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Combating Plastic Pollution in Terrestrial Environment

Challenges and Strategies for a Sustainable Future

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Chapter 14
Heterogeneous Microbial Biofilms: A Promising Solution for Combating Terrestrial Microplastic Pollution

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Abstract Environmental pollution by primary and secondary microplastics is currently recognized as a major threat to planetary health. Transferring among organisms through food webs, they accumulate in humans and other organisms resulting in detrimental effects across the biosphere. Bacteria and fungi are known to degrade petroleum hydrocarbons. Heterogeneous communities of microbial biofilms benefit from an extended genetic repertoire and consequent metabolic and survival capacity. Our previous work demonstrated high efficiency degradation of hexadecane and crude oil by naturally occurring fungal-bacterial biofilms. These studies provide a platform for investigating the use of biofilms for combating microplastic pollution in terrestrial environments. Microorganisms were isolated from a municipal landfill in Sri Lanka. Fungal–bacterial communities that appeared during screening formed a biofilm during static culture in hexadecane and crude oil at 1% as sole carbon source. Biofilm formation was confirmed by scanning electron microscopy. Degradation of alkanes in cultures was quantitatively estimated using GC-MS. In silico investigation of putative *Aspergillus flavus* alkane monooxygenases was carried out using

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the NCBI protein database and computational tools such as PSI-BLAST, SWISS MODEL and Autodock Vina. A biofilm-forming consortium comprising *Bacillus cereus* and *A. flavus* demonstrated highly efficient synergistic degradation of the alkanes in hexadecane and crude oil in liquid culture (~99% in 7 days). Several alkane monooxygenases were identified in *A. flavus* as distal homologs of bacterial long chain alkane monooxygenases LadA and AlmA and the short to medium alkane monooxygenase Cyp52. Our results suggest that heterogeneous microbial biofilms are promising candidates for the application of 'green solutions' to combating terrestrial microplastic pollution. Future directions for consideration are improvement of the bioremediation potential of (polluted) soils through inclusion of plastic degraders in the soil microbiota. Development of stable bioformulations of biofilm forming microbes is the next step. Exploitation of the identified extracellular fungal alkane monooxygenases as biocatalysts in bioremediation formulations needs to be additionally considered.

Keywords Biofilm · Heterogeneous · Microplastic · Bioremediation · Alkane degradation

14.1 Introduction

Plastics are a global pollutant that have now become a threat to planetary health. Their low production cost, durability and convenience have led to their extensive use and disposal in the domestic sector. In particular, single use plastics such as grocery bags, food containers, cutlery, water bottles and straws constitute a significant proportion of urban waste. Even though some plastics may be recyclable, most of the plastic waste (~95%) is not recycled (Zhou et al., 2022a) due to a variety of reasons including inadequate infrastructure for waste management, as well as cost of collection, sorting and separation and cleaning (Vogt et al., 2021). As a result this waste becomes accumulated in garbage dumps or enters waterways and oceans.

Plastics are manufactured from petrochemicals originating from crude oil. Their chemically inert hydrocarbon structure makes them recalcitrant compounds, taking 100–1000s of years to degrade through natural weathering processes (Zhou et al., 2022a). Through these physical, chemical and biological processes, plastics disintegrate into smaller fragments. Although macroplastic contamination is easily visible, a large proportion of contamination comprises microplastics which are defined as fragments of plastic <5 mm in diameter. Plastic fragments <1 μm in diameter are further defined as nanoplastics. As such, beyond the easily visible problem of plastic pollution, lies an even larger, 'hidden' threat to living organisms (Wang et al., 2022), in the barely visible-invisible category. Microplastic contamination is thus becoming an emerging global concern.

Microplastics found in the environment may be of primary or secondary origin. Primary microplastics are those which are synthesized directly for use and subsequently discarded (eg: beads found in certain personal care products). Microplastic

beads also known as nurdles, which form the seed plastic for manufacturing industries may also pollute the environment through accidental spills. Secondary microplastics are those which are derived through degradation of larger plastics through combined mechanical breakage and natural weathering processes (Akdogan & Guven, 2019). Microplastic fibres from synthetic textiles are released into the environment during washing as well as recurrent abrasion during wear (Napper & Thompson, 2016). While more dense plastics entering the terrestrial environment may become buried and thereby remain in the soil, lighter plastics and microplastics may be carried across land by the wind and eventually end up in water bodies.

