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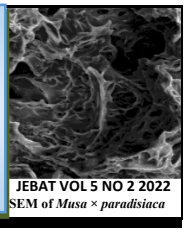

Beyond Culturing Approach for Accessing Hydrocarbon-Degrading Microbes in Petroleum Hydrocarbon Polluted Soils: A Perspective

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ABSTRACT

Petroleum hydrocarbon-polluted environments contain massive diversity of microbes capable of transforming or reducing hydrocarbon concentrations, and this has consequently led to an interest in the cultivation screening for microbial potentials to remediate petroleum hydrocarbon-polluted lands. Conversely, the reliance singly on culturing approach for the discovery of various hydrocarbon-degrading bacteria without probing for its hydrocarbon degradative capabilities has now become rampant in some research communities, and in most cases may not be justifiable. Besides, vast microbial communities with hydrocarbon-degrading potentials are eluded with the conventional method. Opportunely, the advent of culture-independent approaches such as molecular techniques and next-generation sequencing (NGS) technology has shifted the paradigm of research, now focusing on contemporary and advanced trending ways to discover the uncultivable microbial communities and assess their functional roles in the environment. To ascertain that microorganisms cultured from polluted environmental samples are factual hydrocarbon-degrading strains, a microbiologist needs to investigate beyond just culturing and probe further for the hydrocarbon-degrading prowess by choosing from various arrays of the culture-independent approaches. Consequently, this counters the questionability of only the cultivation approach and explores the vast recompenses of the latter approach when coupled. This perspective review exposes the huge gap in the application of the lone conventional culturing technique for retrieving the uncultured communities, particularly the hydrocarbon-degrading group while hinting at complementary alternatives for improved research and scientific evidence-driven and justifiable study inference.

INTRODUCTION

Recent studies have focused on petroleum hydrocarbon pollution of urban soils, as this has garnered public health concern due to their potential toxicity, mutagenicity and carcinogenicity [1, 2], and this is mostly a result of the increasing urban-level hydrocarbon pollutants concentration in the environment [3, 4, 5]. Petroleum hydrocarbon-contaminated urban soils are increasing as a result of environmental pollution mostly from anthropogenic sources that include crude oil exploration and exploitation, especially in oil-rich nations [6, 7]. Also, among the causes are man-made affiliated such as oil spills, burning of fossil fuel, oil tanker accidents, indiscriminate discharge, offloading, storage tank leakage, lubrication and automobile repair services and others [7-9]. Nonetheless, airborne combustion sources remain paramount sources of petroleum hydrocarbon contamination in the environment due to increased urbanization [10, 11].

Soil and groundwater are vital pathways often contaminated with crude oil and refined petroleum products that include petroleum motor spirit (PMS), diesel, jet fuel, paraffin, and other

petroleum derivatives like motor oil and other lubrication oils. Consequentially, these are chief sources of petroleum hydrocarbons in the polluted environment [1, 4, 12]. Petroleum hydrocarbons ranging from the simplest aliphatic hydrocarbon to multiple fused-ring hydrocarbons or polycyclic aromatic hydrocarbons (PAHs) are the main hydrocarbon pollutant components of crude oil and refined petroleum products [1, 4]. As a result, contamination by refined petroleum products or petroleum derivatives could significantly increase the hydrocarbon load in the environment. In corroboration, Fuchs et al. [4] stated that a vast range of petroleum hydrocarbons abound in the environment as pollutants of concern, among are Benzene, Toluene, Ethylbenzene and Xylene, (BTEX) which are typical aromatic hydrocarbons from petroleum derivatives such as gasoline. The PAHs have also become widespread and ubiquitous contaminants in various ecosystems [1].

Suitably, relevant studies (e.g., Brooijmans et al. [13], 14] have widely reported the existence and proliferation of some crucial microorganisms capable of utilizing or degrading petroleum hydrocarbon contaminants in the environment. Even though, many bioremediation studies have demonstrated successful methods (e.g., physical, chemical and biological approaches [15, 16]) for reclaiming petroleum hydrocarbon-contaminated land. However, bioremediation with petroleum hydrocarbon-degrading microbial consortium is generally accepted as cheap, efficient and eco-friendly technology compared with other methods [10, 17, 18]. Based on this, researchers to date have adopted different microbiological techniques to access or retrieve much diversity of hydrocarbon-degrading microbes from contaminated land or environmental samples.

Among the approaches is the antique traditional culturing method which mostly involves petri dish cultivation or flask culturing of microorganisms of environmental relevance. Though this culture technique is useful to evaluate the physiology, metabolic properties and functions, microbial phenotypes, metabolite extractions and others [19], however, several constraints of this conventional approach could be a stumbling block in retrieving larger chunks or major diversity of microorganisms of the uncultured communities which include the hydrocarbon degraders. Currently, there is an over-dependency on this cultural approach to retrieve hydrocarbon-degrading bacterial communities without complementing its flaws to justify inference. On the contrary, current advanced research has mostly relied on culture-independent approaches to access hydrocarbon degraders, hydrocarbon-degrading strain improvement and other hydrocarbon degradation studies for enhanced bioremediation of polluted land.

