). The TIC spectrum displayed 22 different prominent peaks for straight chain alkanes (C9–C30) and less prominent peaks for the branched alkanes Pr and Py. An unresolved fraction (URF) was denoted by unresolved low-intensity peaks observed between *n*-alkanes C12 and C24. The TIC spectrum of the control flask (without microbial inoculum) during 7 days of

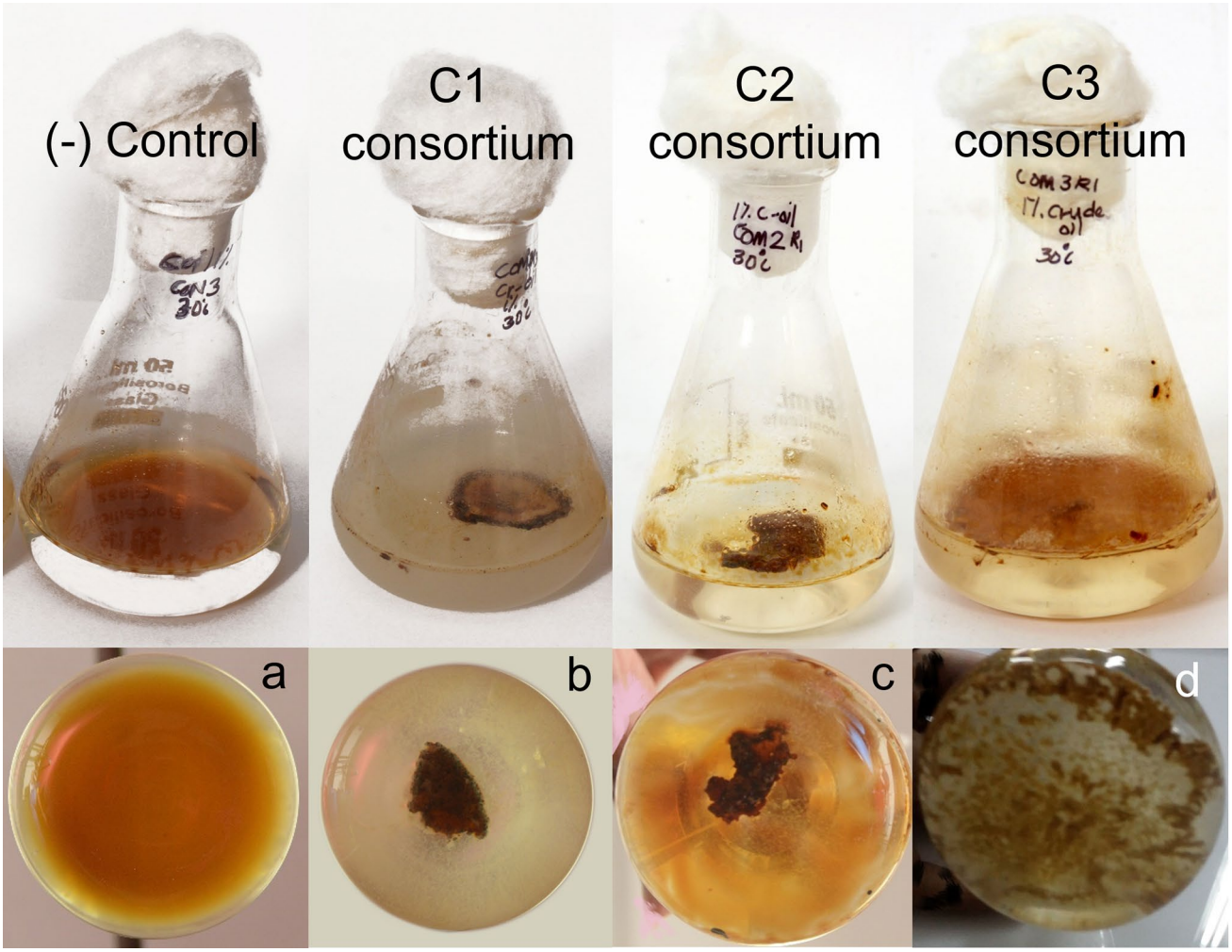


**Fig. 1** Removal of crude petroleum oil by C1, C2, and C3 consortia; **a**–**d** bottom view of each flask

incubation showed only 19 different peaks of straight chain alkanes (C12–C30) and peaks for Pr and Py. Accordingly, C9 (nondance), C10 (decane), and C11 (undecane) were completely evaporated. The rest of the alkanes were partially evaporated (39 ± 14%) during the 7 days (Fig. 2).

### Biotic removal of crude petroleum oil by *Aspergillus–Bacillus* biofilm and its community counterparts

For quantitative analysis of crude petroleum oil degradation, individual members of C1 consortium, *Aspergillus* sp. MM1 and *Bacillus* sp. MM1 were cultured separately in their monoculture form as well as in co-culture to obtain the *Aspergillus–Bacillus* biofilm. The degradation was studied by GC–MS analysis after 7 days of incubation.

The *Aspergillus–Bacillus* biofilm utilized straight chain alkanes (C12–C30) of crude petroleum oil to an extent of 99 ± 0.2% within 7 days and respective peaks in the TIC spectrum for the branched alkane Pr and the URF were undetectable (Fig. 3a).

