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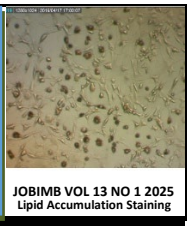

Hydrocarbon-Utilising Microorganisms for *In Vitro* Degradation of Perfluorohexane Sulphonate (PFHxS)

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ABSTRACT

Perfluoroalkyl substances are emerging environmental pollutants of increasing concern that are used extensively in industry. They are highly recalcitrant and pose environmental and public health hazards. This study is among the few assessing the degradation of perfluorohexane sulphonate (PFHxS) in laboratory-based studies using indigenous hydrocarbon-degrading fungi and bacteria from Nigerian soils. The isolates were enumerated and characterised via standard microbiological techniques. The bacteria were further identified using an Analytical Profile Index®20E kit. The utilisation capacity and tolerance indices of the isolates were determined via media dilution. A total of 4 fungal and 8 bacterial isolates were identified for study. *Pseudomonas putida* (27.5%) and *Klebsiella* (15.0%) dominated amongst the bacteria, while *Alternaria* (28.0%) and *Aspergillus niger* (36.0%) dominated amongst the fungi. The greatest mean abundance after exposure to PFHxS was seen with *Trichoderma* sp. (300–500 SFU/mL) and *Alternaria* sp. (900–1000 SFU/mL). Amongst the bacteria, the highest mean counts were found for *Bacillus cereus* (109–670 CFU/mL), *Pseudomonas putida* (267–720 CFU/mL), and *Serratia marcescens* (312–610 CFU/mL). There were statistically significant differences ($p \leq 0.05$) in the abundance of the bacteria and fungi both between groups and within groups. From the tolerance indices, the bacteria tolerated PFHxS better than the fungi. Only *Alternaria* was able to degrade PFHxS amongst the fungi, while all the bacterial isolates showed PFHxS degradation capacity except *Escherichia coli* and *Flavobacterium*. From the results, it can be concluded that organisms like *Alternaria*, *Bacillus cereus*, *Pseudomonas putida,* and *Serratia marcescens* would be useful bioresources in the environmental decontamination of PFHxS.

INTRODUCTION

Anthropogenic activities are responsible for the introduction of per- and polyfluoroalkyl substances (PFAS) into the environment. These groups, consisting of about 10,000 different compounds, occur in high levels throughout the environment. They are a diverse group of man-made compounds used extensively in industry. They have been associated with the production of pesticides, cosmetics, paper, fire- and waterproof products like non-stick cookware, waterproof fabrics, foams for fire extinguishers, food packaging, leather treatment, and carpets [1,2]. Structurally, these compounds contain at least a single perfluorinated methyl or methylene group. This structure endows them with chemical stability and oil and water resistance, which, in turn, accounts for their exceptional bioaccumulation, biomagnification, and environmental persistence [3,4]. Living organisms take up PFAS via exposure to air and dust in heavily polluted environments. Humans and higher animals may also be exposed to PFAS through direct consumption in food and drinking water [5]. These compounds impair immune, endocrine, cardiac, metabolic, and reproductive function in humans and higher organisms. They have also been linked to loss of cellular metabolic function and disrupted metabolic processes in lower organisms [6,7].

Their environmental half-lives are substantially greater than the average environmental contaminant. PFAS like perfluorooctanoic acid (PFOA) and perfluorooctane sulphonic acid (PFOS) have estimated half-lives of 1.48 – 5.10 years and 3.40 – 5.70 years, respectively, in human tissue, while

perfluorohexane sulphonate (PFHxS) (used in the current study) has a half-life of about 2.84 – 8.50 years [4]. PFOS has a half-life of 41 years or more in aquatic ecosystems and 114 days in the air. PFOA, similarly, records a half-life of over 92 years in water and 90 days in air [8]. Long-chain PFAS (containing 7 or more carbons) will typically have longer half-lives and bioaccumulation capacity than the short-chain PFAS (containing 6 or less carbons); however, the short-chain PFAS, like PFHxS, show greater environmental mobility and enhanced solubility, which increases their bioavailability.

The remarkable recalcitrance of the long-chain PFAS, their toxicity and slow elimination from living tissue resulted in a ban on production and commercial use in more developed nations. Short-chain PFAS and PFAS mixtures are still employed in industrial activities worldwide. These short-chain compounds, therefore, occur more regularly in the environment. This raises concerns for toxicity to organisms and diminished ecosystem function [9–11]. Perfluorohexane sulphonate (PFHxS) is a short-chain, 6-carbon, completely fluorinated alkane containing a sulphonic acid group attached. PFAS are employed as an industrial surfactant and for the manufacture of food packaging, extinguisher foams, and stain-resistant textiles [6].