This review emphasized the inadequacies of singly adopting the traditional culturing approach for accessing hydrocarbon-degrading bacteria without further justifying their hydrocarbon degradative capabilities via other complementary culture-independent techniques for a purposeful rational inference. Also, encouraged the application of several advanced non-culturing approaches to explore and retrieve massive untapped communities of hydrocarbon degraders in different polluted environments.

Pollution by petroleum hydrocarbons in motor oil

Concerning petroleum hydrocarbon pollution from refined product derivatives like motor oil, numerous gallons of spent motor oil are generated in various automobile workshops nationwide and subsequently discharged indiscriminately into the environment [9, 20], consequently worsening urban soil pollution. In a related study, Onuoha et al. [21] related that soil contamination with spent motor oil has become predominant in most oil-producing and industrialized countries due to a lack of effective regulatory policies on environmental pollution prevention and control. In the case of Nigeria, indiscriminate disposal of spent diesel and motor oil into drainages, water channels and soils is a common practice [22]. Furthermore, the unregulated setting up of automobile workshops on agricultural land by auto mechanics due to space challenges has also increased soil pollution by these petroleum product derivatives [23].

Motor oil, also termed engine oil principally functions as cleaning oil for motor engines, essentially, lubricating the moving parts and preventing corrosion of motor engines;also improves sealing and cooling of the engine via heat elimination away from the moving parts [24]. Also, motor oil is applied to grease automobile engine parts to keep them running smoothly [25]. Based on its composition, literature has referred to the composition of engine oil as complex, Butler and Mason [26] stated that engine oil contains a complex blend of hydrocarbon, other organic compounds and some organometallic components. However, other studies like Corsico et al. [27] have highlighted its major constituents as a mixture of hydrocarbon compounds, relating that it's mainly blended using base oils composed of hydrocarbons.

Importantly, a typical unused motor oil consists of hydrocarbon compounds having between 18 to 34 carbon atoms per molecule, with a complex mixture of hydrocarbons which amount to 80 to 90 % of its volume, and performance-enhancing additives making up the remaining 10 to 20 % of its volume [12]. Mohammed et al. [28] clarified the chemical constituent difference between fresh or unspent motor oil and spent motor oil, where he reported that the constituent of spending is a higher percentage of aromatic and aliphatic hydrocarbons, sulphur, nitrogen and metals (e.g., Zn, Mg, Pb, and Ca) than the unspent motor oil.

Motor oil, when spent or used in motor engines is transformed in terms of physical properties, and the chemical constituents could vary though retaining the large percentage of hydrocarbon mixture - this makes the exact composition of spent motor oil un-definitive and chemical constituents may vary depending on the extent of usage. The spent motor oil is transformed after use due to the breakdown of additives, contamination with combustion products, and build-up of metals (namely, Lead, Copper, Zinc, Magnesium, Zinc, Cadmium) arising from the wear and tear of the running engine [9, 25, 29]. However, Irwin et al. [31] reported that the foremost components of spent engine oil are aliphatic and aromatic hydrocarbons namely, phenol, naphthalene, benzo (a) anthracene, benzo (a) pyrene, and fluoranthene. Agarry and Oladipupo [32] also termed spent motor oil is a mixture of PAHs, additives and heavy metals such as lead, vanadium, Nickel, Arsenic mercury and other constituents from the wear and tear of engine parts.

Among the implication of soil contamination with motor oil, Odjegba and Sadiq [33] reported that spent engine oil and its heavy metals constituents can alter soil biochemistry, microbial properties, soil pH, oxygen and soil nutrient availability. This is aside from changing the physical and chemical properties of the soil [34]. Additionally, spent engine oil damages the soil and soil native microflora as a result of poor aeration, lowering of soil pH and immobilization of soil nutrients [36].

Microorganisms capable of degrading hydrocarbon components of refined petroleum products

Numerous bacteria strains possess the capability to completely mineralize simple hydrocarbons (e.g., linear alkanes) if these bacteria own all the required enzymes for the targeted hydrocarbon substrate [37, 38]. Asserting that hydrocarbons are essential sources of carbon and electron donor for crucial hydrocarbon-utilizing microbial communities, Singleton et al. [39] relate that polycyclic aromatic hydrocarbons (PAHs) are found in the most fused aromatic hydrocarbon-polluted environment in concentrations to support microbial growth as a carbon source.

