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Selection of new fungal strains and development of a
microbial consortium for the bioremediation of
complex hydrocarbon mixtures

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Preface

This thesis results from my PhD in Earth and Environmental Sciences (Cycle XXXIII), supported financially by ENI S.p.A.. The experiments were carried out partly at the Mycology Laboratory of the University of Pavia, located at the Botanical Garden, and partly at the ENI S.p.A. laboratories in San Donato Milanese (MI). All phases of the project were supervised by Prof. Solveig Tosi of the Department of Earth and Environmental Sciences of the University of Pavia and Dr Giovanna Carpani of the Department of Research and Technological Innovation of ENI, Environmental Technologies Laboratories (TEAMB), ENI S.p.A.. Some experimental activities were carried out in collaboration with Dr Federica Corana and Dr Barbara Mannucci of the Centro Grandi Strumenti of the University of Pavia.

This PhD research has deepened my knowledge of the infinite fields of fungal organisms and experience the collaboration between different organizations and researchers, both national and international, all having different working methods and requirements. The collaboration with ENI, in particular, has undoubtedly been a fundamental experience for my growth as a researcher, as it has allowed me to experience at first hand the work in a laboratory outside the University and to observe more closely, the working dynamics of a large company.

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Abstract

Excessive release of hydrocarbons and their derivatives into the environment has harmful effects on the ecosystems and the organisms them populating. One of the threats is represented by the treatment of hydrocarbons in the oil industry. Large tanks are used to store raw materials and intermediate or finished products. The sludge and heavy oil deposits accumulated in these tanks need be cleaned periodically, mainly manually. The procedure is dangerous, time-consuming, labour-intensive, and expensive. An excellent alternative is bioremediation, which exploits the metabolic activities of microorganisms to degrade toxic compounds in the in tanks that eventually can pollute the environment. In this context, a microbial consortium with a wide array of enzymatic abilities is more effective in degrading hydrocarbons than the use of a single microorganism.

This PhD project aimed to develop a fungal-bacterial consortium with a high biodegradation potential against a complex hydrocarbon mixture, in order to find a more sustainable, low-impact and cost-effective method to treat contaminated surfaces and soils. In this thesis, I present the results of both fungal and bacterial strains. The study on fungi has been carried out by myself and most of the works on bacteria were carried out by ENI S.p.A.. Therefore, the results on the bacterial activity are not detailed.

Chapter 1 presents a general introduction on the thesis, detailing the problem of petroleum hydrocarbon pollution and the bioremediation solutions. I dedicated a most of the first year of my PhD at searching and studying the scientific literature, which has been fundamental for writing this chapter and to develop all the procedures presented in the present thesis. I reported here general information about the structure of petroleum hydrocarbons, its toxicity, and the problem posed by the oil industry. Then, I focused on the comparison between conventional remediation methods and bioremediation, with a focus on the use of bacteria and fungi for hydrocarbons remediation. At the end of Chapter 1, two published articles I produced during my thesis are reported: one is a review dealing with the degradation of aliphatic hydrocarbons by fungi; the other presents the results of our study on the abilities of two fungal strains, *Aspergillus oryzae* and *Mucor irregularis* to degrade hydrocarbons.

Chapter 2 focuses on the isolation and identification of microbial strains useful to build a microbial consortium. This part was developed during the first year of my PhD. Twenty-nine fungal strains and 50 bacterial strains were isolated from different contaminated substrates from a Pakistan gas plant (ENI S.p.A.). Fungi were identified through a polyphasic approach combining morphological and traits.

In Chapter 3, I present the results on the screening of the isolated fungal strains for their abilities to grow on a hydrocarbon complex mixture as the sole carbon source. I also present the tests I used to evaluate the enzyme production of the fungal strains I have selected. To this end I have employed enzymatic colorimetric tests to verify the ability of strains to produce ligninolytic enzymes, heavily involved in the hydrocarbon degradation. From these tests, I selected 8 strains potentially useful to be further tested.

Chapter 4 presents the tests I used to evaluate the hydrocarbon-degrading capacity of the fungal strains I have selected. To this end, during the second year of my PhD, I analysed the composition of hydrocarbons of the contaminated substrates before and after fungal action through gas chromatography analyses. At the end of the chapter I reported an article I published about the potential of *Trichoderma* sp. in bioremediation.

Chapter 5 describe the steps I have taken to assemble the fungal-bacteria consortium and to study its efficacy. An antibiosis test was performed to verify the absence of antagonistic effects among the fungal strains. Consequently to this test, I have assembled the fungal-bacteria consortium combined the 6 fungal strains (*Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Curvularia aerea* F28, *Fusarium solani* F39 and *Fusarium chlamydosporum* F41) and the 28 bacterial strains selected by ENI.

The effectiveness of the consortium was assessed through gas chromatography analysis by evaluating the hydrocarbon composition in the substrate *pre-* and *post-* treatment with the consortium. The consortium proved to be efficient in degrading the complex substrate utilising all the available hydrocarbons fractions within a week. Finally, I have inquired the best method for consortium preservation, to find the most suitable technology for preserving and transporting it over long distances. Freeze-drying proved to be the most suitable method.

Chapter 6 reports the experimental trials performed in the third and final year of my PhD. With respect to what I foresaw for my research, the difficulties posed by the COVID-19 pandemic forced us to modify the work plans for this last year. Unable to demonstrate the efficacy of the consortium with field tests, I confirmed the consortium potential through an ecotoxicological test.

In Chapter 7, general conclusions on the results obtained and future perspectives are given. Undoubtedly, further investigations are needed to optimise the performance of microorganisms and to complete the analyses with field tests. However, this work demonstrates how isolating autochthonous microbial strains from contaminated substrates and using them to develop a consortium is an efficient methodology for the degradation of complex hydrocarbon mixtures. It also highlights how collaboration between different

Abstract

researchers with different training and experience, specialised in various scientific fields, is essential to find greener and low environmental impacts methodologies.

Riassunto

Il rilascio di idrocarburi e dei loro derivati nell'ambiente ha effetti nocivi sugli ecosistemi e sugli organismi che li popolano. Recentemente è sorto un ulteriore problema causato dal trattamento degli idrocarburi nell'industria petrolifera, dove grandi serbatoi vengono utilizzati per lo stoccaggio sia di materie prime che di prodotti intermedi o finiti. I fanghi e i depositi di olio pesante che si accumulano nei serbatoi devono essere puliti periodicamente, di solito con mezzi manuali. La procedura è pericolosa, richiede molto tempo, molta manodopera e spesso l'utilizzo di frazioni leggere del petrolio o di altri solventi per risultare efficace; non sono trascurabili oltretutto i costi di tali procedure. Un metodo alternativo a questi trattamenti convenzionali è il biorisanamento, che prevede l'uso di microrganismi per degradare i componenti tossici presenti in un substrato. In particolare, l'uso di un consorzio microbico con una vasta gamma di abilità enzimatiche è più efficace nel degradare gli idrocarburi rispetto all'uso di un singolo microrganismo.

Questo progetto di dottorato ha avuto lo scopo di sviluppare un consorzio funghi-batteri con un alto potenziale di biodegradazione nei confronti di miscele complesse di idrocarburi, al fine di trovare un metodo più sostenibile, a basso impatto ambientale e più economico per trattare superfici e suoli contaminati. In questa tesi, presento sia i

risultati ottenuti per i ceppi fungini sia per quelli batterici, ma la maggior parte dei lavori sui batteri sono stati svolti da ENI S.p.A. e quindi non sono riportati in dettaglio.

Il capitolo 1 rappresenta l'introduzione generale della tesi e tratta dell'inquinamento da idrocarburi petroliferi e del biorisanamento. Ho dedicato buona parte del primo anno di dottorato allo studio della letteratura scientifica, che è stata fondamentale per la stesura di questo capitolo. Sono riportate informazioni generali sulla struttura degli idrocarburi petroliferi, la loro tossicità sugli esseri viventi e sull'ambiente, e i problemi legati all'industria petrolifera. In seguito vengono analizzati i metodi di risanamento convenzionale, il biorisanamento e in particolare l'uso di batteri e funghi nei processi di biorisanamento degli idrocarburi. Alla fine del Capitolo 1, sono riportati due articoli da me prodotti: uno sulla degradazione degli idrocarburi alifatici da parte dei funghi e l'altro sulle capacità di *Aspergillus oryzae* e *Mucor irregularis* contro gli idrocarburi.

Il capitolo 2 si concentra sull'isolamento e l'identificazione di ceppi microbici utili per costruire un consorzio microbico. Questa parte è stata sviluppata durante il primo anno del mio dottorato. Ventinove ceppi fungini e 50 ceppi batterici sono stati isolati da diversi substrati contaminati di un impianto di gas del Pakistan (ENI S.p.A.). I funghi sono stati identificati attraverso un approccio polifasico che combina l'analisi morfologica e molecolare.