Microplastics in the environment may be inhaled or ingested directly by humans and animals (Lwanga et al., 2016; Wu et al., 2022). Living organisms have been reported to accumulate microplastics in various cells and tissues leading to a potential health hazard. These microplastics may be transferred among organisms through food webs and become accumulated in humans, resulting in detrimental effects on health. Inadvertent ingestion may also occur through the use of plastic food and drink packaging.

14.2 Use of Microorganisms for Bioremediation

Microorganisms, both bacteria and fungi are known to colonize and degrade petroleum hydrocarbons. They have been investigated extensively as a potential bioremediation strategy for polluted environments. An advantage of microbial bioremediation is its potential as an 'in situ' strategy with either or combined use of bioaugmentation and biostimulation.

Microbial biodegradation of microplastics in soil has been investigated through microcosm studies, particularly in the past few years (Sarker et al., 2021; Sun et al., 2022). However, it is noted that individual organisms are generally limited in their ability to utilize the entire spectrum of alkanes and additional components present in petroleum products (Jayasena & Perera, 2021). Mixed communities of microbes, on the other hand, benefit from an extended genetic repertoire and consequent metabolic capacity. Therefore, the development of heterogeneous microbial communities for degradation of hydrocarbons has been a popular area of investigation (Malik and Ahmed, 2012; Hamzah et al., 2013; Poddar et al., 2019).

However, development of microbial consortia for bioremediation can be challenging due to possible antagonistic behavior among the participating organisms which might in turn affect the rate of biodegradation. This is clearly demonstrated in the experiments of Patowary et al. (2016) who described the development and investigation of 14 different consortia from five selected bacterial isolates obtained from three different hydrocarbon-contaminated sites. The organisms included both biosurfactant producers as well as non-producers. While all five isolates used in the study were selected based on efficient growth on hydrocarbons, biodegradation of total petroleum hydrocarbon varied among the consortia, while just one combination demonstrated relatively higher rates of degradation (~68%) after three

weeks, compared to the others which varied between ~40 and 51%. These experimental results reflect the impact of the interaction between the microorganisms in the community. More recently however, it has been shown that hydrocarbon-degrading bacteria may benefit from the use of non-degrading fungi in a mixed community (Chen et al., 2024). In this study, the fungus was shown to improve accessibility to the alkanes by adsorbing it onto the mycelium.

Heterogeneous communities of microbes that form naturally in the environment, particularly those that form biofilms may provide a promising strategy for terrestrial bioremediation. The formation of multispecies biofilms is a widespread phenomenon in natural environments where organisms co-exist and partake in mutually beneficial interactions. Such indigenous communities are likely to have developed optimal adaptation behaviors for survival, especially under harsh conditions. Microbial populations form biofilms on both biotic and abiotic solid surfaces as well as at oil–water interfaces.

Biofilms are three dimensional, dynamic microbial communities comprising single or multiple species attached to a biotic or abiotic surface and embedded in a matrix of extracellular polymeric substances (EPS). The EPS often comprises polysaccharides, proteins, glycoproteins, glycolipids and sometimes extracellular DNA as well. Biofilm formation has been shown to involve the expression of distinct genes indicating an adaptation to a particular mode of life (Mounier et al., 2014). The biofilm structure enables the intercellular exchange of metabolites, signal molecules, as well as genetic material among the organisms in the biofilm. The organisms embedded in the polymeric matrix are further protected from predators and antimicrobial agents, making them upto thousand times more resistant to antibiotics than planktonic bacteria.

Due to the ability of microorganisms to form biofilms on particulate substrates, biofilm formation has been considered as a strategy for combatting pollution (Sivadon et al., 2019). Thus, exploiting such naturally occurring consortia is likely to provide more promising results over synthetically developed consortia.