Various naturally occurring environmental microorganisms can degrade aromatic hydrocarbons and PAHs [40, 41], and this is backed by several studies among which Goyal and Zylstra [42] cloned novel hydrocarbon-degrading genes from natural strains into environmentally relevant microorganisms for improved bioremediation. The profiling and characterisation of the autochthonous microbial communities are highly valuable to assess their potential biodegradation capacity of the contaminated site - as many indigenous microbes of polluted soil and water are capable of degrading oil and hydrocarbons, and bacteria are the most active agents in petroleum degradation designated as primary degraders of oil contaminated environment [13]. Kleindienst et al. [43] reported that petroleum hydrocarbons in the polluted environment are degraded by indigenous bacteria as a result of microbial energetic and carbon requirements for growth and reproduction, this is aside from the need to relieve physiological stress triggered by the pollutant's presence in the environment. Other key studies (e.g., [19, 44, 45]) also widely emphasised that the vital components enabling bacterial degradation of hydrocarbons from petroleum sources are mainly a result of their various distinctive enzymatic properties or degradative gene capabilities.

Consequently, due to their highly valued relevance, microbes are now being screened, isolated and utilized to transform and degrade food, agricultural, pharmaceutical and industrial waste products [46]. A largely promising technology applied in recent years is the use of microbial agents like bacteria to tackle environmental pollutants as it's mostly eco-friendly and depicts a low-cost budget [18, 19]. To depict the high-scale research significance of this scope, several relevant studies have credibly exposed petroleum hydrocarbon-degrading microbial communities from diverse environments. The members of phyla *Proteobacteria*, *Actinobacteria* and *Acidobacteria* are widely reported as hydrocarbon degraders in diverse polluted soil and water environment [11, 47, 48], and relevant studies have identified bacteria capable of degrading petroleum hydrocarbon from more than 79 genera [49]. Among these hydrocarbon-degrading bacteria with enzymatic prowess include genus *Rhodococcus, Achromobacter, Mycobacterium, Acinetobacter, Burkholderia, Alkanindiges, Pseudomonas, Alteromonas, Dietzia, Arthrobacter, Streptobacillus, Marinobacter, Pandoraea* and others [45, 46, 50, 51]. In a related study, Yakimov et al. [52] revealed the dominance of some marine bacteria referred to as obligate hydrocarbonoclastic bacteria (OHCB) such as *Alcanivorax, Cycloclasticus, Marinobacter, Oleispira, Thallassolituus* after environmental contamination with petroleum oil.

Other important PAH-degrading bacteria genera include *Streptomyces, Mycobacterium, Bradyrhizobium, Burkholderia, Micromonospora, Pseudomonas, Spingomonas, Comamonas, Pseudonocardia, Actinoplanes, Nocardia, Rhodococcus, Nocardioides, Methylibium, Bacillus, Solirubrobacter, Porphyra, Frankia*, using different hydrocarbon-degrading enzyme system and present in high and minute abundance in petroleum hydrocarbon polluted environment [11, 53-61].

Petroleum hydrocarbon degradation:genes, enzymes and pathways

Various microbes have been associated with the breakdown of different sorts of hydrocarbons (aliphatic, aromatic and PAHs), as they possess the vital genetic capabilities for the hydrocarbon breakdown processes [13, 62]. Noteworthy, hydrocarbon-degrading microorganisms possess vital hydrocarbon-degrading genes that encode for the enzymes catalyzing the degradation reaction of hydrocarbon contaminants to less toxic and simpler metabolites [59]. For mention, the Genus *Pseudomonas* possess hydrocarbon-degrading genes associated with the PAH-biodegradative pathway highly homologous to the cluster of *nah* genes (naphthalene-degrading genes) that have been cloned from NAH7 plasmid harboured by *Pseudomonas putida* G7 [53]. As such, mixed populations and consortia of microorganisms with overall broad enzymatic capacities can transform, utilize or degrade complex mixtures of hydrocarbons in the soil [11, 59, 63]. Again, some microorganisms are capable of metabolizing multiple hydrocarbon compounds using one or multiple enzyme systems [64], thus earliest hydrocarbon degradation studies (e.g., [63, 65]) have crucially established that the essential roles of microorganisms in the degradation of hydrocarbon pollutants are predominantly attributed to their wide-range enzymatic functions and capabilities. Soil bacterial community possessed the capabilities to degrade PAHs, and this is most evident due to the abundance of PAH-ring hydroxylating (PAHRHD) genes in Gram-negative and Gram-positive bacteria in PAH-amended and un-amended control soils [66]. Therefore, the path for the removal of hydrocarbons in the environment is considered to be via microbial transformation and degradation [8].

Microorganisms have shown exceptional biochemical and physiological versatility for degradation processes in different conditions such as oxic and anoxic [59]. So, due to their diverse genetic capabilities, there exist various enzymatic hydrocarbon-degrading roles and multifaceted pathways in varied conditions for certain hydrocarbons ranging from aliphatic to PAHs - it must be acknowledged that this review is only focused on some oxygenase enzyme systems used by microorganisms for degradations of aromatics and PAHs. In oxic degradation conditions, petroleum hydrocarbon degradation pathways have been demonstrated to use oxidizing reactions, though pathways may vary greatly due presence of specific oxygenases in different species of bacteria [46]. Habe and Omori [67], reported that the initial degradation of PAH has been extensively studied in bacteria, and this begins with the initial oxidation of one of the benzene rings to a cis- dihydrodiol intermediate by the activity of multi-component ring-hydroxylating dioxygenases (RHD).