The monoculture of *Aspergillus* sp. MM1 was also able to remove straight chain alkanes (C12–C30) and the branched alkane Pr within 7 days. Removal of the branched alkane Py by *Aspergillus–Bacillus* biofilm as well as the *Aspergillus* sp. MM1 monoculture was similar, being 47 ± 15 and 47 ± 29%, respectively, in 7 days (Fig. 3). The URF, however, was not completely removed by *Aspergillus* sp. MM1 monoculture (Fig. 3b).

Visual observation of the cultures revealed that a few oil droplets were still present in *Aspergillus* sp. MM1 monoculture compared to the consortium, after 7 days (Fig. 4).

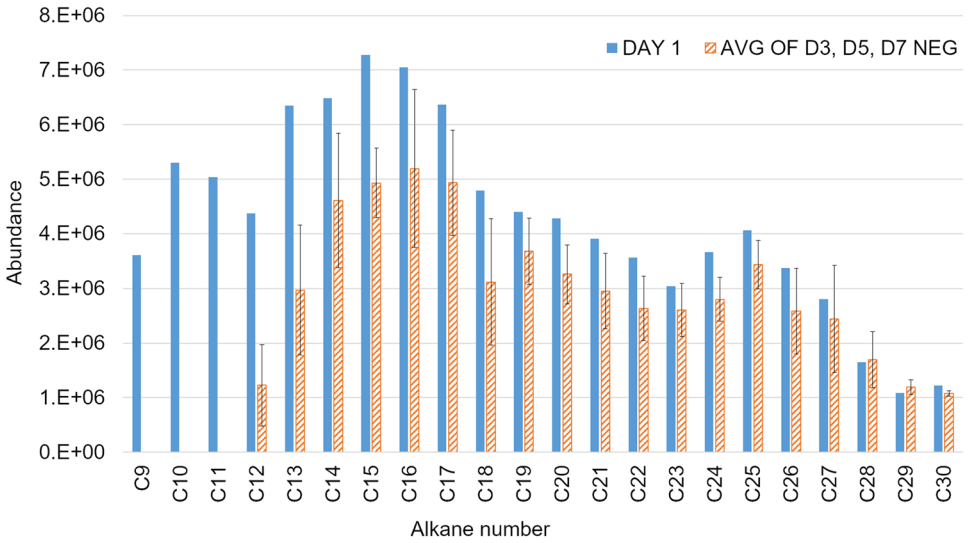


**Fig. 2** Abiotic removal of crude oil components. Error bars represent standard deviations ($n = 3$)

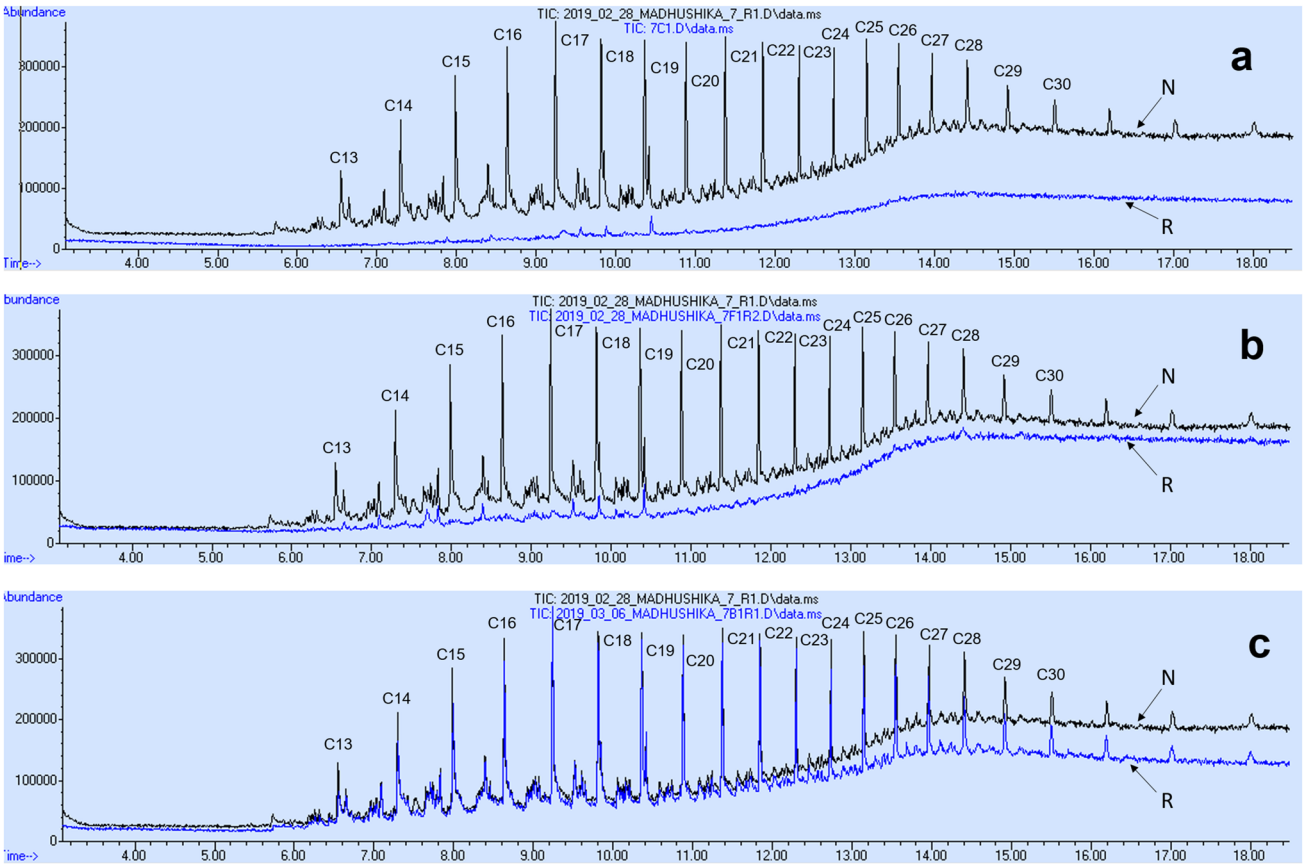