Nel capitolo 3, presento i risultati dello screening dei ceppi fungini isolati per la loro capacità di crescere su una miscela complessa di idrocarburi come unica fonte di carbonio. Vengono presentati anche i test usati per valutare la produzione di enzimi dei ceppi fungini selezionato. A questo scopo sono stati impiegati test enzimatici colorimetrici per verificare la capacità dei ceppi di produrre enzimi ligninolitici, fortemente coinvolti nella degradazione degli idrocarburi. Da questi test, ho selezionato 8 ceppi potenzialmente utili per essere ulteriormente testati.

Il capitolo 4 presenta i test utilizzati per valutare la capacità di degradazione degli idrocarburi dei ceppi fungini selezionati. A questo scopo, durante il secondo anno del mio dottorato, ho analizzato la composizione degli idrocarburi dei substrati contaminati prima e dopo l'azione dei funghi attraverso analisi gascromatografiche. Alla fine del capitolo ho riportato un articolo che ho pubblicato sul potenziale del *Trichoderma* sp. nel biorisanamento.

Il capitolo 5 descrive i passi compiuti per assemblare il consorzio funghi-batteri e studiarne l'efficacia. È stato eseguito un test di antibiosi per verificare la compatibilità dei ceppi fungini. Il consorzio funghi-batteri è stato quindi assemblato combinando i ceppi fungini selezionati (*Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Curvularia aeria* F28, *Fusarium solani* F39 and *Fusarium chlamydosporum* F41) e i 28 ceppi

batterici selezionati da ENI. L'efficacia del consorzio è stata valutata mediante analisi gascromatografica, valutando la composizione degli idrocarburi nel substrato prima e dopo il trattamento con il consorzio. Il consorzio si è dimostrato molto efficiente nella degradazione del substrato, utilizzando tutti gli idrocarburi disponibili entro una settimana. Infine, sono stati testati metodi di conservazione del consorzio al fine di individuare quello ottimale per conservare e trasportare il consorzio su lunghe distanze. La crioconservazione è risultata ottimale per la conservazione mentre liofilizzazione il metodo più adatto per il trasporto.

Il capitolo 6 riporta la parte sperimentale eseguita nel terzo e ultimo anno del mio dottorato, svoltosi soprattutto con le difficoltà poste dalla pandemia COVID-19, che ci ha costretto a modificare i piani di lavoro che avevamo previsto per quest'ultimo anno. Non potendo dimostrare l'efficacia del consorzio con prove sul campo, il potenziale del consorzio è stato confermato attraverso un test ecotossicologico.

Nel capitolo 7, sono riportate le conclusioni generali sui risultati ottenuti e le prospettive future. Indubbiamente, sono necessarie ulteriori indagini per ottimizzare le prestazioni dei microrganismi selezionati e completare le analisi con test sul campo. Tuttavia, questo lavoro dimostra come l'isolamento di ceppi microbici autoctoni di substrati contaminati e il loro utilizzo per sviluppare un consorzio sia

una metodologia efficace per la degradazione di miscele di idrocarburi complessi. Evidenzia anche come la collaborazione tra diversi ricercatori con diversa formazione ed esperienza, specializzati in vari campi scientifici, sia essenziale per trovare metodologie sempre più verdi e a basso impatto ambientale.

Aims of the project

This PhD research aimed to develop a fungal-bacterial consortium to clean petroleum industry machinery and bioremediate extraction well sites. In order to fulfil the main goal, the project was planned for three years (2017-2020) and structured to achieve the following objectives:

- i. reviewing the existing literature on hydrocarbon microbial degradation;
- ii. isolating and selecting microbial strains useful to build the consortium;
- iii. evaluating the enzymes production and the hydrocarbon-degrading capacity of selected fungal strains;
- iv. assembling a fungal-bacteria consortium and inquiring about its efficacy;
- v. disseminating the results through the publication of scientific papers and by participating in international congresses.

Chapter 1: Introduction

This chapter presents state of the art on petroleum hydrocarbons, the problems related to their release in the environment, and the available bioremediation technologies. Firstly, I describe hydrocarbons, their toxicity and the problems arising from their processing in the oil industry. Then, I review the principal aspects of hydrocarbons remediation techniques, with a major focus on bioremediation and mycoremediation, i.e. the use of yeasts and pluricellular fungi in bioremediation processes

1.1 Petroleum hydrocarbons and their toxicity on living beings

1.1.1 General characteristics of petroleum hydrocarbons

The term "hydrocarbons" denotes any class of organic compounds composed only of carbon (C) and hydrogen (H). The carbon atoms come together to form the backbone of the compound, and the hydrogen atoms attach to them in many different configurations. Depending on the type of configuration the chemical structure takes, they are classified as aliphatics and aromatics (Fig. 1.1) (source: IUPAC).

Aliphatic hydrocarbons are divided into three main groups according to the types of bonds they contain: alkanes, alkenes, and alkynes. Alkanes have only single bonds, alkenes contain carbon-

carbon double bonds and alkynes contain carbon-carbon triple bonds (Fig. 1.2). Aliphatics can also be straight or branched, cyclic or acyclic (Petrov, 2012).

Aromatic hydrocarbons, or arenes, contain a benzene ring: a ring of six carbons with alternating double and single bonds that impart different and unique properties to a molecule, including stability. These hydrocarbons can be monocyclic or polycyclic (PAHs) (Fig. 1.2), depending on the number of benzene rings in their chemical structure (Carey, 2020).

Petroleum is a complex mixture of hydrocarbons, some organo-metallo constituents, and usually nitrogen, sulfur and oxygen in some amount (Chandra *et al.*, 2013; Lamichhane *et al.*, 2016; Varjani, 2017). It is produced by the thermal decay of buried organic material over millions of years, and petroleum-derived products are the primary energy source for industry and our daily lives. Petroleum hydrocarbons are categorized into four fractions: (a) alkanes (mostly in the range C₁-C₄₀) that represent the highest percentage; (b) aromatics, including compounds such as benzene, toluene, ethylbenzene and xylenes (BTEX) and PAHs; (c) resins, consisting of compounds having nitrogen, sulphur, and oxygen dissolved in oil; (d) asphaltenes, large and complex molecules that are colloiddally dispersed in crude oil (Abdel-Shafy and Mansour, 2016; Varjani, 2017; Mikhailenko and Baaj, 2019).

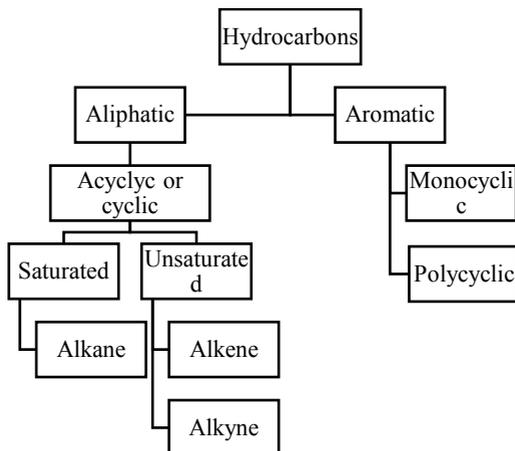


Figure 1.1. Hydrocarbons classification based on chemical structure

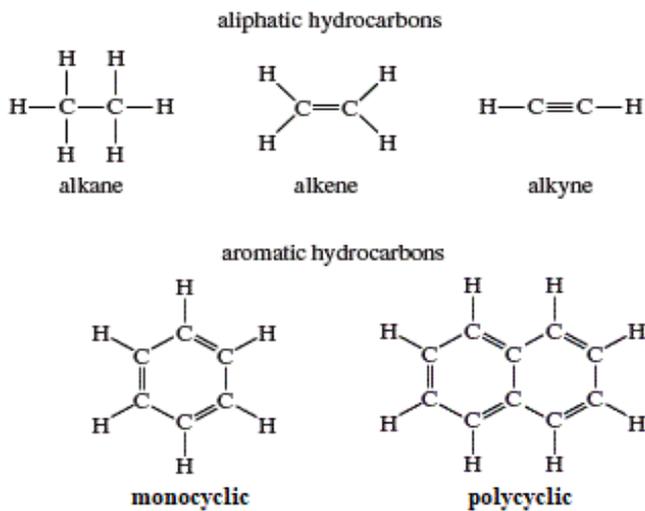


Figure 1.2. Structures of representative hydrocarbons.

1.1.2 Petroleum hydrocarbons toxicity on animals

Many petroleum constituents are recalcitrant and highly toxic for living organisms and the environment (Chandra *et al.*, 2013; Souza *et al.*, 2014; Meckenstock *et al.*, 2016). BTEX and PAHs cause the major problems, as some are carcinogenic (the tendency to induce cancer), genotoxic (the ability to damage the genetic information of an organism), cytotoxic (capacity to provoke cell damage) or ecotoxic (harmful effects for the ecosystems in general) (Anyakora, 2007).