Accordingly, hydrocarbon-degrading bacteria oxidize the PAHs to cis-dihydrodiols by incorporation of both atoms of an oxygen molecule, and the cis-dihydrodiols are further oxidized, to aromatic dihydroxy compounds such as catechols then channelled through ortho- or meta-cleavage pathways [68]. Summarily, Harayama and Rekik [69] earlier relate that the enzymes which catalyze such initial oxidation reaction of PAHs are oxygenase enzymes which are classified into two groups namely monooxygenase and dioxygenase - these enzymes are widely distributed in nature and involved in both biosynthesis and biodegradation process [69]. The monooxygenases catalyze the insertion of one atom of dioxygen into an aromatic ring to yield phenols [70].

Also, Gibson et al. [71] relayed the pathway where one of the dioxygenases known as the ring-hydroxylating dioxygenases catalyses the initial step of the PAH deoxygenation by destabilizing the aromatic ring to form *cis*-dihydrodiols, and the second dioxygenase group called the ring cleavage dioxygenases further oxidizes the destabilized aromatic ring leading to cleavage of the aromatic ring to form compounds such as catechol, protocatechuate and gentisate products. Under an anoxic environment with depletion of oxygen (such as aquifers), the degradation of hydrocarbons is accomplished by anaerobic bacteria using other electron acceptors (nitrate, sulfate, and ferrous iron and others), and more importantly, specific anaerobic biochemical pathways are purportedly involved [11, 72]. Thus, several anaerobic hydrocarbon degradation studies (e.g., [73-75]) have demonstrated PAH degradation under denitrifying and sulfate-reducing anaerobic conditions using nitrate and sulfate as alternative electron acceptors. Nieman et al. [76] have also reported that ethylbenzene, naphthalene, pyrene, phenanthrene, and others are degradable and mostly completely oxidized to CO_2 in denitrifying conditions.

Approaches for accessing hydrocarbon-degrading microorganisms from petroleum hydrocarbon-polluted soils

Environmental microbiologists have adopted several techniques for accessing and assessing the microbial world to detect their existence and investigate their functional roles in their natural and artificial environment. Several of these methodologies have become basic microbiological techniques in our today's research while others stand out as novelty and advancements in this field of microbiology. Regardless, only seasoned microbiologists are much acquainted with and suitably relish the varied peculiarities of any chosen approach to achieve and satisfy its research goal. Based on the interest in the cultivability and uncultivability of focused microorganisms, this review simply considers the various techniques into two groups of culture-dependent and culture-independent approaches regardless of their distinctiveness and methodological values and differences.

The traditional cultivation approach:a culture-dependent technique

Since the dawn of microbiology, microbiologists have relied primarily on traditional culturing to study microbes in a petri dish and obtain pure cultures, and this then became essential for the characterization of diverse microorganisms in the laboratory [77]. Additionally, this long-aged petri dish culturing of microbes has been used for studying the physiology, functions and diversity of different microorganisms [19]. It has been established that microorganisms ubiquitously flourish in their diverse natural ecological habitat, and one of the ancient microbiological techniques for accessing microbial communities and proving their existence in various habitats is to demonstrate their growth or isolate them in a foreign environment. This foreign environment which is external to their native habitat is mostly provided in form of an artificial culture media containing nutrients and growth factors for the cultivation of microorganisms. Thus, conventional culturing of microbes relies on the ability of members of Eubacteria and Fungi to utilize nutrients present in laboratory culture media for their growth and metabolism.

Generally, defined culture media with known organic or chemical nutrient composition among which are dextrose, tryptone, malt extract, soya extract, beef extract, peptone, and glucose primarily serve as sources of Carbon and energy for microbial growth and metabolic activities. Others may contain certain ingredients such as salts, a variety of minerals and others required to grow specific microbes. However, for the isolation of hydrocarbon-degrading microorganisms, many studies have used different culture media such as Bushnell Haas or compounded a mineral salt medium (containing only mineral salts and buffers) devoid of a carbon source to cultivate these important microbial communities. Essentially, an alternative C source mostly the targeted hydrocarbon substrate must be provided in the media in form of hydrocarbon crystals, liquid form, dissolved in a carrier gas, or C sources delivered via hydrocarbon components in crude oil or its refined products like diesel. By insinuation, it is commonly anticipated that only hydrocarbon degraders using the hydrocarbon as a C source could be isolated from such medium;consequently, many studies have solely banked on this approach to isolate suspected hydrocarbon degraders and identify biochemically without evidently confirming the capability of the isolates to genuinely transform or degrade hydrocarbon pollutants.