The challenge of PFHxS biodegradation has been linked the typical inhibitory nature of the organohalides, particularly organofluoride, because the carbon-fluoride bond (C–F) is remarkably stable. In addition, organofluorides, like most other organohalide compounds, tend to exert an inhibitory effect on microbial enzymes [12–15]. The current approaches to treatment for PFAS are relatively expensive and somewhat disadvantageous to the environment. Techniques like adsorption using activated carbon have been known to eventually reintroduce the PFAS into the environment over time [16,17]. Other methods like incineration at extreme temperatures and chemical decomposition are not only ineffective and environmentally damaging, but also present a health risk to humans and animals [18,19].

Green solutions with minimal to no environmental impact are preferred. Biodegradation lies at the heart of this new approach to the environmental elimination of PFAS, one that offers low cost and ensures environmental sustainability while removing these high-priority pollutants. Microorganisms are known to adsorb the PFAS prior to degradation. This characteristic has been used in biological wastewater treatment and composting [14,20]. Hu and Scott [21] report that in spite of their environmental and public health significance, there is a paucity of studies on the specific microorganisms or enzymes involved in the breakdown and elimination of PFAS. This study was designed to address this gap by investigating microbial degradation of Perfluorohexane sulphonate (PFHxS) by isolates from hydrocarbon-contaminated Nigerian soil.

MATERIALS AND METHODS

Materials and Sample Collection

Soil samples were collected from mechanic workshops in Aluu, Choba and Rumuosi areas of Rivers State, Nigeria. Soil was collected up to a depth of 15 cm. The samples were transported to the Environmental Laboratory in sterile black polyethylene bags. The soil samples from each area were mixed together to form a composite, then screened using a 2 mm sieve to remove foreign objects, stones, large particles, and debris. The 99 % pure analytical grade perfluorohexane sulphonate (PFHxS) used in the study was purchased from Sigma Aldrich, USA.

Enumeration of Hydrocarbon-Utilising Microorganisms

This was done using the enrichment approach employed by [22]. About 1 g of soil was added to 9 mL of water and agitated thoroughly to ensure mixing. The mixture was then serially diluted. Suitable dilutions were inoculated onto suitable medium (as elaborated above) via the spread plate technique using 0.1 mL aliquots of the dilutions. The bacterial isolates were cultivated on Bushnell-Haas medium modified with 2% crude oil, while fungi were cultivated on potato dextrose agar (Merck, Germany) amended with 2% crude oil and 2.5 mg/L streptomycin to prevent bacterial growth. Incubation was at 37 °C ± 2 °C for 48 h and 25 °C ± 2 °C for 4 – 6 days for bacteria and fungi, respectively.

At the end of the incubation period, visible colonies on the plates were tallied and expressed as colony-forming units per gram of soil (CFU/g). The plates were enveloped in aluminium foil to prevent photodegradation. Plates with visible colonies over 300 colonies were not included in the results. Discrete colonies on the primary plates were transferred onto freshly prepared sterile medium to obtain pure isolates, which were then stored on slants to be used for further investigation.

Characterisation of Isolates

The fungal isolates were identified via their morphological and microscopic characteristics according to Larone [23] and Dugan [24]. Microscopic investigation was carried out by mounting a minute portion of hyphae on a clean slide using a sterile inoculating needle and then staining with lactophenol blue before observation under the microscope. The bacterial isolates were initially analysed for their microscopic and biochemical properties, including Gram staining, nitrate, indole, coagulase, starch hydrolysis, oxidase, catalase, citrate utilization, sugar fermentation, urease, triple sugar iron agar test, methyl red, and Voges-Proskauer tests [25]. Further bacterial characterisation was carried out with an Analytical Profile Index®, API–20E identification kit (BioMerieux Inc., USA). The kit was used according to the manufacturer's instructions.

PFHxS Utilisation Assay

The pure bacterial isolate on a slant was transferred into sterile nutrient broth in an Erlenmeyer flask using a sterile wire loop. This was incubated at 37 °C for 48 h with agitation, after which 1 mL was again transferred into fresh broth and incubated at 37 °C for 24 h. Spores from the pure fungal isolates were transferred into fresh sterile potato dextrose broth (PDB) using a sterile inoculating needle and incubated with agitation at 25 °C ± 2 °C for 4 days, after which 1 mL of the culture was transferred into fresh PDB. Sub-culturing for both fungi and bacteria was done for three cycles to ensure the use of actively growing cells in the study [26].