Microplastics found in the environment comprise polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), or polyvinyl chloride (PVC), or mixtures of these along with various additives such as stabilizers, colorants, plasticizer and flame retardants that are often included into products. Their chemical properties, particularly their chemical inertness have impacted the environment, affecting the health of living organisms across the biosphere. The slow rate of degradation is the main challenge that currently limits microbial bioremediation technology.

The chemical structure of polyethylene is among the simplest among the synthetic plastics (Fig. 14.1). They are non-polar, high molecular weight saturated hydrocarbons composed of polymers of two carbon units (ethylene) having the chemical structure $(C_2H_4)n$. Their hydrophobicity and lack of functional groups in its chemical structure make them highly inert and therefore recalcitrant in the environment.

Degradation of alkanes requires the initial activation of a terminal or subterminal carbon through oxidation. It is known that both biotic as well as abiotic factors such as enzymes and UV irradiation have the ability to initiate the degradation of alkanes

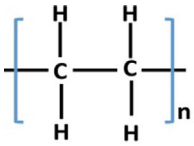

Fig. 14.1 Basic chemical structure of polyethylene

such as those found in polyethylene. However, the polyethylene fragments need to be reduced to ~10–50 carbons for enzyme action (Restrepo-Flórez et al., 2014). Thus, the photo-oxidative fragmentation of plastics is an important initial step in the degradation process. The UV-mediated photo-oxidation also leads to the increase of carbonyl residues which have been shown to be utilized by *Rhodococcus ruber* during degradation of polyethylene (Orr et al., 2004). The biodeterioration and biodegradation of polyethylene and methods of investigation have been comprehensively reviewed by Restrepo-Flórez et al. (2014).

14.3 Use of Heterogeneous Microbial Biofilms for Degradation of Liquid Alkanes

The need for high efficiency biodegradation of plastics continues to drive the exploration of novel microbial species and strains as well as the investigation of genetically modified microorganisms and mixed microbial communities with a high capacity for degradation of hydrocarbons. The use of genetically modified organisms is aimed at overcoming the challenges of acclimatization of microorganisms in new environments, which may sometimes occur (Rebello et al., 2021).

Our laboratory investigations using liquid petroleum products, namely hexadecane and crude oil, demonstrated the ability of three naturally occurring fungal–bacterial communities that formed biofilms (*Aspergillus—Bacillus* Consortia, designated ABC1, 2 and 3) to metabolize alkanes with remarkable efficiency, when provided as the sole source of carbon in minimal media (Perera et al., 2019, 2021).

Microorganisms capable of degrading hydrocarbons were isolated from samples of waste polyethylene with attached soil that were collected from a municipal landfill in Colombo district in Sri Lanka. Organisms capable of utilizing hydrocarbons as a sole carbon source were purified by enrichment culture (1% hexadecane in Bushnell and Haas minimal medium) at 40 °C followed by plating of serially diluted cultures to obtain isolated colonies.

This initial screening process led to the isolation of three naturally occurring fungal–bacterial communities that appeared during culture and they were further investigated for hydrocarbon degradation potential. All three communities were observed to form a biofilm at the oil–water interface, when grown as static cultures. The individual organisms in the communities were identified through phenotype, biochemical characteristics as well as molecular level analysis (16S ribosomal gene in bacteria or internal transcribed spacer (TS) region in fungi). The bacteria in the three

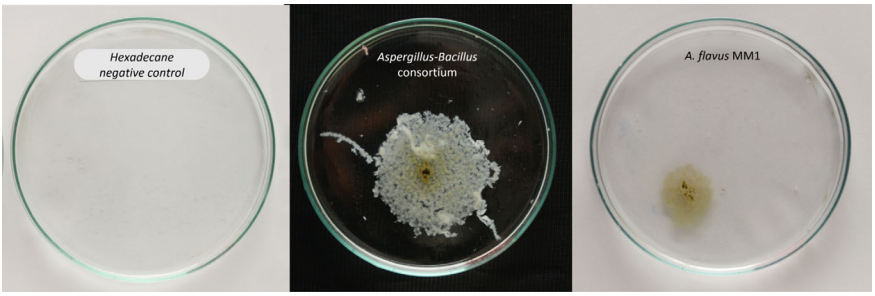

Fig. 14.2 Growth of ABC1 and *A. flavus* MM1 monoculture in 1% *n*-hexadecane in minimal medium (liquid medium) after 14 days (*Note* cultures were grown in conical flasks and poured into petri dishes for better visualization after the incubation period)