Even though culturing technique allows for isolates to be purified and preserved for further studies such as cloning and transformation, plasmid curing, strain improvement, co-culture bioaugmentation (bioremediation), production of bioactive compounds (such as biosurfactants) and others. Inappropriately, this technique has weighty constraints, among which is that it does not capture the entirety of microbial communities since the uncultivable majority will not grow on laboratory media [78, 80], thus the majority of hydrocarbon-degrading microorganisms would be evaded. Studies have attempted to mitigate these limitations, as adopted by optimizing growth conditions namely, temperature, pressure and variety of growth media [81, 82]. Still, this approach could only retrieve a few diversity of microbial communities as expatiated in the next sub-heading.

The uncultured majority and culturable minority

Microbial ecologists have emphasized the beauty of identifying diverse microorganisms, especially from their natural ecological habitat, and mostly cherish the possibility of capturing an entire microbial community. Here, microbial diversity is the focal consideration in this scope of the culturability stance of the microbial world.

Diverse species of microorganisms have been cultivated on different culture media, portraying the ability to proliferate on artificial medium – this group of microorganisms are termed culturable microbes. Nevertheless, relevant studies have established this cultivable population represents the minority [80, 84], as only 1 % or less of microorganisms have been studied by culturing method [85, 86]. On the contrary, a significantly larger diversity of microbes representing the majority communities is recalcitrant to conventional petri dish culturing or isolation even while using rich assorted growth medium and adopting current culturing techniques, thus branded the 'unculturable' though with varied perspectives of reasoning.

Based on the literature, more than 99 % of the possible microorganisms are still undiscovered via culturing [87], so termed uncultured majority;besides only a minor portion are culturable by recent techniques [84, 86, 88, 89]. Microbiologists have logically and widely reflected and reported on the culturability of the majority via cultivation technique and inferred the term ‘unculturable’ have been differently misconstrued, and this implies the context of unculturability is divergent among microbiologist. While some conceived this as microbes that cannot be grown on culture media, others like Stewart [90] reasonably opined the term “unculturable” indicates that current laboratory culturing techniques are unable to grow a given bacterium in the laboratory. Also, seasoned microbiologists have reasonably argued that these communities

are not actually unculturable but microbiologist hasn't clearly understood their specific growth requirements to culture them emphasizing the growth requirements are mostly unknown consequently they remain as uncultured communities. Others have argued that knowledge of growing fastidious microbes is deficient in culturing these communities. Therefore, the perception assuming the uncultured communities are factually 'unculturable' in the context of 'cannot be cultured' should be expunged, as several studies such as Connon and Giovannoni [91], Kaeberlein et al. [92] and Rappe´ et al. [93] have shown that previously tagged uncultured microorganisms can be cultured in pure culture when provided with the chemical constituents of their natural environment or habitat. Based on this, the unculturable group could instead be termed 'yet-to-be-cultivated' [94].

Researchers have established the reason for their unculturability, as Stewart [90] stated that microbiologists often fail to reproduce the essential features or aspects (growth conditions) of their natural environment. Also, Simu and Hagstrom [95] emphasized why bacteria may be uncultivable and among which are lack of the rightly needed nutrients, inappropriate growth temperature combination, symbionts, atmospheric gas composition, presence of inhibitory compounds and others. It may be understandable why microbiologists have under-tapped the very large and diverse uncultured microbial communities;as conventional culturing of microorganisms is mostly laborious, time spending, and importantly selective and partial for growing exact microorganisms [96]. Therefore, the inability to surmount the traditional culturing challenges resulted in the majority of microbial communities remaining uncultured, and further explain why most studies could isolate only a few hydrocarbon-degrading microorganisms via traditional culture technique from polluted environmental samples.

Culture-independent approach

Even though the ancient discovery of the culturing of microbes in a petri dish is a classical landmark still currently sustained as the earliest method applied by a microbiologist for the study of microbial ecology, physiology, preceding diversity and functions [19], this hasn't been without its downsides. For instance, the taxonomic identity of the microbes associated with any specific process or function has commonly been restricted to that small portion of the microbial community cultivated [97]. Moving forward, microbiology has evolved from the earliest culturing technique to advanced culture-independent techniques to majorly circumvent the constraints of the culturing approach for microbial diversity and ecology study, and pollutant metabolism [11].