The replicated set-up for the utilisation assay consisted of 3 sets of flasks per PFHxS concentration being considered, and 4 sets altogether per organism tested. Each set of four flasks consisted of PFHxS-amended broth (potato dextrose broth for fungi and nutrient broth for bacteria) at application levels of 100 ppm, 1000 ppm, and 2000 ppm and a control experiment (0 ppm) consisting of medium with no PFHxS. This was the setup for each organism. Exactly 1 mL of a 24-hour-old broth culture of the test organism was introduced into the PFHxS-amended medium in the flasks and the control. Incubation conditions were as previously stated for bacteria and fungi. The changes in microbial abundance were monitored.

Tolerance Index for the Isolates

The 48h tolerance indices for the isolates were ascertained by comparing the abundance of the isolates in contaminant-tainted medium after 48h incubation against that in the control (uncontaminated) medium as shown in Equation (1) adapted from [26].

$$TI_{48} = \frac{\text{Bacterial abunadance in contaminated medium}}{\text{Bacterial abundance in Control study}} \quad \text{(Equation 1)}$$

Where: TI_{48} = 48-hour tolerance index

Statistical Analysis

The data obtained was analysed using SPSS® 24.0 (IBM, USA) and Microsoft Excel 2016. Analysis of variance (ANOVA) assessed for statistically significant differences between and within groups. Basic measures of central tendency and dispersion were also determined.

RESULTS AND DISCUSSION

Out of all the isolates obtained, a total of eight bacteria and four fungi were identified. *Pseudomonas putida* (27.5%) and *Klebsiella* spp. (15.0%) dominated amongst the bacteria, while *Alternaria* (28.0%) and *Aspergillus niger* (36.0%) dominated amongst the fungal isolates, as shown in **Fig. 1**.

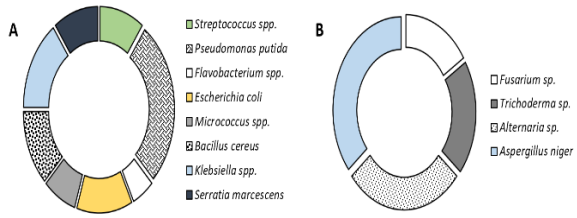

Fig. 1. Distribution of hydrocarbon-degrading (A) bacterial and (B) fungal soil isolates.

The macroscopic and microscopic characteristics of the fungal isolates are outlined in **Table 2,** while Figures 2 and 3 depict the abundance of bacteria and fungi, respectively at the end of the 7-day study. The increased abundance obtained in the presence of PFHxS compared to the control study indicates that the microorganism is utilising PFHxS as a source of carbon and energy, thereby degrading it [27,28]. The bacterial isolates generally fared better than the fungal isolates with regard to utilisation of PFHxS.

Table 1. Morphology and microscopic characteristics of fungal isolates.

Macroscopy	Microscopy	Probable genera
Fast growing white colony with cotton surface, white on reverse	Thick-walled septate hyphae with macroconidia	*Fusarium* sp.
Light green cottony mycelia with visble concentric rings and white margin, non-cracked green reverse,	Septate and hyaline hyphae with globose conidia growing close together and branched conidiophores	*Trichoderma* sp.
Fast growing, Initial grey colour on surface turned blackish grey with a white margin	Transverse and longitudinal septate hyphae bearing brownish conidiophores	*Alternaria* sp.
Light yellow margin, dark brown woolly mycelia, dark spores, yellow on reverse,	Visible septate and hyaline hyphae with slender conidiophores and brown rounded conidial heads	*Aspergillus niger*

For both the bacteria and fungi, the greatest abundance was seen at 100 ppm (compared to 1000 ppm and 2000 ppm). Statistically significant differences ($p \leq 0.05$) were obtained in bacterial abundance from one organism to the other and from one PFHxS concentration to the other.