**Fig. 3** TIC spectrum of residual crude oil (R) (blue) compared to the negative control (N) (black) after 7 days of incubation; **a** *Aspergillus–Bacillus* biofilm, **b** *Aspergillus* sp. MM1, and **c** *Bacillus* sp. MM1

The removal of aliphatic components of crude petroleum oil by monoculture of *Bacillus* sp. MM1 was only $20 \pm 4\%$ in 7 days (Fig. 5).

## Synergistic association of *Aspergillus* sp. MM1 and *Bacillus* sp. MM1 in *Aspergillus–Bacillus* biofilm in degradation of crude petroleum oil

The GC–MS analysis indicated that the degradation of crude

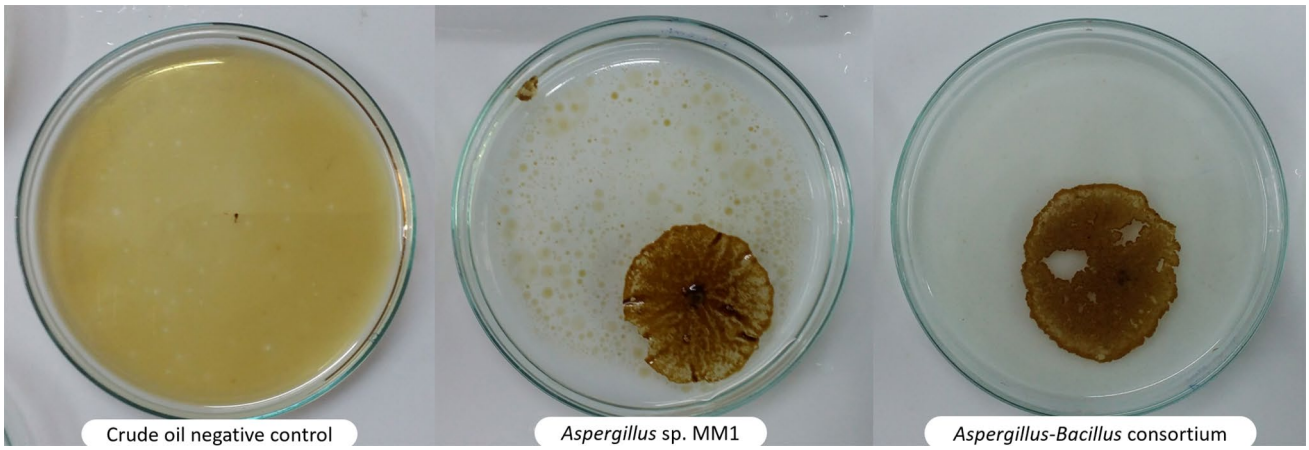


**Fig. 4** Growth of *Aspergillus–Bacillus* biofilm and *Aspergillus* sp. MM1 monoculture in crude petroleum oil in 7 days (cultures were grown in conical flasks and poured into petri dishes for better visualization)