Animals (included human beings) are exposed to pollutants mainly through three routes (a) dermal (by skin contact), (b) inhalation (through respiration), (c) ingestion (eating contaminated food) (Kim *et al.*, 2013; Yang *et al.*, 2015; Zafra *et al.*, 2015). The primary effects of exposure to petroleum hydrocarbons are central nervous system (CNS) depression and polyneuropathy.

Monoaromatic hydrocarbons and PAHs have less solubility in water and are highly lipophilic, and due to this property, they can cause damages to internal organs, particularly adipose tissues. Moreover, long-term exposure to PAHs has the potential to induce malignant skin tumours as PAHs have a high affinity for the nucleophilic centre of macromolecules like RNA, protein, and DNA (Costa *et al.*, 2012; Desforges *et al.*, 2016). Some aromatic hydrocarbons are also known to be teratogenic toxic compounds (Perera *et al.*, 2005; Khairy *et al.*, 2009; Aramandla *et al.*, 2010). Teratogenicity is known as malformation in developing an embryo or

fetus (Perera *et al.*, 2005). A high level of benzo[a]pyrene is responsible for congenital disability and decreased body weight during the progeny (Perera *et al.*, 2005), and if PAHs enter the body during pregnancy, they can cause low birth weight, premature delivery, mentally abnormal child and heart malformations (Ng *et al.*, 2009; Aramandla *et al.*, 2010). Aromatic hydrocarbons can also manifest acute effects such as vomiting, eye irritation, nausea, and diarrhoea (Ng *et al.*, 2009; Varjani, 2017).

Concerning BTEX compounds, the carcinogenic and immunologic effects of benzene and its isomers are well known. For instance, trimethyl benzene may cause nervousness, tension, and anxiety (Davidson *et al.*, 2020). Chronic exposure has also been related to respiratory problems, such as asthma and lung infections, and leukaemia in children and adults (Gordian *et al.*, 2010; Heinrich, 2011; Chandra *et al.*, 2013). Toluene, easily found in gasoline, nail polish, spray paint, and other everyday products, can cause headache, nausea and drowsiness cardiac arrhythmia (Vitale and Gutovitz, 2018). Xylene is a compound with great value in the research and chemical industry. However, its vapour is absorbed rapidly via the lungs, and the exposure to high concentration has narcotic effects on the human body, leading eventually to neuropsychological dysfunction with respiratory tract impairment (Joshi and Adhikari, 2019).

Other compounds, such as *n*-hexane, *n*-heptane and cyclohexane, induce peripheral nerve damage, narcosis and irritation of the eyes and mucous membranes after repeated and prolonged exposure (Valentini *et al.*, 1994; Sendur *et al.*, 2009; Joshi and Adhikari, 2019). For all these negative effects, the International Agency for Research on Cancer (IARC) has classified benzo[a]pyrene as carcinogenic to humans (Group 1), cyclopenta[cd]pyrene, dibenz[a,h]anthracene, and dibenzo[a,l]pyrene, as probably carcinogenic to humans (Group 2A), and benz[j]aceanthrylene, benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, benzo[c]phenanthrene, chrysene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, indeno[1,2,3-*cd*]pyrene, and 5-methylchrysene, as possibly carcinogenic to humans (Group 2B) (IARC, 2020).

Concerning environmental consequences of hydrocarbons release, aquatic and marine organisms are the most affected by the presence of oil in their habitats. Some PAHs are extremely toxic for these organisms: long-term impacts include developmental abnormalities of sea animals such as jaw reductions, lack of pigmentation and unfused skulls (Van Meter *et al.*, 2006; Alonso-Alvarez *et al.*, 2007; Varjani, 2014).

Several studies on wild fish have connected the occurrence of hepatic neoplasms and neoplasia-related toxicopathic liver lesions to PAH exposure (Collier *et al.*, 2013), while Vignet *et al.* (2016) found

that PAHs might disrupt fish reproduction. Moreover, the heavier fractions of hydrocarbons can accumulate in sediments, affecting benthic organisms there living, causing sub-lethal to lethal effects (Khan *et al.*, 2005; Du *et al.*, 2012). The major risk of petroleum contamination in the marine environment is bioaccumulation. Evidence exists for the preferential accumulation of hydrocarbons in the organs of marine vertebrate and invertebrates, such as mussels, oysters, and fish, with impact on their health status and fitness (Scarlett *et al.*, 2007; Inam *et al.*, 2012; Mojiri *et al.*, 2019). This phenomenon is facilitated by the high lipophilicity of certain hydrocarbons and their resistance to metabolic break-down, making them able to penetrate and accumulate in the fatty tissues (Mackay and Fraser, 2000; Macdonald *et al.*, 2002). Marine mammals, among which many protected species, are particularly vulnerable to the bioaccumulation of hydrocarbons pollutants (Abdel-Shafy and Mansour, 2016; Yu *et al.*, 2019).

1.1.3 Petroleum hydrocarbons toxicity on plants and algae

The phytotoxicity of petroleum and its derivatives has been known for decades (Currier and Peoples, 1954). The phytotoxicity effect of petroleum in the aquatic environment has already been reported for microalgae and macroalgae species, seagrasses, wetland plants, and mangroves since the early 1970s (Straughan, 1970; Cooper and Wilhm, 1975). The information showed that exposure to oil causes

a depressive effect on algal photosynthesis probably due to the alterations in light intensity and quality below the surface oil layer (Wilbur, 1969; Soares *et al.*, 2017). Affected organisms also underwent significant improvements in quality and abundance following contamination abatement (Perkins, 1968; Lewis and Pryor, 2013). This is probably because the oil acts as a selective agent, selecting the organisms best adapted to the contaminated environment and preventing the survival of uncompetitive ones (O'Brien and Dixon, 1976).

Direct effects of contamination comprise coating and suffocation: oil form a physical barrier coating roots and leaf and making them more difficult to absorb nutrients and perform photosynthesis (Ahmed *et al.*, 2018; Odukoya *et al.*, 2019). In addition, oil limits the plants' growth in height and leaf area as it decreases the availability of essential nutrients such as nitrogen and oxygen (Odukoya *et al.*, 2019). Oil can also cause various sublethal effects on enzyme systems, photosynthesis, respiration, protein, and nucleic acid synthesis (Lewis and Pryor, 2017). Furthermore, seed germination of several species is negatively affected by volatile and hydrophobic aromatics like benzene, indene, naphthalene, styrene, toluene, and xylene isomers (Henner *et al.*, 1999).

When oil spillage occurs in a terrestrial environment, hydrocarbons occupy air-filled pore spaces. This phenomenon impacts oxygen transfer, causing oxidative stress. Oxidative stress

causes considerable metabolic changes in plant tissues, as plants can produce high concentrations of reactive oxygen species (ROS), such as superoxide (O_2^-), peroxide (H_2O_2), hydroxyl radicle ($*OH$), and singlet oxygen ($^1[O_2]$), which cause cell damage. Moreover, several PAHs and their derivatives are known to bioaccumulate and produce ROS in plant tissues. The extent of plant cell's response to oxidative stress is usually evaluated based on the concentration of H_2O_2 , cellular DNA damage, and enzyme activities (Chen *et al.*, 2013; Kuppusamy *et al.*, 2020). This same effect can occur in aquatic environments when the oil floats less dense than water to form a thin surface film (Lewis and Pryor, 2013).

1.2 Environmental impact of petroleum hydrocarbons

1.2.1 Introduction routes of petroleum hydrocarbons in the environment

Environmental contamination by petroleum hydrocarbons is a major concern in the contemporary world. It has disastrous consequences for humans and for the biotic components of the ecosystems. In the last decades, there has been a growing public awareness and concerns about the effects of petroleum hydrocarbons releasing into the environment.

Tanker oil spillage and oil spill accidents have always been the primary cause of petroleum hydrocarbons environmental pollution.

The International Tanker Owners Pollution Federation Limited (ITOPF) maintains a database of oil spills from tank vessels containing information on accidental spillages of hydrocarbon oil since 1970. Data reported how the number of large spills (>700 tonnes) decreased significantly over the last few decades (Fig. 1.3). The yearly average recorded in the 2010s was 1,8 spills, less than 1/10 of the average recorded in the 1970s. Furthermore, before the 1990s, the oil spillage accidents were more frequent and severe while, after 1990, this number remained relatively low and falling year by year. For the year 2020, fortunately, zero large spills (>700 tonnes) were recorded, but three medium spills (7-700 tonnes) occurred. Although the data is an improvement on previous decades, much still needs to be done to ensure that these accidents do not happen again. The causes of large spills were various, but 50% occurred while the vessels were underway in open water due to collisions and groundings. If the vessels underway in inland or restricted waters, these same causes account for 99% of spills (Fig. 1.4).