Amongst the bacteria, *Escherichia coli* and *Flavobacterium* seemed unable to effectively degrade PFHxS, as the growth obtained in the presence of the contaminant was significantly lower than that obtained in the control study. Both isolates showed only minimal growth at 1000 ppm and did not grow at all at 2000 ppm PFHxS. Although *Streptococcus* grew at all the contaminant levels, it showed only negligible growth in the presence of PFHxS. The growth levels obtained for *Streptococcus* at 100 ppm were not significantly different from the control, while its abundance at 1000 ppm and 2000 ppm was significantly lower than the control experiment. The greatest abundance for the PFHxS-degrading bacterial isolates was seen at 100 ppm for all except *Pseudomonas putida* [C], which thrived best at 1000 ppm PFHxS. The most effective PFHxS degrading bacteria at 100 ppm compared to the control were *Bacillus cereus*, *P. putida,* and *Serratia marcescens* (**Fig. 2**).

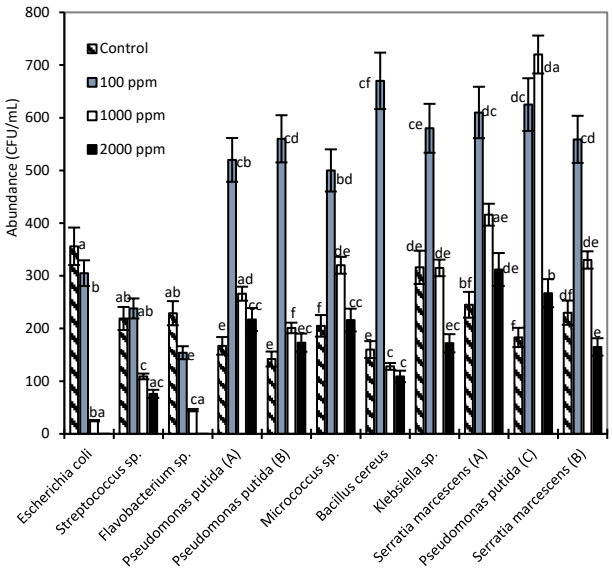

Fig. 2. Bacterial abundance following exposure to perfluorohexane sulphonate (PFHxS) over a 7-day period. Bars represent standard deviation from mean; Values represent mean ± SD of three biological replicates; different letters indicate statistically significant differences at $p \leq 0.05$; Neither *E. coli* nor *Flavobacterium* sp. showed growth at 2000 ppm PFHxS

The greatest abundance in the presence of PFHxS was seen with the fungal isolate, *Alternaria*. In the control studies, the highest counts was obtained with *Trichoderma* spp. Based on comparisons with the Control studies, only *Alternaria* seemed to be effectively utilising PFHxS as a carbon and energy source; having mean counts of up to 900 SFU/mL and 1000 SFU/mL at 100 ppm and 1000 ppm respectively. *Aspergillus niger* showed negligible utilisation capacity. With the remaining two fungal isolates, abundance in the presence of PFHxS was much lower than in the control (set-up devoid of PFHxS contamination). None of the fungal isolates grew at 2000 ppm. There were statistically significant differences in the abundance of the different fungal species from one concentration to the other and from one fungal genera to the other at 95 % confidence interval ($p \leq 0.05$) as depicted in **Fig. 3**.

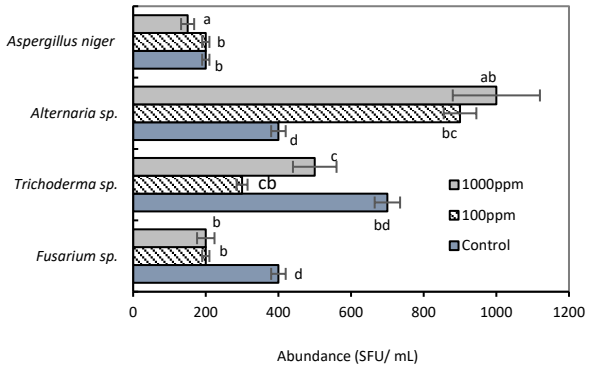

Fig. 3. Fungal abundance after 7 days' exposure to different concentrations perfluorohexane sulphonate (PFHxS). Bars represent the standard deviation from the mean; Values represent mean ± SD of three biological replicates; different letters indicate statistically significant differences at $p \leq 0.05$; none of the fungal isolates showed growth at 2000 ppm PFHxS.