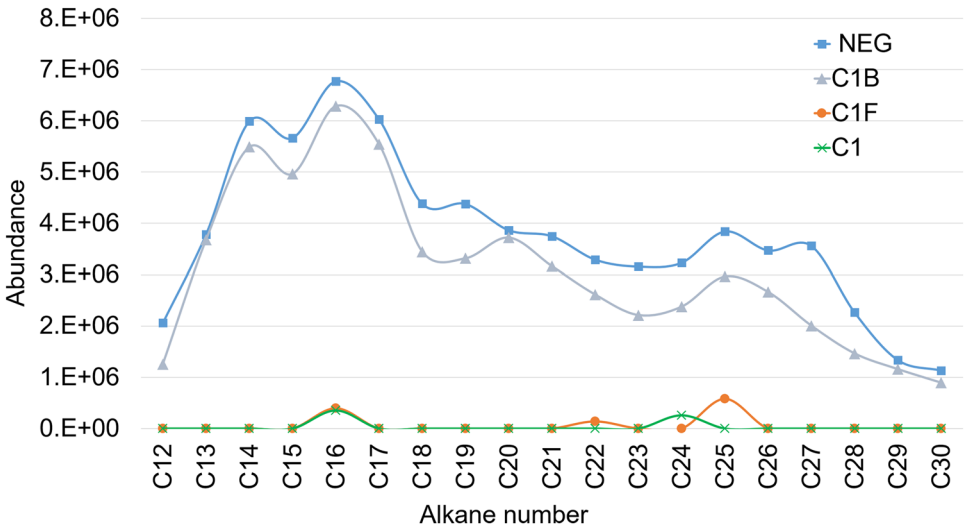


**Fig. 5** Residual crude oil fractions (C12-C30 alkanes) at day 7 at 30 °C for the *Aspergillus–Bacillus* biofilm (C1) and its counterparts, *Aspergillus* sp. MM1 (C1F) and *Bacillus* sp. MM1 (C1B)

petroleum oil by the *Aspergillus* sp. MM1 in monoculture was similar to when it was co-cultured with *Bacillus* sp. MM1. However, degradation of crude oil by the *Bacillus* in monoculture was only 20 ± 4%.

Therefore, *Aspergillus* sp. MM1 and the *Aspergillus–Bacillus* biofilm were further tested at day 3 and day 5 during the 7-day incubation period to determine the role of the consortium. The *Aspergillus–Bacillus* biofilm removed 24 ± 1.4% of the aliphatic fraction of crude petroleum oil in three days. The removal increased to 66 ± 7% by day 5 (Figs. 6, 8) and reached 99 ± 0.2% degradation by day 7.

The removal of the aliphatic fraction of crude petroleum oil by *Aspergillus* sp. MM1 monoculture was only 4.1 ± 0.4% by day 3 and 14 ± 6% by day 5, although by the end of 7 days *Aspergillus* sp. MM1 was able to remove the aliphatic fraction of crude petroleum oil by 98 ± 2%, (Figs. 7, 8).

## Discussion

The resistance of fungi towards adverse environments and persistent contaminants make them suitable candidates to remediate crude petroleum oil contaminations. However, efficiency is limited by their slower growth rate compared to bacteria. *Penicillium* and *Aspergillus* spp have shown efficient degradation of crude petroleum oil. *P. citrinum* showed the ability to degrade 77% of crude petroleum oil (13.35%; w/v) at 28 ℃ in 23 days [28]. *Aspergillus* sp. RFC-1 degraded 60.3% crude petroleum oil (250 mg/L) at 30 °C in 7 days [17]. *A. niger* utilized 95% of crude petroleum oil (2% v/v) at 25 ℃ in 28 days [16].

In our study, a previously isolated *Aspergillus–Bacillus* consortium and monocultures of its constituents, *Aspergillus* sp. MM1 and *Bacillus* sp. MM1, were tested for crude petroleum oil degradation. The naturally occurring,