Simply put, an approach is indicative of being culture-independent if it excludes the antique traditional petri dish cultivation of microorganisms or any cultivation of the sort. In so doing, microorganisms are being studied on an advanced level and this has resulted in many leading-edge discoveries in the field of microbiology. Numerous crucial studies (e.g., [11, 57, 60, 98, 99]) have demonstrated the application of culture-independent approaches for hydrocarbon-degradation and bioremediation studies and pollution monitoring. Among the culture-independent approaches are molecular techniques including hydrocarbon-degrading gene targeted Polymerase chain reaction (PCR), Quantitative polymerase chain reaction (qPCR), Denaturing gradient Gel Electrophoresis (DGGE), Reverse transcriptase-denaturing gradient gel electrophoresis (RT-DGGE), Terminal restriction fragment length polymorphism (T-RFLP), the –omics approaches (Genomics, Metagenomics, and Metatranscriptomic), Stable isotope probing, Next generation sequencing (NGS) technology and bioinformatics (**Table 1**). These culture-independent approaches are not limited to only the detection and characterization of microbial communities degrading hydrocarbons but importantly allow for digging deeper into microbial ecology, diversity, microbial community profiling, microbial biochemistry and pollutant degradation [39, 100-104]. Others include evaluation of community structure in a complex polluted environment, determination of the abundance of microbial biomarkers, hydrocarbon-degrading gene quantification, detection of the diversity of hydrocarbon-transforming microbes, and allowing for cogent assessment of enzymatic and genetic capabilities of petroleum hydrocarbon-degrading communities [94, 105-109, 157]. The majority of afore-mentioned applications remain unachievable with culturing approach thus relevance is predominantly restricted to isolation, biochemical characterization and detection of phenotypic morphologies while neglecting to probe for their hydrocarbon degrading potentials.

The interest in identifying microorganisms in a different environment and logical evaluation of their genetic functionality without laboratory cultivation has shown a prompt rise in the application of metagenomics [19]. This implies direct isolation or extraction of microbial genomic DNA from environmental samples and cloning into an environmentally relevant bacterium as a transformant;alternatively, applying sequencing technology could circumvent limitations of direct culturing and increase the chances of gaining access to the uncultured communities. Metagenomics and a few other -omics methods are tremendously valued in assessing the biodegradation prospect or potentials in environmental samples [110, 111], and have become one of the culture-independent methods designed to gain access to the physiology and genetics of uncultured organisms [19].

Another is stable isotope probing (SIP), an elegant technique for elucidation of the functional and metabolic capabilities of the uncultivated microbes which focuses on the incorporation of isotopically labelled stable substrates into microbial biomass, consequently revealing the identity of the community actively assimilating the label [112]. Essentially, SIP has massive potential for recognizing microorganisms responsible for the degradation of xenobiotic compounds in natural environments and remediation systems [113], thus while detecting microorganisms, it confirms their ability to degrade pollutants. Therefore, establishing the link between the function and identity of microorganisms without the need for media culturing has seen a swift increase in the adoption of stable isotopes to investigate biological processes [114].

The trending adoption of molecular techniques like PCR and real-time PCR for accessing hydrocarbon-degrading microbial communities is highly plausible as it provides conclusive genetic competence of microorganisms isolated from polluted soil or water. Many studies have targeted and detected assorted hydrocarbon-degrading genes such as monooxygenases and dioxygenase genes from different environmental samples using PCR, and others have detected the abundance of PAH ring-hydroxylating dioxygenase genes via qPCR. Additionally, Chikere [115], also detailed some of these microbiological and molecular techniques useful for assessing hydrocarbon-degrading microbial communities and applicable to the bioremediation of hydrocarbon and non-hydrocarbon pollutants. These, among others, are some of the culture-independent approaches that could prove the hydrocarbon-degrading potential or capability of microorganisms isolated from polluted environmental samples.

Table 1. Culture-independent techniques with relevant reference studies.

Culture-independent techniques	Reference studies
Gene-targeted PCR	[102, 106, 116, 117]
Real-time PCR (qPCR)	[106, 116, 118, 119]
Metagenomics/Gene-target metagenomics Denaturing gradient gel electrophoresis (DGGE)/Denaturing Temperature gradient gel electrophoresis (DGGE)	[19, 108, 109, 111, 117, 120, 121] [100, 101, 122]
DNA/RNA-Stable isotope probing (DNA/RNA-SIP) including DGGE-enabled SIP and Stable isotope probing coupled with metagenomics	[39, 97, 112, 113, 123, 124, 125]
Amplified Ribosomal DNA Restriction Analysis (ARDRA)	[126, 127]
Metatranscriptomics	[128, 129, 130, 131]
Terminal restriction fragment length polymorphism (T-RFLP)	[132, 133, 134, 135]
Next-generation sequencing technologies (e.g., 16S Amplicon sequencing, Illumina shotgun metagenome sequencing, sequencing), Gene-targeted sequencing.	[103, 109, 136-141]

Appraisal of culturing and culture-independent approaches for genuinely accessing hydrocarbon-degrading bacterial communities

Having expatiated on the conventional culturing and culture-independent approaches for retrieving and assessing microbial communities having the gene capabilities to degrade hydrocarbon, their appraisal to justifiably achieve the target goal is only indispensably normal. Essentially, this is a valid pointer and could advise researchers on both approaches while portraying the approach's level of advancement to align current and trending realities in this field of research. To begin with, the rampancy of studies on the isolation of hydrocarbon-degrading bacteria from refined petroleum product polluted soils (in most cases, diesel or spent motor/engine oil polluted soils) adopting culturing techniques must be admitted, and most of which are highly debatable based on today's advanced research cognizance.