Certainly, oil spills are the most significant and severe source of petroleum hydrocarbons in the environment. However, hydrocarbons can enter the environments through several other routes: spillages and leakage from underground tanks, unplugging of oil wells, abandoned oil refinery sites, but also fuel combustion, pyrolytic processes, waste incinerators, domestic heaters and industry (Souza *et al.*, 2014; Varjani and Upasani 2017; Kuppusamy *et al.*, 2020).

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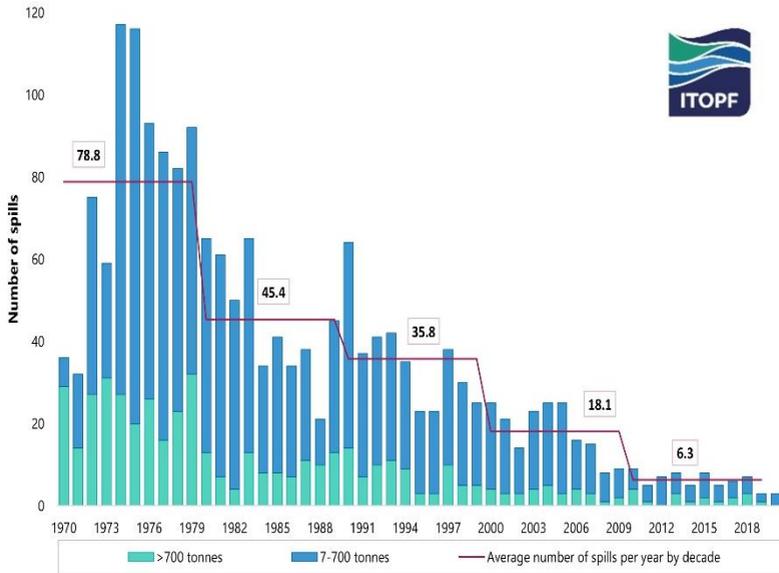


Figure 1.3. Number of spills (>7 tonnes) from 1970 to 2020 (itopf.org).

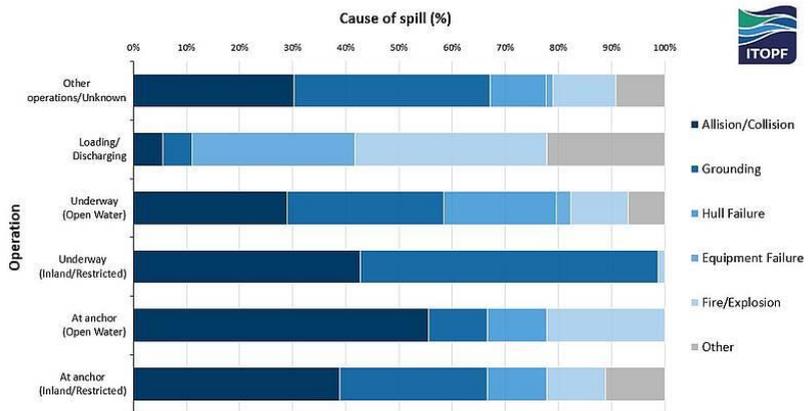


Figure 1.4. Primary causes of large spills from 1970 to 2020 (itopf.org).

1.2.2 Influence of petroleum hydrocarbons on microbiomes

The microbiome represents all the microbial population and communities present in a particular environment that are directly or indirectly related to its functions (Teng and Chen, 2019). Environmental factors and soil conditioning, (i.e. soil moisture, soil compositions, *etc.*) directly influence microbial community structure and activities. Similarly, also, the presence of hydrocarbon contaminants influences microbiomes. Changes in the microbial population largely depend on the hydrocarbon concentration, the residence time in the soil, and the soil properties (Lauber *et al.*, 2009). For example, it has been shown that microbial structure is altered in aged contaminated soil compared to freshly contaminated soil, since the presence of hydrocarbons alters the fungal and bacterial communities (Abbasian *et al.*, 2015, Mustafa *et al.*, 2016; Galazka *et al.*, 2018). Schwarz and coauthors (2019) reported how, in sandy soils, the most contaminated test pit had high fungal diversity, with a dominance of *Aspergillus* and Eurotiales species, taxa already known for their hydrocarbon-degrading abilities. Yakimov *et al.* (2007) and Brooijmans *et al.* (2009) showed that bacterial genera *Oleispira*, *Marinobacter*, *Thalassolituus*, *Alcanivorax*, and *Cycloclasticus* were present in the test soil at low or undetectable levels before pollution but were found to dominate in oil-polluted sites. Galitskaya *et al.* (2021) also demonstrated that soils with high petroleum contents (6 and 25%) led to an increase of hydrocarbon-degrading bacteria. A

similar trend was observed for fungi capable of decomposing lignin and cellulose, thus suitable for the degradation of hydrocarbons, as will be shown in section 1.6.

It is clear from these studies that hydrocarbons act as selective agents. When soils are exposed to contaminants, the indigenous microbiome is influenced in composition and diversity, and microorganisms are selected so that only the most suitable species can survive (Galazka *et al.*, 2018; Teng and Chen, 2019).

1.3 The oil industry-related issues

In 2019 the oil industry produced 95,192 thousand of barrel per day of petroleum products (source: BP.com). In the last ten years, the production trend has never stopped growing, and although the value had slightly decreased in 2019, this shows how much our lives still depend on fossil resources (Fig. 1.5). The world's largest oil producer has always been the Middle East. However, in recent years, North America has been increasing its production by thousands of barrels per day, from over 22,000 in 2019 to almost 25,000 in 2020 (Fig. 1.6).

The petroleum industries can be divided into two components: the upstream industry (extraction and production) and the downstream industry (refining and marketing). Each of these stages poses risks to the environment and workers, but the refining and distribution processes are more prone to accidental oil spills during operation and maintenance (Verma *et al.*, 2001). Drilling, the process in which a hole

is made on the earth to allow subsurface hydrocarbons to flow to the surface, is also a critical step. The primary way drilling activities can impact the environment is through the drill cuttings and the drilling fluid used to lift the cuttings from the well. Secondary impacts can occur due to the air emissions from the internal combustion engines used to power the drilling rig (Pathak and Mandalia, 2012).

Oily wastes are one of the most significant solid wastes generated in the oil industry. It is a complex emulsion of various petroleum hydrocarbons, water, heavy metals and solid particles. A considerable amount of this waste is generated by the oil industry during all operation phases (Xu *et al.* 2009). Generally, a higher refining capacity is associated with a larger amount of oily sludge production. It has been estimated that one ton of oily sludge waste is generated for every 500 tons of crude oil processed (Tahhan *et al.*, 2011; de Silva *et al.*, 2012). Oily wastes and sludges accumulated in tanks gained much attention, especially because they need to be cleaned periodically, usually by manual mean. The procedure is dangerous, time-consuming, laborious and expensive. In addition, the substances resulting from cleaning must be specifically eliminated because they are high in hazardous substances.

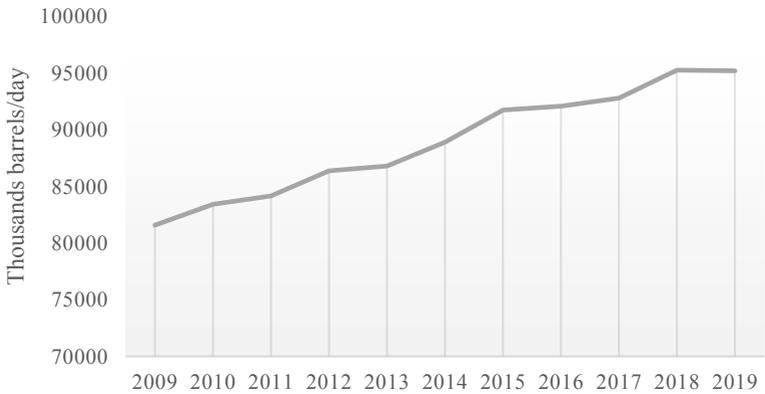


Figure 1.5. Petroleum production trend in years 2009-2019, expressed as thousands of barrels per day (source: BP.com).

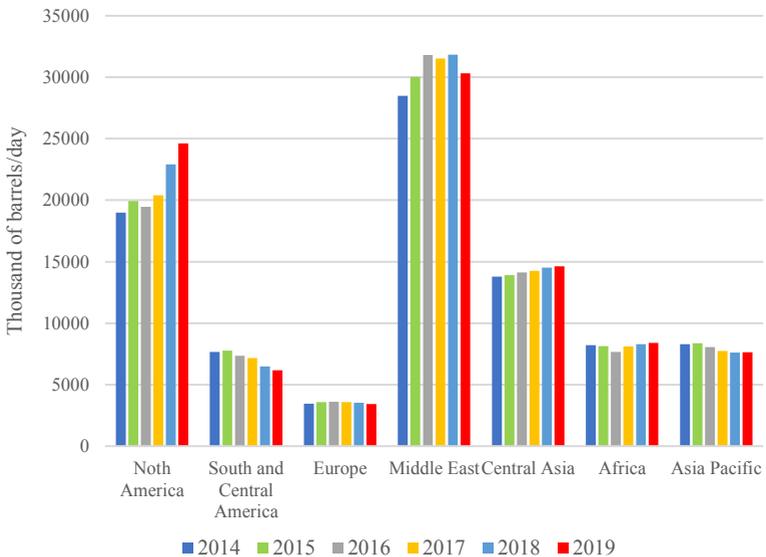


Figure 1.6. Petroleum products produced in the different world's area in the years 2014-2019 (thousands of barrel /day) (source: BP.com).