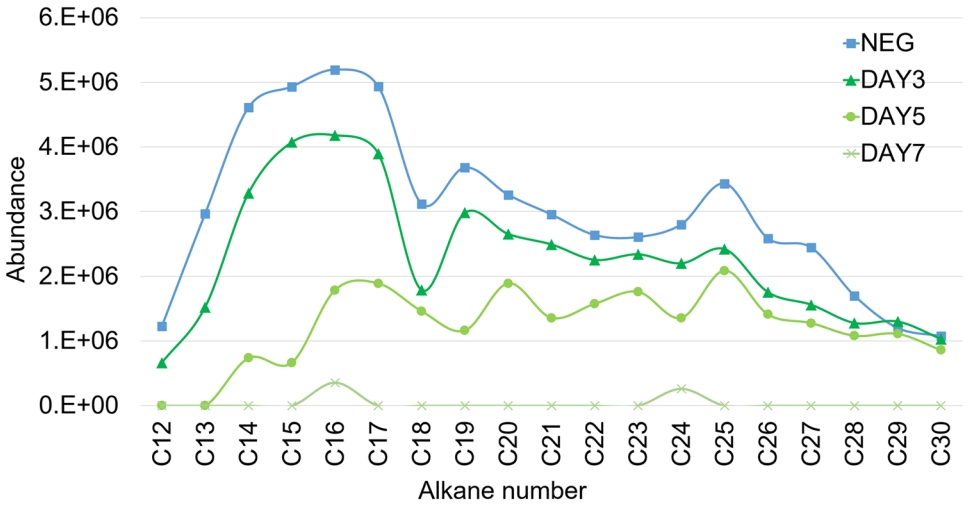


**Fig. 6** Removal of crude oil by *Aspergillus–Bacillus* biofilm at day 3, day 5, and day 7

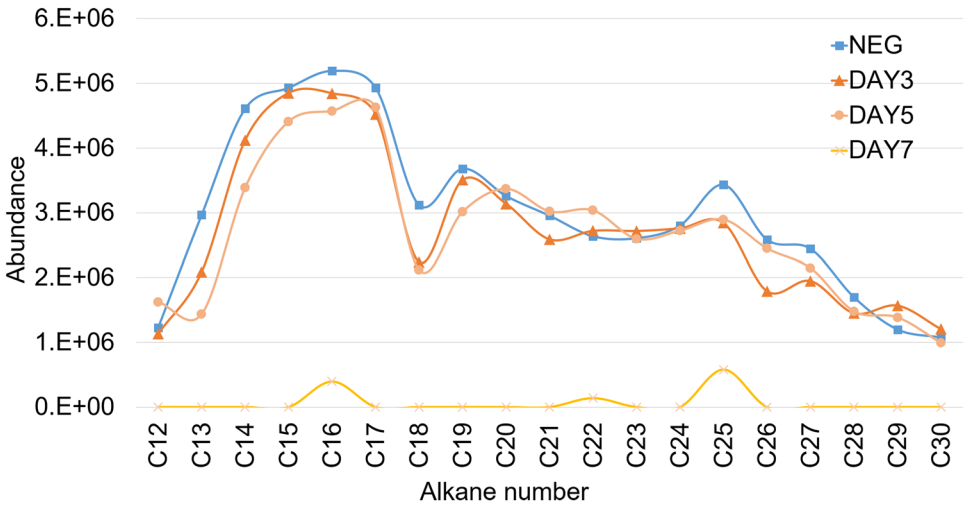


**Fig. 7** Removal of crude oil by *Aspergillus* sp. MM1 at day 3, day 5, and day 7

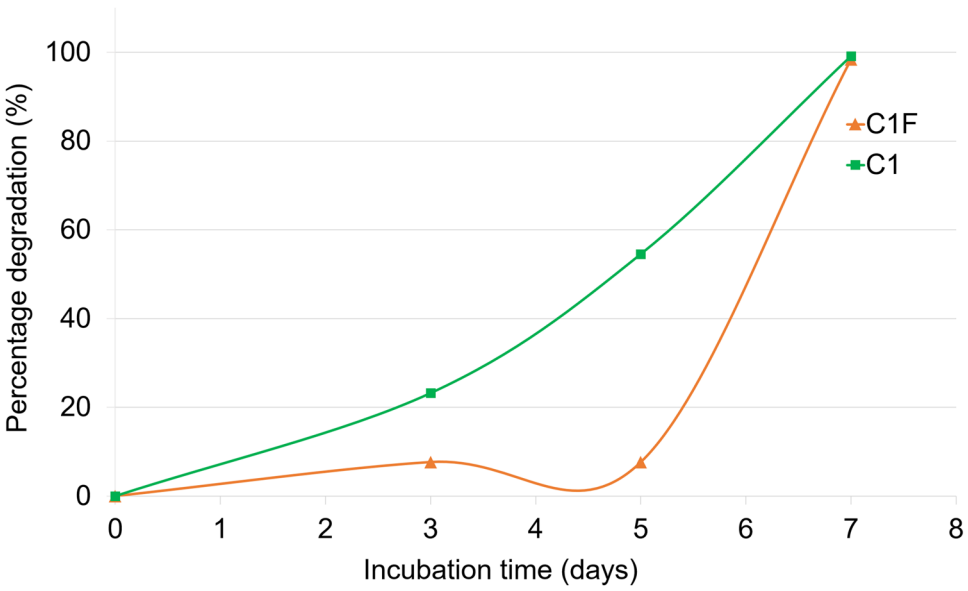


**Fig. 8** Percent degradation of crude oil aliphatic fraction by *Aspergillus–Bacillus* biofilm (C1) and *Aspergillus* sp. MM1 (C1F). Error bars represent standard deviations ($n = 2$)

biofilm-forming *Aspergillus–Bacillus* consortium was previously isolated for its ability to degrade hexadecane [25].

An inoculum of *Aspergillus* sp. MM1 ($5 \times 10^3$ fungal spores/mL) as well as *Aspergillus–Bacillus* consortium ($5 \times 10^4$ bacterial cells/mL, $5 \times 10^3$ fungal spores/mL) in a static liquid culture were able to remove $99 \pm 0.2\%$ of aliphatic fraction of 160 mg of sterile crude petroleum oil (8.25 g/L; 1% v/v) in minimal medium within 7 days at 30 ℃.

GC–MS analysis of the negative control revealed the abiotic removal of crude petroleum oil in static liquid cultures during the incubation. It was ~39%, due to complete (*n*-alkanes; (C9-C11) and partial evaporation (*n*-alkanes; C12–C30) (Fig. 2). This is accountable for the abiotic loss of the carbon source inside the culture flask. Evaporation of crude petroleum oil and petroleum products has been experimentally shown to be up to 45% within a few days [29]. Our results are in accordance with the previous reports.

Crude petroleum oil is a complex mixture of hydrocarbons. The major fraction is saturates (*n*-alkanes) and the remaining fractions are naphthenes, aromatics, resins, asphaltenes, [2, 3] etc. However, crude petroleum oil composition is highly variable depending on the origin and the refining process [30–32] it undergoes.