Some study scenarios have cultured and identified microorganisms from polluted soils using a common nutrient-rich culture media containing a carbon source, yet inferred the isolates degrade the hydrocarbon substrate provided in form of engine oil. Others have solely relied on the use of mineral salt media, Bushnell Hass or related media while adding motor oil or diesel as a hydrocarbon source. Consequently, attributing all the cultured bacteria identified via biochemical methods to the degradation of the spent engine oil, diesel or other hydrocarbons without further probing for the hydrocarbon-degrading competence of the isolates. In this case, it is reasonably presumed that isolates may be utilizing any hydrocarbon or non-hydrocarbon organic constituents of the refined petroleum products until the capability of these isolate to degrade hydrocarbon is proven scientifically.

In another scenario, only a few advanced studies better endeavored to detect and monitor the loss of hydrocarbons components using Differential Scanning Fluorimetry (DSF), Mass Spectrometry (MS), Gas Chromatograph Flame Ionization Detector (GC-FID), Gas Chromatography-Mass Spectrometry (GC-MS), High-Performance Liquid Chromatography (HPLC) during incubation and inferred the disappearance of the hydrocarbon components substantiate hydrocarbon degradation by isolated bacteria. By realistic insinuation, the aforementioned instances are only assumptions of hydrocarbon degradation claims if there is no convincing scientific or research backing of hydrocarbon degradation capabilities of bacteria communities isolated as would be elaborated further. Firstly, it should be reminded that adopting only a conventional culturing method to isolate hydrocarbon-degrading bacteria from polluted soil is highly disadvantaged as this method practically elude the majority of bacteria communities since many studies have established that only 1 % or fewer communities could be cultured [80,142], this simply implies that very bulk remaining percentage are uncultivable away from their natural habitat while this does not rule out their genetic existence and metabolic relevance in such environment as exposed by sequence-driven metagenomic studies (e.g., [143]). Besides, the isolation or cultivation approach cannot thoroughly depict the entire microbial communities in the environment and thus failed in capturing the full spectrum of microbial population and diversity [19, 143-146].

By implication, the chances of retrieving many populations and a huge diversity of hydrocarbon-degrading microorganisms via culturing are not very probable, as Hugenholtz and Pace, [147] stated that technique limit analysis to only microbes culturable under laboratory conditions. Rondon et al. [143] also stated that the metagenomes of the total microbiota found in the natural environment encompass massively more genetic information than contained in the cultivable subset. However, this doesn't entirely rule out the possibility especially when the suspected minority communities isolated are further investigated to ascertain the claim of hydrocarbon degradation. On the contrary with metagenomics and SIP, it enabled researchers to study uncultivable bacteria and link potential functional roles to microbes in their habitat [11, 148] without the need for their cultivation.

Secondly, adopting a cultural technique requires a result-promising experimental design that would essentially alleviate the constraint of detecting few cultivable communities, so by providing suitable growth and environmental conditions as explained by Simu and Hagstrom [149] and Stewart [90] for the growth of the target communities could increase the chance of success. Bodor et al. [94] suggested the modification of isolation culture media formulation in terms of substrates and nutrient requirements could increase the possibility of isolating the target hydrocarbon-degrading community. This begins but doesn't end with the use of a no Carbon source culture media and provision of an alternative C source in form of hydrocarbon pollutant substrate while ensuring microbes does not rely on other C nutrient from the soil inoculum.

In another way, the earliest efforts to isolate formerly uncultured bacteria communities were accomplished via mimicking natural conditions which are reduced nutrient and inoculum sizes, and prolonged incubation period [91, 150]. In other studies, methods like One-factor-at-a-time (OFAT) were deployed where other growth or environmental factors (e.g. C source, nutrient, pH, temperature, and oxygen availability) are optimised in the laboratory to ensure the growth of hydrocarbon-degrading bacteria, though limited to a single bacteria culture. Another important step adopted in most SIP studies (e.g., [11, 113]) is the attempt to mimic their natural environment by initial culturing in a microcosm setup enriched with hydrocarbon substrate to increase the chance of isolating the target microbial communities. Mostly, a time-course experimental design is considered with a longer incubation period to firstly rid-off the communities using readily available C source in the soil inoculum and this is monitored from the heterotrophic plate count

as other communities relying on hydrocarbon C substrate will mostly emerge afterwards in later incubation period. Thirdly, the monitoring of the loss of hydrocarbon components (such as total petroleum hydrocarbon, total aliphatic hydrocarbon, total aromatic hydrocarbon and PAHs) during periodic incubation is a very plausible step. Many studies (e.g., Muhammad et al. [151]) have included the investigation of hydrocarbon concentration reduction via analytical chemistry techniques like Mass spectrometry (MS), GC-MS, HPLC, and GC-FID. While this is an important requirement to confirm the degradation or loss of hydrocarbon components of diesel or spent motor oil and to help deduce the degradation curve and percentage degradation. However, careful replication of samples and adoption of control experiments is crucial for this step to avoid any bias extrapolation. Importantly, it must be recognized that not all hydrocarbon reduction during incubation are as a result of microbial degradation, hydrocarbon loss also occur via volatilization, adsorption, photolysis, and chemical degradation [152, 153], nonetheless, microbial degradation is the major process causing hydrocarbon concentration reduction [153]. Therefore, the hydrocarbon reduction check is only another indication of hydrocarbon utilization by microbial communities which should serve as a caller for hydrocarbon-degrading gene probing for confirmation.