1.4 Conventional treatments and bioremediation processes for hydrocarbons remediation

The removal of petroleum hydrocarbons from the environment can be performed through physical, chemical, and biological processes (Das and Chandran, 2011; Cole, 2018). The treatment choice depends on the nature of the contaminant, the area to be treated, and the action time available. Nowadays, biological degradation processes are generally preferred to conventional treatment because they are environmentally friendly, cost-effective, and simple to apply (Gadd, 2001; Chandra *et al.*, 2013; Varjiani, 2017). Here, an overview of the treatment actually most employed is presented.

1.4.1 Conventional treatments

Conventional physical-chemical treatments can be performed *in-situ* or *ex-situ*, depending on the type of contaminant, the contamination severity, and the type of substrate to be treated.

In-situ treatment technologies consist of treating the soil directly on-site without removing it. *In-situ* treatments consist of processes like advection (the transport of chemical constituents by groundwater movement, with evident criticalities regarding environmental sustainability), adsorption (process allowing the separation of organic compounds through chemical bonding with inorganic compounds) and volatilisation (the process by which a

volatile compound passes from a liquid/solid-state into a vapour/gaseous phase) (Chandra *et al.*, 2013; Varjani and Srivastava, 2015).

Ex-situ technologies consist of removing the contaminated substrate from its original site and treat it in mobile, semi-mobile or portable systems near the contaminated site (on-site treatment) or in permanent systems in another location (off-site treatment). There are different types of *ex-situ* treatments such as chemical inactivation (the use of potassium permanganate and/or hydrogen peroxide as a chemical oxidant to mineralize non-aqueous contaminants such as petroleum), the incineration of the contaminated substrate and soil washing (Chaudhry *et al.*, 2005; Farhadian *et al.*, 2008; Varjani and Srivastava, 2015). Soil washing is one of the most used techniques because it can be used to treat a wide range of contaminants. It is a water-based process for scrubbing soils to remove contaminants in two ways: (i) by dissolving or suspending the contaminants in a wash solution, or (ii) concentrating them into a smaller volume and remove it (fr.gov). These methods are expensive due to the cost of excavation and transport of large quantities of contaminated materials (Singh *et al.*, 2017; Cole, 2018). In addition, the water used for treatment must be specifically discharged or remediate and the risk of accidental groundwater contamination remains high.

Overall, despite their effectiveness, both *in-situ* and *ex-situ* treatments have disadvantages that cannot be ignored. They often

require chemicals in the operational phases, thus introducing more potentially hazardous substances into an already contaminated environment. Besides, incomplete degradation of oil compounds can often occur, forming and releasing into the environment substances that may be even more harmful than the original pollutant. The criticality of conventional treatments and increased public awareness has encouraged alternative, less expensive, energy-efficient, and green biological technologies.

1.4.2 Bioremediation

Bioremediation is a process in which metabolic and enzymatic capabilities of microorganisms, algae, and plants are employed to detoxify or mineralise pollutants, ultimately leading to their degradation (Varjani and Upasani, 2013; Ron and Rosenberg, 2014; Wilkes *et al.*, 2016; Sharma *et al.*, 2018). Bioremediation techniques can be applied on a wide variety of xenobiotic compounds (insecticides, pesticides, dyes, heavy metals, plastics and hydrocarbon), and they are preferred to conventional methods because are eco-friendly, cost-effective, simple to apply, and socially acceptable (Varjani, 2017; Vikrant *et al.*, 2018; Verma and Kuila, 2019). Despite these positive aspects, bioremediation processes have some critical issues, such as the need to adapt the process e to the

individual site to be treated), that need to be taken into consideration. The main pros and cons are listed in Table 1.1.

The most promising bioremediation processes are the use of microorganism (especially fungi and bacteria) because they are ubiquitous, can grow in a wide range of environmental conditions and possess sets of enzymes that can act on various substrates (Srivastava *et al.*, 2014; Abatenh *et al.*, 2017; Singh *et al.*, 2017). As for the conventional treatments, bioremediation through microorganisms can be classified into two types: *in-situ* bioremediation and *ex-situ* bioremediation (Marykensa, 2011).

Ex-situ bioremediation involves treating contaminated soils or water once removed from its initial site (Megharaj *et al.*, 2014). This can be done using two methods; slurry-phase and solid-phase (Aislabie *et al.*, 2006). In slurry-phase, bioremediation, water and other reagents are mixed within the contaminated soil in a bioreactor that provides the ideal growth conditions for microorganisms. Nutrients and oxygen can be added to the system to improve the degradation rate. Once the process is ended, water is separated, and the soil is replaced. Solid-phase bioremediation involves the use of an above-ground treatment site to treat contaminated soil. The treatment site is equipped with an aeration system, and all the physical-chemical parameters (nutrients, oxygen, pH, moisture, and heat) need to be controlled to maximize the bioremediation efficiency. The methods used with solid-phase bioremediation are land farming, windrows, and

soil bio piles (Van Deuren *et al.*, 2002). Land-farming is a methodology in which the contaminated soils are mixed with soil amendments (soil bulking agents and nutrients) and then tilled into the earth. The material is periodically aerated so that the contaminants could be degraded, transformed, and immobilized by microbiological processes (Sharma, 2020). Windrows rely on the periodic turning of piled polluted soil to enhance bioremediation by increasing degradation activities of indigenous microorganisms present in polluted soil (Sharma, 2020). Finally, a bio-pile is a technology in which excavated soils are mixed with soil amendments, formed into compost piles, and enclosed for treatment (Sharma, 2020).

In-situ bioremediation is the treatment of polluted substrate at the site where it was present, reducing costs and without the need to remove the soil (Sharma *et al.*, 2018). Different bioremediation techniques can be performed *in-situ*. When bioremediation occurs on its own, it is termed “natural attenuation”; however, when nutrients are added to stimulate microbial action, the process is referred to as “biostimulation”. When specific microorganisms are introduced to treat a pollutant, the process is termed “bioaugmentation” (Abatenh *et al.*, 2017; Singh *et al.*, 2017).

Many researchers have reported successful biostimulation processes with the addition of the appropriate nutrients that improves the metabolic activity of indigenous microorganisms (Yu *et al.*, 2005; Suja *et al.*, 2014; Smith *et al.*, 2015). On the other hand, autochthonous

bioaugmentation, based on the re-inoculation in polluted sites of indigenous microorganisms previously enriched under laboratory conditions, enhances microbial activities and improves the degradation rate (Dueholm *et al.*, 2015). For this method to be effective, it is essential to isolate the microorganisms directly from the contaminated environment (D'Annibale *et al.*, 2006; Spina *et al.*, 2018).

Due to the complexity of some hydrocarbon pollutants, a microbial consortium composed of microorganisms (e.g. fungi-fungi, fungi-bacteria) with different metabolic capacities is more efficient in hydrocarbon degradation than a pure culture (Das and Chandran, 2011; Varjani *et al.*, 2013; Priya *et al.*, 2015; Ebadi *et al.*, 2017). More information about the use of consortia in bioremediation is reported in the Introduction of Chapter 5.

Table 1.1. Advantages and disadvantages of bioremediation techniques.

| Advantages | Disadvantages |
|--|---|
| Natural process | Highly specific |
| Cost effective | Research is needed |
| Eco-friendly and sustainable | Often takes longer than conventional treatment |
| Applicable on a wide range of contaminants | Limited to those compounds that are biodegradable |
| The residues are usually harmless product | The residues may be more persistent |
| Can be carried out on site | Regulatory uncertainty |
| Nonintrusive | |
| Not use any dangerous chemicals | |

1.5 Bacterial bioremediation of hydrocarbon

Bacteria are widely distributed in water (fresh/marine), soil, and air and are reported among the primary degraders and the most active biodegradation agents (Varjani *et al.*, 2013). Since microbial communities of polluted areas adapt to their environment, it is not surprising that bacteria isolated from polluted hydrocarbon substrates can often utilise/degrade them (Batista *et al.*, 2006; Ron and Rosenberg, 2014; Varjani and Upasani, 2016b). The degradation of

petroleum hydrocarbon pollutants by bacteria has been well-documented and several metabolic pathways have been elucidated (Hendrickx *et al.*, 2006; Abbasian *et al.*, 2015; Meckenstock *et al.*, 2016; Wilkes *et al.*, 2016). In table 1.2 are reported the most common bacterial species able to degrade hydrocarbons.