Aromatic compounds give a low fragmentation in gas chromatography (GC) coupled with mass spectrometry (MS) leading to low-intensity peaks. The use of GC coupled with flame ionization detector (FID) or selected ion monitoring mode (SIM) enables high levels of signals even at lower concentrations of highly specific compounds such as aromatics, cycloalkanes, etc. [33, 34]. In our study, residual crude petroleum oil was extracted by liquid–liquid extraction with DCM. Then, the extracted crude petroleum oil containing both aliphatic and aromatic fractions [33] was analyzed through GC–MS. Some of the unresolved low-intensity peaks observed in the TIC spectrum between *n*-alkane C12 and C24 in the negative control (BH medium + 1% crude petroleum oil) (Fig. 3) might correspond to aromatics and cycloalkanes present in the crude petroleum oil extract.

GC–MS analysis of the residual crude petroleum oil of the *Aspergillus–Bacillus* biofilm confirmed the removal of $99 \pm 0.2\%$ of aliphatic fraction of crude petroleum oil and the unresolved fraction (URF) in 7 days. *Aspergillus* sp. MM1 in its monoculture was able to remove the aliphatic fraction (*n*-alkanes; C12 to C30) in crude petroleum oil within 7 days but was unable to remove the URF (Fig. 3b). Accordingly, the removal of crude petroleum oil by both the *Aspergillus–Bacillus* biofilm and the monoculture of *Aspergillus* sp. MM1 in 7 days was similar except for the URF. However, residual oil droplets were observed in *Aspergillus* sp. MM1 monoculture compared to the consortium after 7 days (Fig. 4). This apparent discrepancy may account for components (resins, asphaltenes, etc.) which are undetectable by the adapted GC–MS protocol.

*Bacillus* sp. MM1 removed only $20 \pm 4\%$ of the aliphatic fraction (*n*-alkanes; C12–C30) of crude petroleum oil in its monoculture in 7 days. According to the day 7 results, *Aspergillus* sp. MM1 was shown to possess the ability to completely remove aliphatic fraction (*n*-alkanes; C12–C30) of crude petroleum oil equally to that of the consortium by day 7, implying that the *Bacillus* sp. MM1 in the consortium may not serve any significant role in the biodegradation process.

However, interestingly, in the GC–MS profiles of the *Aspergillus–Bacillus* biofilm through day 3 and day 5 up to 7 days, a gradual decrease in the aliphatic fraction of the residual oil in the culture was observed ($24 \pm 1.4\%$ in 3 days, $66 \pm 7\%$ in 5 days, and $99 \pm 0.2\%$ in 7 days). In contrast, removal of the aliphatic fraction of crude oil by the monoculture of *Aspergillus* sp. MM1 was minimal up to day 5 and it was able to utilize most of the crude petroleum oil (~80%) in the medium only after the $5^{th}$ day of incubation. This clearly shows a 5-day lag phase in the fungal monoculture (Fig. 8).These results indicate that when the fungus was in association with the bacterium in the biofilm, the lag phase was reduced.

Slow growth rate of fungi is the reason for exhibiting low degradation rates of petroleum hydrocarbons despite the ability to utilize a wide range of substrates by a single fungal species [14]. It has been reported that in the presence of *B. subtilis*, the fungal genes involved in chitin and ergosterol synthesis were up-regulated [24]. Chitin and ergosterol are two main components in fungal cell walls, and they determine the living fungal biomass [35]. Therefore, it can be hypothesized that although the *Bacillus* sp. MM1 monoculture did not degrade crude petroleum oil in 7 days (under static conditions), when it was in the consortium it enhanced the fungal growth, thus increasing its capacity for utilization of the crude petroleum oil.

In the present study, the *Aspergillus–Bacillus* biofilm removed > 50% of crude petroleum oil in 5 days compared to the *Aspergillus* sp. MM1 monoculture, in which the removal is minimal up to day 5. This proves that the *Bacillus* sp. MM1 plays an important role in overcoming this lag phase barrier of the fungus when it is in the consortium. Further, the rapid biodegradation of as much crude oil as possible in a relatively short period of time in contaminated sites is extremely important to minimize the detrimental effects on fauna and flora.

Our *Aspergillus–Bacillus* biofilm has previously been shown to degrade *n*-hexadecane in a synergistic manner. Moreover, this consortium demonstrated the formation of a biofilm in oil–water interface in the liquid medium, attaching *Bacillus* cells on to *Aspergillus* mycelia [25].

It has been reported that *B. subtilis* and *A. niger* have the ability to alter their metabolism during their interaction with each other [24]. Antimicrobial defence mechanisms of both organisms decreased upon attachment of bacilli to *Aspergillus* hyphae. Genes related (putatively) to the production of antifungal and antibacterial molecules were shown to be down regulated in *A. niger* and *B. subtilis*, respectively during their attachment. This indicated the possibility to persuade a mutualistic symbiosis in biofilm formation of *Aspergillus* and *Bacillus* combination.

Our results indicate the previously reported synergism demonstrated between *Bacillus* sp. MM1 and *Aspergillus* sp. MM1 for hexadecane biodegradation [25] is extended to the degradation of crude petroleum oil. Thus, being in a consortium with the compatible partner organisms rather than alone, will be beneficial either for one organism or for both, while producing a synergistic outcome. This can be defined as 'cooperate synergy' in microbial communities, where mutualism is not required for the survival of either organism, but where they develop a synergistic outcome when they are together.