communities were *Bacillus* spp and *Lysinibacillus* spp while the fungi comprised *Aspergillus* spp. The two organisms in ABC1 (*Aspergillus-bacillus* consortium 1) were further characterized as *Bacillus cereus* and *Aspergillus flavus* MM1. ABC1 consortium was selected for further investigations based on its efficient degradation of crude oil and the simplicity of the consortium (Fig. 14.2).

The fungus in ABC1 consortium, *A. flavus* MM1 was observed to form the mycelial mat at the oil–water interface. The aqueous phase which became turbid upon initial growth of the bacilli was then observed to clear, concomitant with the development of the fungal mat. Alongside this, a reduction of the layer of hexadecane or crude oil was observed, indicating its likely utilization by the biofilm. Filamentous fungi, such as the isolated *A. flavus* are known to produce hydrophobic proteins facilitating the attachment to hydrophobic surfaces (Kershaw & Tablbot, 1998). This, together with the fact that the bacillus counterpart in the biofilm was an active, motile bacteria, provides explanation to the observations. The bacteria would be capable of overcoming the nutrient limitation in the aqueous minimal medium, by traveling toward the fungus and attaching onto the mycelia, forming a biofilm. Thus, obtaining access to the carbon source.

Biofilm formation in the ABC1 community was confirmed through scanning electron microscopy (Fig. 14.3) in which bacteria were observed on the fungal hyphae embedded in the EPS (Perera et al., 2019). In fungal-bacterial biofilms, the fungal hyphae serve as a physical and metabolic habitat for the interacting bacteria, allowing the bacterial cells to colonize the surface of the hyphae, obtain nutrients that may be directly or indirectly secreted by them, and 'travel' along with the growing hyphae (Guennoc et al., 2018).

Utilization of hexadecane and the alkanes in crude oil by the biofilm as well as the individual organisms when grown in monoculture was quantitatively estimated in ABC1 using gas chromatography-mass spectrometry (GC-MS). Residual alkanes in 7- or 14-day cultures grown at 30 °C without shaking, were extracted into hexane or dichloromethane, quantified by GC-MS and percentage utilization was calculated. Uninoculated culture media was used as control to estimate abiotic loss of alkanes (e.g. through vaporization).

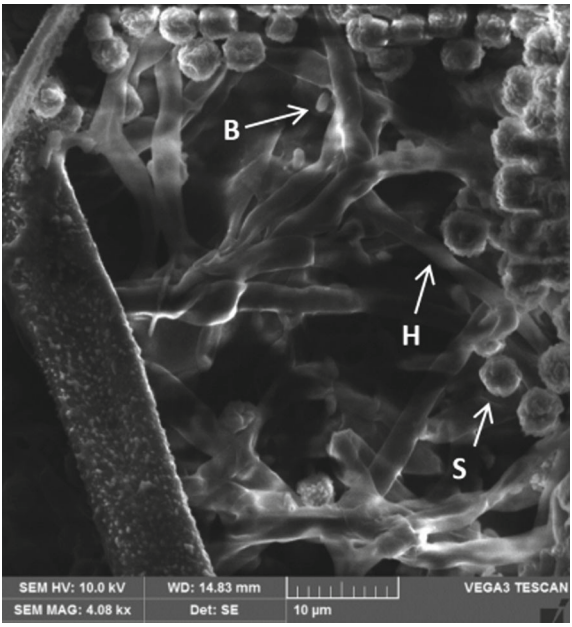

Fig. 14.3 SEM micrograph of *Aspergillus—Bacillus* biofilm. Magnification 4080x. B; *Bacteria,* H; fungal hyphae, S; fungal spores

The ABC1 *Aspergillus-bacillus* biofilm demonstrated highly efficient, synergistic degradation of hexadecane as well as a spectrum of alkanes in crude oil, when provided as the sole source of carbon. Biodegradation of hexadecane by the ABC1 biofilm and the individual organisms in monoculture were estimated in 14-day static cultures by GC-MS analysis. Briefly, residual hexadecane in the culture medium at day 14 was estimated by analysis of the hexane extract and the percentage utilization was calculated.