Fourthly, countless studies have isolated suspected hydrocarbon-degrading microorganisms when there is virtually no literature or data showing the isolates possess any genetic or enzymatic capabilities to utilize or degrade the simplest hydrocarbon pollutant. Some have argued these could be random bugs proliferating especially when contamination issue is factored in – as a poorly done microbiological experiment devoid of basic aseptic technique must reflect or capture microbial contaminants which could somewhat be assumed, hydrocarbon degraders. To counter these queries, most experienced microbiologists wisely screen for the hydrocarbon-degrading potential or capabilities after isolation thus concretizing the inference of the study. For instance, Geiselbrecht et al. [40] isolated marine PAH-degrading *Cycloclasticus* strains (from the Gulf of Mexico) and confirmed their PAH-degrading capabilities whilst comparing them with other *Cycloclasticus* strains.

In this review, it is glaring that reliance singly on conventional culturing technique along with biochemical characterization is not scientifically convincing enough that strains isolated are truly and functionally hydrocarbon-degrading. The main requisite that could justify the inference of such a study is to confirm the hydrocarbon-degrading capabilities of the isolates mostly via culture-independent approaches. The simplest of which is to probe for certain hydrocarbon-degrading genes via gene-targeted PCR or enumeration of PAH ring-hydroxylating dioxygenases (RHD) via qPCR. Evidence-demanding researchers have maintained that even when confirmed that bacterial communities isolated possess hydrocarbon-degrading genes, it must be further investigated if the gene is active and functionally used by the strains to degrade the hydrocarbon pollutant, and SIP culture-independent technique could again be deployed here.

Interestingly, non-molecular approaches such as dioxygenase enzyme activity is a vital method that could be adopted to verify the hydrocarbon-degrading competence of the isolates, as the metabolism or conversion of indole to indigo is used to test for the expression of oxygenase enzymes produced by hydrocarbon-degrading strains during growth on various substrates [154, 155]. Providentially, the hydrocarbon-degrading enzyme system responsible for the formation of indigo mostly consists of more than one enzyme, typically monooxygenases, dioxygenases or hydroxylases [156]. Also, Zocca et al. [155] while studying the biodiversity of culturable PAH-transforming bacteria from an abandoned industrial site adopted a substrate utilization experiment to determine PAH utilization in the medium to ascertain the hydrocarbon-transforming capacity of bacteria isolated. Put together, these shows that research on this subject could go beyond just traditional culturing on Petri dishes and biochemical identification importantly, to justify the inference of the study.

CONCLUSION

No doubt different species of hydrocarbon-degrading bacteria can be isolated from petroleum hydrocarbon-polluted environmental samples via the traditional culturing approach, though a well thought out and structured experimental design is required to put in place fundamental factors (growth and environmental conditions), that could jeopardise the purpose – as this is beyond mere plating of soil samples in a culture media then biochemical identification. A reliable chemistry analysis to monitor the reduction or utilization of hydrocarbons during the period of incubation is only additionally plausible, though appropriate sample replication and adoption of complete experimental controls are crucial for this step. A very vital requirement to this kind of study to make a fair and rational inference is to verify the degradative capabilities or competence of the bacterial species isolated, and this could be achieved by suitable culture-independent approaches, so doing, it is undoubtedly clear the inference generated is genuinely defensible as the isolated microbial communities are certain to have the potential to degrade hydrocarbon when deployed for bioremediation study. Research conclusions drawn on studies focused only on the isolation via culturing and biochemical identification of supposed hydrocarbon-degrading bacteria without convincing hydrocarbon-degrading capability check remain assumptive and questionable. Unless there is a development of a specialized differential media designed to permit visual indication and differentiation of hydrocarbon-degrading bacterial colonies from others (non-hydrocarbon degraders) based on particular chemical reactions such as the conversion of an indicator compound like indole to indigo in the media, especially for PAH dioxygenase producers. Prospects in the study of larger novel diversity of microbes in a petri dish would require innovative culture techniques to capture more diversity of the uncultured community. Among these uncultured or yet-to-be-cultured communities are certainly potential hydrocarbon-degrading microorganisms whose strains could be improved by advanced molecular approach for bioremediation application. Therefore, a more successful application of the conventional culturing technique to access or retrieve hydrocarbon-degrading microbial communities is to complement with a suitable culture-independent technique to genuinely produce an incontestable research output.

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