Based on the 48 h tolerance indices outlined in **Table 2**, the bacterial isolates tolerated PFHxS better than the fungal isolates. All the bacterial isolates displayed very high tolerance of PFHxS ($TI_{48} > 1.0$) at 100 ppm except *E. coli* and *Flavobacterium,* which had high (0.80 – 1.00) and moderate (0.60 – 0.79) tolerance indices, respectively. This buttresses the inability of these two bacteria to effectively degrade PFHxS even at 100 ppm concentration. *E. coli* and *Flavobacterium* showed zero tolerance at 2000 ppm and very low tolerance ($TI_{48} < 0.39$) at 1000 ppm. The *P. putida*, *S. marcescens,* and *Micrococcus* isolates demonstrated very high ($TI_{48} > 1.0$) tolerance at all concentrations of PFHxS. None of the fungal isolates tolerated PFHxS at 2000 ppm ($TI_{48} = 0$). *Alternaria* sp. demonstrated very high tolerance at both 100 ppm and 1000 ppm, while *A. niger* showed very high tolerance at 100 ppm but low tolerance (0.40 – 0.59) at 1000 ppm.

Only a limited number of bacteria and fungi can utilise perfluoroalkyl substances as a nutrient source. The current study revealed that the hydrocarbon-utilising bacteria were better degraders of PFHxS than the hydrocarbon-utilising fungi. This supports the reports of a study on biodegradation of per- and polyfluoroalkyl substances (PFAS) that bacteria typically perform better than fungi during PFAS biodegradation due to the slower metabolic rates and limited enzymatic specificity of fungi [29]. Studies have shown that the bacterial community in soil has better adaptability to environmental stressors than soil fungi. This resilience by the bacteria has been attributed to relatively high growth rates and their unicellular structure. Environmental factors like pH more readily impact fungi, and PFAS are known to strongly increase pH levels, which could impact fungal respiration [30,31].

Furthermore, several species of bacteria, including Bacillus, Klebsiella, and Pseudomonas, produce biosurfactants that serve to enhance their biodegradation capacity [32]. The poor PFHxS utilisation in spite of moderate to very high tolerance exhibited by some of the isolates, like *A. niger* and *Streptococcus*, may indicate that these organisms lack the pre-requisite enzymes to metabolise PFHxS or may require a longer acclimatisation period to adapt to the contaminant. Microorganisms are known to metabolise PFAS like PFHxS by defluorination. This involves cleavage of the carbon-fluoride bond. Microorganisms with the capacity to degrade PFAS have been identified as those that synthesise enzymes with inherent resistance to fluoride inhibition. The microbial enzyme groups implicated include the haloacid dehalogenases that specialize in the hydrolytic cleavage of carbon-halide bonds [21,33]. The fluoroacetate dehalogenases associated with species of *Pseudomonas*, *Moraxella* and *Rhodopseudomonas* are part of this group. They catalyse the cleavage of the C–F bond [21,34,35]. Dehalogenation by *Bacillus* species and *P. putida* has been linked to the production of the cytochrome P450 family of enzymes [12,36]. Both *Bacillus* and *P. putida* stood out as effective utilizers of PFHxS in the current study.

Table 2. Tolerance indices after 48 hours.

	Isolate	Perfluorooctane sulphonamide concentration		
		100 ppm	1000 ppm	2000 ppm
Bacterial Isolates	*Escherichia coli*	0.86	0.07	0.00
	Streptococcus sp.	1.09	0.49	0.34
	Flavobacterium sp.	0.67	0.19	0.00
	Pseudomonas putida (A)	2.00	1.59	1.29
	Pseudomonas putida (B)	2.00	1.42	1.22
	Micrococcus sp.	2.00	1.56	1.05
	Bacillus cereus	2.00	0.80	0.68
	Klebsiella sp.	2.00	0.99	0.54
	Serratia marcescens (A)	2.00	1.69	1.27
	Pseudomonas putida (C)	2.00	2.00	1.46
	Serratia marcescens (B)	2.00	1.43	0.72
Fungal Isolates	*Fusarium sp.*	0.50	0.50	0.00
	Trichoderma sp.	0.50	0.71	0.00
	Alternaria sp.	2.25	2.50	0.00
	Aspergillus niger	1.00	0.50	0.00

KEY: 0.00 (no tolerance) >0.00 – 0. 39 (very low tolerance); 0.40 – 0.59 (low tolerance); 0.60 – 0.79 (moderate tolerance); 0.80 – 1.00 (high tolerance); >1.00 (very high tolerance)

The reduced microbial abundance at higher PFHxS concentrations of 1000 ppm and 2000 ppm as obtained in the current study, confirms the effect of substrate concentration on utilisation and biodegradation rates. The rate of biodegradation typically rises with rising substrate concentrations up to a defined limit, after which further increases in concentration bring about a subsequent decline in biodegradation rates. Contaminant concentration has been found to diminish biodegradation rates, consistent with the present study, where even isolates that displayed strong biodegradation capacity at 100 ppm were unable to effectively break down PFHxS at higher concentrations of up to 2000 ppm. Contaminants have been reported to become more toxic as their concentrations rise, such that at elevated contaminant concentrations, known degrading microorganisms become less effective at adjusting to the new stimulus [37–39].