The ABC1 biofilm demonstrated ~99% utilization of hexadecane (1% in 20 ml at 40 °C) in 14 days. When grown in monoculture however, the fungus utilized ~52% and the bacterium utilized only ~9% hexadecane in 14 days (Perera et al., 2019). Therefore, it is clear that in the biofilm mode, the organisms demonstrate synergistic behavior, where together, the degradation was greater than the sum of their individual degradation.

The ABC1 biofilm, when cultured in 1% crude petroleum oil (8.25 g/L)(CEYPETCO oil refinery, Sri Lanka) in 20 ml minimal medium degraded ~99% of the crude oil within 7 days. The crude oil sample obtained was found to have a range of alkanes from ~C9 to C30 or more (Perera et al., 2021). The fungus demonstrated efficient degradation of the alkanes in crude oil when grown in monoculture as well, reaching degradation levels similar to that observed when in biofilm mode, when estimated at day 7, while the degradation of crude oil by the bacterium was very poor. This begged to question the role of the bacterial counterpart in the degradation of crude oil. In order to obtain answers to this question, the degradation of crude oil was estimated at two additional time points prior to day 7.

Table 14.1 Degradation of straight chain alkanes (C12–C30) in crude oil by ABC community organisms

Organisms	Degradation of alkanes in crude oil		
	Day 3	Day 5	Day 7
ABC1 biofilm	24 ± 1.4%	66 ± 7%	99 ± 0.2%
A. flavus MM1	4.1 ± 0.4%	14 ± 6%	98 ± 2%
B. cereus MM1	–	–	20 ± 4%

These experiments demonstrated that utilization of crude oil by the biofilm was ~ 66% in 5 days compared to 14% by the fungus in monoculture. This demonstrated that although *A. flavus* could degrade the crude oil in monoculture to ~98% by day 7, there was a delay in commencement of the degradation, which was overcome by the presence of the bacillus in biofilm mode (Perera et al., 2021). This observation has implications, particularly if bioremediation is used to mitigate a sudden high exposure (e.g.: due to an accidental oil spill or seepage), where early removal of the contaminant is vital to minimize ecological damage (Table 14.1).

Percentage degradation was calculated by GC-MS analysis of the residual alkanes at the indicated time of incubation (Results of triplicate experiments).

The observation of this rapid degradation of alkanes in the isolated fungus suggested that it processed a repertoire of alkane degrading enzymes, in particular enzymes capable of oxidizing large alkanes (~C30) which are rare in nature.

14.4 Identification of Alkane Oxidizing Enzymes in a Filamentous Fungus

Several aerobic enzyme systems that carry out alkane oxidation have been identified in microorganisms. The methane monooxygenases (Wang et al., 2017) and the related butane monooxygenases oxidize short chain alkanes. The AlkB family of alkane hydroxylases which are found in bacteria, as well as the cytochrome P450 system which is reported in both bacteria and fungi, are known to oxidize medium chain alkanes (~C7–C17). Long chain alkane degrading enzymes, LadA and AlmA systems had been previously reported only in some extremophilic bacteria, such as the thermophile *Geobacillus* and the halophile *Amycolicicoccus* (Feng et al., 2007; Boonmak et al., 2014, Bowman & Deming, 2014). However, investigations in our laboratory and previous research have shown that filamentous fungi are able to degrade long-chain alkanes.

Our research therefore aimed at seeking out the fungal long chain alkane monooxygenases. An in silico investigation of putative fungal enzymes responsible for alkane oxidation was carried out using the *A. flavus* protein sequences in the NCBI non-redundant protein database using a variety of NCBI search tools such

as PSI-BLAST (homolog searches) (Altschul et al., 1997), SWISS MODEL (Waterhouse et al., 2018), I-TASSER (3D modeling) (Zhou et al., 2022b), ORCA (geometric optimization) (Neese, 2012) and Autodock Vina (enzyme-ligand docking).