Although, the *Bacillus* sp. MM1 alone does not demonstrate efficient utilization of crude oil, when it is in the *Aspergillus–Bacillus* consortium, it overcomes the lag time observed when the fungus is in monoculture. Therefore, our results provide a starting point for investigating the mechanisms underlying the reduction of the lag time of *Aspergillus* by bacilli.

## Conclusion

A naturally occurring *Aspergillus–Bacillus* consortium efficiently utilizes the crude petroleum oil in liquid culture by synergistic interaction of the resident fungus and the bacterium. The *Bacillus* itself is inefficient in degradation of crude oil but plays a beneficial role in the consortium. It enables biodegradation to commence earlier as *Bacillus* sp. MM1 overcomes the lag phase of *Aspergillus* when both are in a combination. Consortia comprising microbes that occur naturally together are highly adapted to withstand extreme environments and maintain their persistence through balancing the synergistic and antagonistic effects. However, the density of such naturally existing consortia is generally too low to have a significant effect on bioremediation in the environment. Therefore, isolating efficient consortia, multiplying them *in vitro* and applying back to the environment is a promising concept over developing consortia using microbes selected primarily on their individual efficiency in degradation of the substrate.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00449-021-02534-6.

**Acknowledgements** We thank Ms. K. P. K. R. I. Dilani, Instrument Center—Faculty of Applied Sciences (IC-FAS), University of Sri Jayewardenepura (USJP), Nugegoda, Sri Lanka for technical assistance with GC–MS analysis

**Author contributions** SJ and SW conceptualized and designed the study. MP contributed to the study design, carried out all the experimental work, interpreted the data and wrote the manuscript. SDMC supervised the GC–MS analysis. DW, GS contributed to data interpretation. All authors contributed to manuscript editing and approved the final version.

**Funding** This work was funded by the research grant AP/3/2/2014/RG/12, University of Colombo.

**Availability of data and material** The chromatograms supporting the conclusions of this article are included as ESM1–4. Other datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no conflict of interest.

## References

1. Ezeji UE, Anyadoh SO, Ibekwe VI (2007) Clean up of crude oil-contaminated soil. Terr Aquat Environ Toxicol 1:54–59
2. Tissot BP, Welte DH (1978) The composition and classification of crude oils and the influence of geological factors. Petroleum formation and occurrence: a new approach to oil and gas exploration, 2nd edn. Springer, Berlin, pp 333–377
3. Macías-Zamora JV (2011) Ocean pollution. In: Letcher TM, Vallero DA (eds) Waste, 1st edn. Academic Press, London, pp 265–279
4. Hegazi AH, El-Gayar MS (2017) Role of non-hydrocarbon constituents in crude oils correlation and heavy fractions processing studies. Pet Chem 57:838–842. https://doi.org/10.1134/S0965544117100103
5. Freedman B (2018) Oil spills. In: Environmental science: a Canadian perspective. Dalhousie University Libraries Digital Editions
6. Brusseau ML (2019) Soil and groundwater remediation. In: Brusseau ML, Pepper IL, Charles PG (eds) Environmental and pollution science, 3rd edn. Elsevier, New York, pp 329–354
7. Watson JG (1996) Physical/chemical treatment of organically contaminated soils and sediments. J Air Waste Manag Assoc 46:993–1003. https://doi.org/10.1080/10473289.1996.10467536
8. Jain PK, Gupta VK, Gaur RK, Lowry M, Jaroli DPCU (2011) Bioremediation of petroleum oil contaminated soil and water. Res J Environ Toxicol 5:1–26. https://doi.org/10.3923/rjet.2011.1.26
9. Fondi M, Maida I, Perrin E et al (2016) Genomic and phenotypic characterization of the species *Acinetobacter venetianus*. Sci Rep 6:21985–21997. https://doi.org/10.1038/srep21985
10. Lee DW, Lee H, Kwon BO et al (2018) Biosurfactant-assisted bioremediation of crude oil by indigenous bacteria isolated from Taean beach sediment. Environ Pollut 241:254–264. https://doi.org/10.1016/j.envpol.2018.05.070
11. Elumalai P, Parthipan P, Karthikeyan OP, Rajasekar A (2017) Enzyme-mediated biodegradation of long-chain n -alkanes (C32