The increase in abundance of certain isolates in response to PFHxS compared to the Control experiment, as seen in the current study, signified utilisation of the contaminant by the isolates. These isolates were able to metabolise PFHxS, effectively utilising it as a source of carbon and energy. Research has found that the abundance of bacterial species relative to time in response to a specific compound when it is available as the sole carbon source indicates the ease of biodegradation of the compound [27,28]. There is a directly proportional relationship between microbial abundance and substrate biodegradation rates [38]. This is further corroborated by several studies that have linked boosts in microbial abundance to enhanced biodegradation rates for various emerging pollutants like surfactants, pesticides, and polyethylene [27,28,40].

Comparable to the findings of the current study, reports on modifications in the microbial community of an environment as a result of exposure to perfluoroalkyl substances have shown a general shift towards dominance by members of the Proteobacteria phylum [12,41,42]. The dominant PFHxS

degraders in the present study are of the Proteobacteria phylum. Likewise, exposure to perfluorooctanoic acid (PFOA) and perfluorooctane sulphonate (PFOS) in soil induced respective 16.26 % and 12.87 % increases in the abundance of Proteobacteria while causing a corresponding 14.13 % – 15.32 % decline in the population of members of the Bacteroidota phylum [43]. *Flavobacterium, which showed poor PFHxS degradation capacity in the current study, is of the phylum* Bacteroidota. Somewhat in contrast to these findings, Cerro-Gálvez *et al*. [44] implicated *Flavobacterium*, alongside other bacteria, as degraders of perfluorooctane sulphonic acid (PFOS) in aquatic ecosystems in the Antarctic.

Ezeonuegbu *et al*. [45], in their study on the biodegradation of perfluorooctanoic acid (PFOA) by bacteria and fungi, obtained similar microbial isolates to those found in the present study. Wang and Liu [46] also reported that *Pseudomonas putida* and *Bacillus cereus* effectively degraded the short-chain perfluoroalkyl substance, perfluorooctane sulphonamide (PFOSA); as obtained in the present study. *Pseudomonas parafulva* YAB-1 displayed strong degradation abilities against perfluorooctanoic acid (PFOA) with roughly 58.6 % degradation under aerobic conditions [47]. Microbial genera like *Klebsiella* and *Pseudomonas* spp. are often implicated in the biodegradation of complex organic compounds, mainly because they produce biosurfactants that aid the biodegradation process [32].

Akin to the current study, species of *Alternaria* were seen to predominate in river water sediment contaminated with PFAS emissions, highlighting the ability of this fungal genus to tolerate and utilise PFAS as a nutrient source [48]. The study further reported that fungal abundance correlated significantly with the concentration of PFAS. *Alternaria* sp. was also implicated in the biodegradation of perfluoroalkyl compounds in the delta region of the Yellow River in China [49]. Exposure to perfluorooctanoic acid (PFOA) and perfluorooctane sulphonate (PFOS) has been known to exert a toxic effect on *Aspergillus* and *Fusarium* spp. This corroborates the results obtained in the current study, as both *A. niger* and *Fusarium* showed very limited growth in PFHxS-amended medium compared to the control study. The chitinase enzyme produced by certain fungal species has been credited with their capacity to degrade complex compounds like rubber and polyethylene terephthalate (PET) [50]. This enzyme could also play a role in the breakdown of PFHxS, as obtained in the present study.

CONCLUSION

Short-chain PFAS compounds like perfluorohexane sulphonate (PFHxS), although less bioaccumulative, are more mobile in the environment and, therefore, present unique environmental remediation challenges. This study underpins the ecological role of hydrocarbon-associated microbes in PFAS attenuation and provides baseline data for developing bioremediation consortia targeting PFHxS contamination in tropical environments. Further research into the processes involved in microbial degradation of PFHxS and other PFAS via metatranscriptomic and metabolomic approaches would provide vital insight into the specific enzymes and pathways involved in the biodegradation process. *In situ* field trials are also recommended to confirm the scale-up potential of environmental intervention strategies.

CONFLICT OF INTEREST DECLARATION

The authors declare that there are no known conflicts of interest associated with the paper

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