The publicly available databases at the National Center for Biotechnology Information (NCBI) were screened. Probing of the NCBI protein data bank using Position-Specific Iterative Basic Alignment Search Tool (PSI-BLAST) to search distant homologs, with the query sequences led to the identification of homologs of the flavin-binding long chain alkane monooxygenases LadA and AlmA in *A. flavus* NRL3357.

The LadA homologs formed a family of five enzymes designated Af1-5. Their 3D structures were modeled and all except Af2 were found to accommodate the coenzyme flavin mononucleotide (FMN) in the active site. All four were shown to bind alkanes upto C30 in the active site (Perera et al., 2022) and further confirmation is achieved through the conservation of the active pocket residues with the *Geobacillus thermodenitrificans* LadA (3B9O_A) (Li et al., 2008). This data represents the first demonstration of the presence of distant homologs of the bacterial LadA monooxygenases in a eukaryotic organism.

A similar experimental procedure led to the discovery of AlmA homologs in *A. flavus*. Similar to the LadA family, the AlmA also comprised a multi-enzyme family and in silico analysis of one homolog showed putative binding of alkanes upto C40 (published in abstract form). Our investigations further led to the isolation of the medium chain alkane-oxidizing monooxygenase of the cytochrome P450 family, Cyp52 in *A. flavus* MM1 and alkane binding to the active site was demonstrated in silico (unpublished data).

Together these results clearly show that the fungus *A. flavus* MM1 in the isolated biofilm-forming heterogenous consortium, produces the enzymes required for initiating the oxidation of alkanes. Thus, they have the potential to be exploited for biodegradation of polyethylene.

14.5 Discussion and Future Directions

Our investigations in *Aspergillus flavus* demonstrate that these filamentous fungi have the potential to carry highly active long chain alkane degrading monooxygenases. Unlike bacteria where generally only one enzyme is encoded, the fungal genomes appear to encode multi-enzyme families. Together these enzymes provide efficient alkane-degrading capacity to the organism.

Microbial biofilms are highly stable, dynamic entities often with enhanced characteristics obtained through combined characteristics of the resident organisms as well as horizontal gene transfer. Our results further demonstrated that naturally occurring biofilm forming bacterial-fungal communities with a capacity for highly efficient degradation of liquid petroleum hydrocarbons are present in contaminated soils. It is highly likely that this capacity also extends to degradation of microplastics.

A previous study on biofilm formation by *Marinobacter* spp. demonstrated that it formed a biofilm on the oil–water surface only if it was able to metabolize the oil (Mounier et al., 2014; Klein et al., 2008). Microplastics provide a solid surface for biofilm formation. Indeed, *Rhodococcus ruber* C208 has been shown to form a biofilm on a polyethylene film while utilizing the polyolefin as a sole carbon source (Orr et al., 2004).

Our results (Perera et al., 2019) clearly demonstrated the importance of biofilm formation for rapid degradation of the hydrocarbons. The investigations of oil degradation by *Marinobactor* spp. also demonstrated that when biofilm formation was perturbed by agitation, hexadecane degradation was decreased (Klein et al., 2008; Mounier et al., 2014).

These investigations provide a platform for the investigation of this and similar naturally occurring fungal–bacterial biofilms in combating microplastic pollution in terrestrial environments. Microbial biofilms have the advantage of providing a 'green' solution for purifying contaminated terrestrial environments. It is envisaged that natural microbial biofilms may potentially offer a high efficiency biological system for microplastic degradation. Bioaugmentation of the soil microbiome with such organisms will improve the bioremediation potential of the soil and may provide a path to minimize the accumulation of microplastics in the soil. Exploitation of the identified extracellular fungal alkane monooxygenases for use in bioremediation formulations needs to be additionally considered. This includes the development and optimization of a stable bioformulation for improvement of the bioremediation potential of polluted soils.

Future directions for research and development:

- Investigation of the capacity for microplastic degradation by previously isolated consortia as well as exploration for novel biofilm forming heterogeneous microbial consortia.
- Preparation of a stable bioformulation comprising live organisms for bioaugmentation purposes.
- Investigation of extracellular monooxygenases for potential use as biocatalysts in bioformulations.

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