Hydrocarbon catabolism has long been considered as a strictly aerobic process; however certain microorganisms are reported for their anaerobic degradation (Abbasian *et al.*, 2015; Meckenstock *et al.*, 2016). Various reactions (oxidation, reduction, hydroxylation, and dehydrogenation) are common for both aerobic and anaerobic pathways of microbial degradation petroleum hydrocarbon pollutants (Abbasian *et al.*, 2015; Wilkes *et al.*, 2016).

Table 1.2. Most common hydrocarbon-degrading bacteria.

| Hydrocarbon compound | Bacteria species | References |
|----------------------|---------------------------|--|
| Aliphatics | <i>Acinetobacter</i> sp. | Foght (2008) Mittal and Singh (2009) |
| | <i>Alcanivorax</i> sp. | Harayama <i>et al.</i> (2004) Brooijmans <i>et al.</i> (2009) |
| | <i>Bacillus</i> sp. | Ghazali <i>et al.</i> (2004) Das and Mukherjee (2007) Singh <i>et al.</i> (2013) |
| | <i>Brevibacterium</i> sp. | Leahy and Colwell (1990) Chaillan <i>et al.</i> (2004) |
| | <i>Micrococcus</i> sp. | Roy <i>et al.</i> (2002) Ghazali <i>et al.</i> (2004) |
| | <i>Oleispira</i> sp. | Harayama <i>et al.</i> (2004) Brooijmans <i>et al.</i> (2009) |

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| | Mittal and Singh (2009) Rocha <i>et al.</i> (2011) Singh <i>et al.</i> (2013) Sajna <i>et al.</i> (2015) Varjani <i>et al.</i> (2015) Varjani and Upasani (2016a,b) |
| | Varjani <i>et al.</i> (2015) Kumari <i>et al.</i> (2018) |
| | Batista <i>et al.</i> (2006) Simarro <i>et al.</i> (2013) |
| | Janbandhu and Fulekar (2011) Ghevariya <i>et al.</i> (2011) Nzila <i>et al.</i> (2018) |
| | Mittal and Singh (2009) Janbandhu and Fulekar (2011) Zafra <i>et al.</i> (2016) |
| | Sheng <i>et al.</i> (2009) Simarro <i>et al.</i> (2013) |
| Aromatics | Widdel and Rabus (2001) Mittal and Singh (2009) Simarro <i>et al.</i> (2013) Meckenstock <i>et al.</i> (2016) Zafra <i>et al.</i> (2016) Kumari <i>et al.</i> (2018) |
| | Leahy and Colwell (1990) Salleh <i>et al.</i> (2003) |
| | Urszula <i>et al.</i> (2009) Kumari <i>et al.</i> (2018) |
| | Widdel and Rabus (2001) Hedlund and Staley (2001) Smith <i>et al.</i> (2012) |

1.5.1 Aerobic hydrocarbon biodegradation by bacteria

The fastest and most complete degradation of organic pollutants occurs under aerobic conditions, because of the metabolic advantage of having the availability of O₂ as an electron acceptor. In fact, the first step of hydrocarbons biodegradation is an intracellular oxidative process catalyzed by oxygenases and peroxidases (Das and Chandran, 2011). Several peripheral degradation pathways have been proposed based on oxidative reactions that convert hydrocarbons step by step into intermediates of the central intermediary metabolism: terminal oxidation, sub-terminal oxidation, ω -oxidation and β -oxidation (Das and Chandran, 2011; Abbasian *et al.*, 2015). The proposed steps of the hydrocarbon degradative process are highlighted in Fig. 1.6.

The aromatic hydrocarbons are less biodegradable than saturated hydrocarbons due to the benzene ring. The initial oxidative attack and subsequent cleavage of the benzene ring are the key steps in the degradation of aromatic hydrocarbon pollutants (Hendrickx *et al.*, 2006). The benzene ring is cleaved in different ways by appropriate enzymes (Li and Liu, 2002): intradiol dioxygenases catalyse *ortho*- cleavage, *meta*- cleavage is catalysed by extradiol dioxygenases. Polyaromatic hydrocarbons are degraded in the same way, one ring at a time.

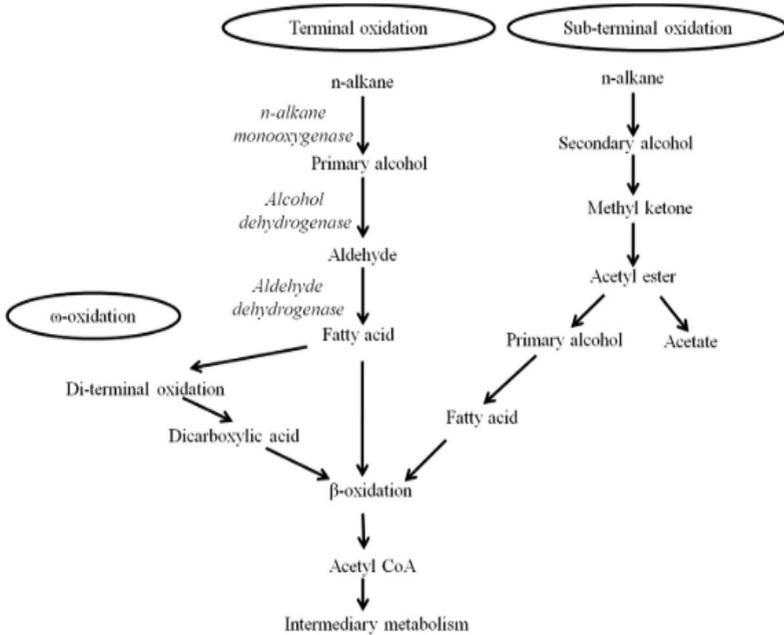


Figure 1.6. Proposed peripheral pathways for *n*-alkane aerobic degradation in bacteria (Varjani, 2017)

1.5.2 Anaerobic hydrocarbons biodegradation

Less information is available about anaerobic degradation because its understanding is recent (Jaekel *et al.*, 2013; Meckenstock *et al.*, 2016; Wilkes *et al.*, 2016). For this reason, it has not yet been possible to define an indicative pathway of this type of degradation. To date, only examples related to the specific anaerobic degradation of certain compounds are available. For example, Wilkes *et al.* (2016)

proposed two biochemical mechanisms, the addition of fumarate and carboxylation, as the initial degradation of alkanes. This because bacteria must have developed oxygen-independent reactions for the initial attack of hydrocarbons. Under anaerobic metabolism, some aromatic compounds are first oxidised to phenols or organic acids, then transformed to long-chain volatile fatty acids, which are finally metabolised to CH₄ and CO₂ (Abbasian *et al.*, 2015; Wilkes *et al.*, 2016).

1.6 Fungal bioremediation of hydrocarbons

The kingdom Fungi comprises eukaryotes with remarkably diverse life histories that make essential contributions to the biosphere, human industry, medicine, and research. Fungi can be single-celled or pluricellular filamentous organisms. They are found in just about any habitat on Earth, but mostly living on soil or plant material (Spatafora *et al.*, 2017; Stajich *et al.*, 2019).

A large group act as saprotrophs, decomposing dead matter and playing an important role, together with bacteria, in biogeochemical cycling. Some are parasitic symbionts of plants causing diseases such as mildews, rusts, scabs or canker and some cause diseases in animals (Carlile *et al.*, 2001). Others are mutualistic representing important symbiosis with plants (mycorrhizae), algae and cyanobacteria (lichens), and with animals (*e.g.* herbivorous, insects).

Fungi are ecologically subdivided based on their life cycles and morphologically based on filamentous or unicellular tallus, the presence and structure of their fruiting body or conidiophores, arrangement, and type of sexual or asexual cells for reproduction (spores or conidia). Digestive esoenzymes are secreted by the cells for breaking down the organic matter into smaller molecules used by the fungus as a nutrient source (Carlile *et al.*, 2001).

Avoiding going into the details of the taxonomic organization fungi can be grossly distinguished in:

- microscopic filamentous fungi (Fig. 1.6A) (popularly called mould) made up of threads (hyphae) that grow at the tip cell that repeatedly creating long and branching chains. The hyphae keep growing and intertwining until they form a network of threads called mycelium (Stajich *et al.*, 2019).
- Macroscopic filamentous fungi (Fig. 1.6B), that can form large fruiting bodies (popularly called mushrooms) holding the sexual spores. The fruiting body comprises tightly packed hyphae usually divided in cap (pileus), stalk, hymenium (Spatafora *et al.*, 2017).
- Single-celled fungi called yeasts (Fig. 1.6C) showing sexual or asexual reproduction. Due to their ease of cultivation and growth speed, yeasts are among the most widely used model organisms for genetic studies. Some yeasts, such as *Saccharomyces*, play an important role in bread production

and alcoholic beverages; other genera (e.g. *Candida* sp.) are important pathogens (Carlile *et al.*, 2001).