and C40) by thermophilic bacteria. 3 Biotech 7:116–126. https://doi.org/https://doi.org/10.1007/s13205-017-0773-y
12. Chen Q, Li J, Liu M et al (2017) Study on the biodegradation of crude oil by free and immobilized bacterial consortium in marine environment. PLoS ONE 12:e0174445. https://doi.org/10.1371/journal.pone.0174445
13. Wang D, Lin J, Lin J et al (2019) Biodegradation of petroleum hydrocarbons by *Bacillus subtilis* BL-27, a strain with weak hydrophobicity. Molecules 24:3021–3036. https://doi.org/10.3390/molecules24173021
14. Sun S, Li S, Avera BN et al (2017) Soil bacterial and fungal communities show distinct recovery patterns during forest ecosystem restoration. Front Microbiol 83:966. https://doi.org/10.1128/AEM.00966-17
15. Wemedo SA, Nrior RR, Ike AA (2018) Biodegradation potential of *Aspergillus Niger* and *Rhizopus arrhizus* isolated from crude oil spilled site in Rivers State. J Environ Sci Toxicol Food Technol 12:49–57. https://doi.org/10.9790/2402-1212014957
16. AI-Jawhari IFH (2014) Ability of Some Soil Fungi in Biodegradation of Petroleum Hydrocarbon. J Appl Environ Microbiol 2:46–52. https://doi.org/10.12691/jaem-2-2-3
17. Al-Hawash AB, Zhang X, Ma F (2019) Removal and biodegradation of different petroleum hydrocarbons using the filamentous fungus Aspergillus sp RFC-1. Microbiologyopen 8:e619. https://doi.org/10.1002/mbo3.619
18. Kato T, Haruki M, Imanaka T et al (2001) Isolation and characterization of long-chain-alkane degrading Bacillus thermoleovorans from deep subterranean petroleum reservoirs. J Biosci Bioeng 91:64–70. https://doi.org/10.1016/S1389-1723(01)80113-4
19. Maamar A, Lucchesi ME, Debaets S et al (2020) Highlighting the crude oil bioremediation potential of marine fungi isolated from the Port of Oran (Algeria). Diversity 12:196–215. https://doi.org/10.3390/D12050196
20. Singh R, Paul D, Jain RK (2006) Biofilms: implications in bioremediation. Trends Microbiol 14:389–397. https://doi.org/10.1016/j.tim.2006.07.001
21. Davey ME, O'toole GA, (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867. https://doi.org/10.1128/MMBR.64.4.847-867.2000
22. Edwards SJ, Kjellerup BV (2013) Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. Appl Microbiol Biotechnol 97:9909–9921. https://doi.org/10.1007/s00253-013-5216-z
23. Seneviratne G, Zavahir JS, Bandara WMMS, Weerasekara MLMAW (2008) Fungal-bacterial biofilms: their development for novel biotechnological applications. World J Microbiol Biotechnol 24:739–743. https://doi.org/10.1007/s11274-007-9539-8
24. Benoit I, van den Esker MH, Patyshakuliyeva A et al (2015) *Bacillus subtilis* attachment to *Aspergillus niger* hyphae results in mutually altered metabolism. Environ Microbiol 17:2099–2113. https://doi.org/10.1111/1462-2920.12564
25. Perera M, Wijayarathna D, Wijesundera S et al (2019) Biofilm mediated synergistic degradation of hexadecane by a naturally formed community comprising *Aspergillus flavus* complex and *Bacillus cereus* group. BMC Microbiol 19:1–9. https://doi.org/10.1186/s12866-019-1460-4
26. Bushnell LD, Haas HF (1940) The utilization of certain hydrocarbons by microorganisms. J Bacteriol 41:653–673
27. Dasgupta D, Ghosh R, Sengupta TK (2013) Biofilm-mediated enhanced crude oil degradation by newly isolated *Pseudomonas* species. Int Sch Res Not 2013:e250749. https://doi.org/10.5402/2013/250749
28. Barnes NM, Khodse VB, Lotlikar NP et al (2018) Bioremediation potential of hydrocarbon-utilizing fungi from select marine niches of India. 3 Biotech 8:1–10. https://doi.org/10.1007/s13205-017-1043-8
29. Fingas MF (1997) Studies on the evaporation of crude oil and petroleum products. II Boundary layer regulation. J Hazard Mater 57:41–58. https://doi.org/10.1016/S0304-3894(97)00051-4
30. WHO (1989) IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans; occupational exposures in petroleum refining; crude oil and major petroleum fuels. IARC Press, Lyon
31. Jeffrey AWA, McLoughlin PW, Pirkle RJ (2016) Application of isotopic compositions in fugitive petroleum product identification and correlation. Stand Handb Oil Spill Environ Forensics Fingerprinting Source Identif Second Ed. https://doi.org/10.1016/B978-0-12-809659-8.00010-3
32. Atlas RM (1975) Effects of temperature and crude oil composition petroleum biodegradation. Appl Microbiol 30:396–403
33. Brewster CS, Sharma VK, Cizmas L, McDonald TJ (2018) Occurrence, distribution and composition of aliphatic and polycyclic aromatic hydrocarbons in sediment cores from the Lower Fox River, Wisconsin, US. Environ Sci Pollut Res 25:4974–4988. https://doi.org/10.1007/s11356-017-0819-z
34. Neff JM, Durell GS (2012) Bioaccumulation of petroleum hydrocarbons in arctic amphipods in the oil development area of the Alaskan beaufort sea. Integr Environ Assess Manag 8:301–319. https://doi.org/10.1002/ieam.1247
35. Ekblad A, Wallander H, Näsholm T (1998) Chitin and ergosterol combined to measure total and living fungal biomass in ectomycorrhizas. New Phytol 138:143–149. https://doi.org/10.1046/j.1469-8137.1998.00891.x

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