The use of fungi to remediate polluted sites, the so-called mycoremediation, is considered a frontier in remediation technologies. They act as pioneer species for the renaturalization of impacted environments and as fundamental degraders of harmful compounds in polluted sites. Indeed, fungi can colonize and exploit very complex and toxic substrates, including crude oil and its by-product. Fungi can withstand even extreme conditions, exploiting substrate characterized by low moisture and low nutrient concentrations (Tiquia-Arashiro and Grube, 2019). Furthermore, fungi are often favoured by acidic pH and contribute to further acidification by secreting organic acids (Ruijter *et al.*, 2002; Mancera-López *et al.*, 2008). The outstanding potential of fungi to remediate toxic and recalcitrant compounds lays especially in their peculiar trophic mode: while hyphae physically penetrate the substrate, reaching inaccessible interstices, they release powerful enzymes that break down complex polymers into molecules more accessible to uptake. Thanks to apical hyphal growth, fungi can penetrate the soil matrix more efficiently than bacteria (Dix and Webster, 1995). Compared to bacteria, filamentous fungi show some advantages in the transport or translocation of essential substances, including nutrients and water, and the pollutant itself, over significant

distances (Harms *et al.* 2011; Furuno *et al.* 2012; Worrich *et al.* 2018). Furthermore, fungal mycelia can act as “highways”, facilitating the transport of pollutant-degrading bacteria over distance in soil, thus enhancing bioremediation (Kohlmeier *et al.* 2005; Banitz *et al.* 2013). According to the literature, the degradation of petroleum hydrocarbons is mainly performed by Basidiomycota and Ascomycota, and less to Mucoromycota or other phyla (Hatami *et al.*, 2018; Prenafeta-Boldù *et al.*, 2019).

Hydrocarbon degradation is strictly related to lignocellulolytic activity (Ghosal *et al.*, 2016). Fungi can degrade lignin are mainly known as white-rot fungi; when the lignin is degraded, the cellulose component remains, and the wood appears white. (Deshmukh *et al.*, 2016). The extracellular ligninolytic enzyme system has been directly linked to the biodegradation of petroleum hydrocarbons, especially PAHs (Lang *et al.*, 1998; Wang *et al.*, 2009). More information on ligninolytic enzymes is reported in paragraph 1.6.2, but many authors have demonstrated the fungal action on hydrocarbons. For instance, the basidiomycetes *Pleurotus ostreatus* has been reported to degrade almost 80–95% of all PAHs present in contaminated soil in 80 days (Steffen *et al.*, 2007), and *Ganoderma lucidum* degrade 99.65% of 20 mg/L of phenanthrene and 99.58% of pyrene in mineral salt broth after 30 days of incubation at 27 °C (Agrawal *et al.*, 2018). Among Ascomycota, Covino *et al.* (2015) showed that strains of *Fusarium*, *Pseudallescheria*, *Penicillium*, and *Aspergillus* could degrade up to

79% aliphatic hydrocarbons in contaminated soils. Furthermore, *Candida tropicalis* and *Aspergillus clavatus* degrade up to 70% of PAH component in diesel, crude oil and engine oil in 16 days at 28° C (Mbachu *et al.*, 2016). As for Mucoromycota, few records are available. This is not surprising as they are known as “sugar fungi”: they are pioneer species able to utilise simple and ready to use sugars as a carbon source (Spatafora *et al.*, 2016). However, I have demonstrated the hydrocarbon-degrading ability of *Mucor irregularis* in a paper published in the journal *Microorganism* present the end of this chapter. This work opens interesting scenarios in this neglected phylum of fungi.

Other most reported fungal strains involved in hydrocarbon degradation are listed in table 1.3.

Table 1.3. Most common hydrocarbon-degrading fungi listed according to the taxonomic classification of Spatafora *et al.* (2017).

| Phylum | Fungal species | References |
|---------------|-------------------------|---|
| Basidiomycota | <i>Bjerkandera</i> sp. | Kotterman <i>et al.</i> (1998) Gramss <i>et al.</i> (1999) |
| | <i>Corioloopsis</i> sp. | Gómez <i>et al.</i> (2006) Cambria <i>et al.</i> (2008) |
| | <i>Pleurotus</i> sp. | Bernicchia (2005) Pozdnyakova <i>et al.</i> , (2006a,b) Bernicchia and Gorjon (2010) Drevinskas <i>et al.</i> (2016) |
| | <i>Trametes</i> sp. | Bernicchia and Gorjon (2010) |

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| | <i>Phanerochaete</i> sp. | Barclay <i>et al.</i> (1995) Song (1999) Hiratsuka <i>et al.</i> (2005) Wang <i>et al.</i> (2009) |
| | <i>Aspergillus</i> sp. | ZoBell (1946) Chaillan <i>et al.</i> (2004) Covino <i>et al.</i> (2015) Zafra <i>et al.</i> (2016) |
| | <i>Candida</i> sp. | Chaillan <i>et al.</i> (2004) Mbachu <i>et al.</i> (2016) Mikolasch <i>et al.</i> (2019) |
| | <i>Cladosporium</i> sp. | Jun <i>et al.</i> (1972) Weber <i>et al.</i> (1995) Potin <i>et al.</i> (2004) |
| | <i>Fusarium</i> sp. | Chaillan <i>et al.</i> (2004) Covino <i>et al.</i> (2015) |
| Ascomycota | <i>Penicillium</i> sp. | ZoBell (1946) Chaillan <i>et al.</i> (2004) Covino <i>et al.</i> (2015) Govarathanan <i>et al.</i> (2017) |
| | <i>Pseudallescheria</i> sp. | April <i>et al.</i> (1998) Covino <i>et al.</i> (2015) |
| | <i>Purpureocillium</i> sp. | Mikolasch <i>et al.</i> (2019) Benguenab and Chibani (2020) |
| | <i>Trichoderma</i> sp. | Zafra <i>et al.</i> (2016) Daccò <i>et al.</i> (2020) |
| | <i>Yarrowia</i> sp. | Chaillan <i>et al.</i> (2004) Hassanshahian <i>et al.</i> (2012) |
| Mucoromycota | <i>Mucor</i> sp. | Dan <i>et al.</i> (2006) Mikolajczyk <i>et al.</i> (2015) Asemoloye <i>et al.</i> (2020) |
| | <i>Rhizopus</i> sp. | Elshafie <i>et al.</i> (20017) Lotfinasabasl <i>et al.</i> (2012) |



Figure 1.6. A) Example of filamentous fungi. From the left: *Pseudallescheria boydii*, *Trichoderma harzianum*, *Fusarium solani*, *Fusarium chlamydosporum*.
B) Example of a macroscopic fungi (*Mycena* sp.) (source: acremar.it).
C) Example of a yeasts. From the left: *Candida* sp. and *Rhodotorula* sp.

1.6.1 Fungal degradation of aliphatic hydrocarbons

I have detailed this aspect in a review paper that has been published in the journal *International Biodeterioration and Biodegradation* and reported at the end of this chapter.

1.6.2 Fungal degradation of aromatic hydrocarbons

In recent years, the fungal biodegradation of polycyclic aromatic hydrocarbons (PAHs) has been reviewed extensively in recent years (Cerniglia and Sutherland 2010; Harms *et al.* 2011; Ghosal *et al.* 2016; Kadri *et al.* 2017). The decomposition of aromatics has been proposed to involve different enzymatic pathways that vary depending on the fungal species (Cerniglia and Sutherland 2010; Prenafeta-Boldù *et al.*, 2019). The degradation mechanisms also vary with the structure of the PAH molecule to be processed. For example, high molecular weight PAHs are degraded more slowly than low molecular weight PAHs, probably because they have a lower water solubility, a slower uptake into the cells, a insufficient ability to induce degradative enzymes, and consequently, a lower energy yield for growth (Kanaly and Harayama, 2000).

As already mentioned in paragraph 1.6, ligninolytic fungi have always been considered promising candidates for PAH degradation, as they produce extracellular ligninolytic enzymes. Lignin is a large, heterogeneous, amorphous polymer composed of phenylpropane subunits with a high content of benzene-oxygen rings, ether bonds and abundant C-C side chains. Although less complex than lignin, PAHs consist of fused benzene rings, which is why ligninolytic enzymes can also attack PAHs (Gadd, 2001; Steffen *et al.*, 2003). Three major classes of ligninolytic enzymes designated manganese-dependent peroxidases (EC 1.11.1.13) (MnPs), lignin peroxidases (EC 1.11.1.14)

(LiPs), and laccases (EC 1.10.3.2) (Lac) are important in the fungal degradation of petroleum hydrocarbons (Silva *et al.*, 2009). LiPs are known to oxidise different phenolic aromatic compounds and various non-phenolic compounds. The high redox potential of LiPs (around 1.2 V at pH 3) makes these enzymes capable of oxidising substrates that are not oxidised by other peroxidases (Sigoillot *et al.*, 2012). A LiP from *Phanerochaete chrysosporium* has been shown to oxidise pyrene (Song, 1999), benzo[a]pyrene (Barclay *et al.*, 1995; Hammel, 1995), and several other PAHs (Hiratsuka *et al.*, 2005). Similarly, LiPs from *Bjerkandera* sp. oxidise anthracene (Gramss *et al.*, 1999; Kotterman *et al.*, 1998). *Phanerochaete chrysosporium* and *Irpex lacteus* oxidise PAHs and anthracene in the presence of Mn^{2+} and H_2O_2 , employing MnPs (Bogan *et al.*, 1996; Baborová *et al.*, 2006). A blue Lac of *Pleurotus ostreatus* oxidises PAHs with three to five aromatic rings in the presence of artificial mediators; however, a modified form (yellow laccase) produced by the same species catalyses the same PAHs' oxidation as the blue laccase but does not require a mediator (Pozdnyakova *et al.*, 2006a,b). The Lac from *Trametes versicolor*, *Coriolopsis rigida*, and *Rigidoporus lignosus* oxidise PAHs in vitro in the presence of artificial mediator compounds, such as 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxy benzotriazole (HBT) (Cambria *et al.*, 2008; Gómez *et al.*, 2006). A general aromatic hydrocarbons pathway is shown in figure 1.6. Ligninolytic enzymes oxidise aromatics to form transient PAH

diphenols, easily autoxidised to quinones. More specifically, in the presence of endogenously formed H_2O_2 , LiP oxidises veratryl alcohol, an endogenously generated low-molecular-mass redox mediator, which in turn carries out one-electron oxidations of nonphenolic aromatics to form aryl cation radicals. These radicals initiate a chain of random oxidative chemical reactions that result in a variety of aliphatic and aromatic oxidised products. MnP performs H_2O_2 -dependent oxidation of Mn^{2+} to Mn^{3+} , stabilised by fungal chelators such as oxalic acid. Chelated Mn^{3+} acts as a low-molecular-mass redox-mediator that attacks phenolic structures, while other recalcitrant aromatic compounds are oxidised via cation radicals (Cerniglia and Sutherland, 2010; Prenafeta-Boldù *et al.*, 2019).

However, non-ligninolytic fungi can be useful in PAHs degradation. The intracellular enzyme cytochrome P450 (CYP) monooxygenase is also involved in the degradation of aromatic hydrocarbons (Masaphy *et al.*, 1996; Bezalel *et al.*, 1997). CYPs commonly act as terminal monooxygenases in a range of biochemical reactions by catalyzing the transfer of molecular oxygen to various cellular substrates (Mansuy, 1998; Sono *et al.*, 1996). The CYP system catalyses the ring-epoxidation of the aromatic structure to form arene oxides, which can either undergo enzymatic hydration by epoxide hydrolase to trans-dihydrodiols or rearrange non-enzymatically to form phenols (Sariaslani, 1991). Hydroxylation products can undergo further detoxification by O-conjugation to various intermediate forms,

including methyl, glucoside, glucuronide, sulfate, and xyloside forms. These molecules are more soluble and can eventually be excreted (Fig. 1.6) (Prenafeta-Boldù *et al.*, 2019). CYPs are characterised by possessing an active-site cavity unusually large compared to other CYP analogues from mammals and bacteria. This characteristic would explain why the enzyme has an extraordinarily low substrate specificity can act on many different compounds (Prenafeta-Boldù *et al.*, 2019). Although some examples are reported in literature (Capotorti *et al.*, 2010; Marco-Urrea *et al.*, 2015), few fungal CYPs have been functionally characterized compared to analogous in plants and animals. However, the investigation of fungal CYPs is fundamental because it may offer opportunities to exploit their catalytic functions, increasing their biodegradation abilities (Shin *et al.*, 2018).

All the described metabolic processes are primarily co-metabolic, which means that hardly aromatics support the fungal growth as sole carbon sources, but fungi may co-metabolise them to a wide variety of oxidised products and, in some cases, to CO₂. These metabolites are generally less toxic than the original substrate, depending on the biotransformation process (Prenafeta-Boldù *et al.*, 2019).

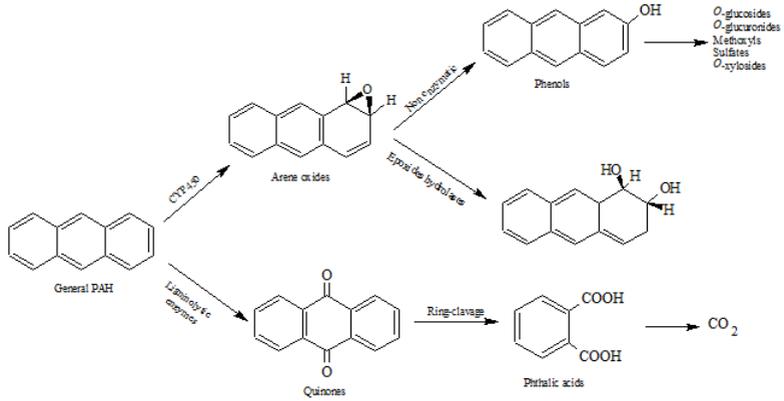


Figure 1.6. Generalized pathways for the PAHs degradation by fungi (modified from Cerniglia and Sutherland, 2010).

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**Paper I. Key fungal degradation patterns, enzymes
and their applications for the removal of
aliphatic hydrocarbons in polluted soils: a
review**

Key fungal degradation patterns, enzymes and their applications for the removal of aliphatic hydrocarbons in polluted soils: a review

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Abstract

Many saturated or unsaturated aliphatic hydrocarbons in fuels and their derivate pose considerable hazards to biological receptors due to the formation of toxic and carcinogenic metabolites. Currently, “bioremediation” (the use of living organisms to mitigate environmental pollution) is gaining public attention due to its cost-effectiveness and environment-friendly strategies. Understanding of biodegradation mechanisms is of high ecological significance; it relies on the use of indigenous microorganisms to transform/mineralise hydrocarbon contaminants. Fungal degradation processes have been well proven to degrade many hydrocarbons as they possess different cassettes of enzymes which enable them to degrade and utilise different hydrocarbons as sole carbon/energy sources. However, there is still a dearth of knowledge on fungal abilities for degradation of aliphatic hydrocarbons with respect to aromatics. The present paper is a review of the main results published on the ability of fungi in degrading aliphatic hydrocarbons. Aliphatics have been considered in particular for their abundance in the crude oil mixture and its derivate. The paper focuses on the biochemical processes and mechanisms involved in the degradation, analysing the differences observed in micromycetes (mainly *Ascomycota*) and macromycetes (*Basidiomycota*). The application of fungi in bioremediation in field-scale experiences is also discussed in light of environmental constraints.

Keywords: Pollution; bioremediation; fungi; enzymes; aliphatic hydrocarbons

1. Introduction

There are many expectations on fungi, bacteria and mixotrophic protists to remove toxic recalcitrant compounds from the environments in a sustainable manner.

Fungi, thanks to their strong and peculiar biodegradative actions, seem to be particularly promising. This is all the more true in the soil context where saprotrophic fungi are in their chosen environment (Dix & Webster, 1995). Above all the hyphal network of filamentous fungi exhibits an apical growth mode which enables them to penetrate in soil matrix more efficiently than bacteria. The mechanical forcing by hyphae allows fungi to reach inaccessible interstices where contaminants are difficult to remove (Dix and Webster, 1995). Thanks to the variety of degradation modes, fungi can exploit substrata even characterised by low moisture and low nutrient concentrations and several species can overcome dry periods by cryptobiosis (Magan, 1997). Finally, fungi are often favoured by acidic pH and contribute to further acidification by secreting organic acids (Ruijter *et al.*, 2002; Mancera-López *et al.*, 2008).

Hydrocarbons are one of the polluting compounds that fungi are capable to attack, although they are particularly recalcitrant. Many native fungi, residing in polluted environments, have developed adaptive mechanisms by which they are able to utilise hydrocarbons as the sole carbon source (Gadd, 2001; Asemoloye *et al.*, 2017a,c). Hydrocarbons are the main constituents of fossil fuel, i.e. natural gas, fossil carbon and petroleum. The crude oil, specifically, contains hundreds of different hydrocarbons ranging from C₁ (methane) to over C₃₀. Analysis of Saturate, Aromatic, Resin and

Asphaltene (SARA) highlights that aliphatic hydrocarbons in petroleum are almost exclusively alkanes; apart from gases, the major components are open-chain linear or branched alkanes and cycloalkanes (also known as naphthenes). Aromatic hydrocarbons are typically abundant in the heaviest fractions and provide monomers to resins (which also include aliphatic unsaturated monomers) (Aske *et al.*, 2001; Mikhailenko and Baaj, 2019). The so-called asphaltenes, important in asphalt production, are composed by polycyclic (as well as heterocyclic) aromatic structures insoluble in n-alkanes but soluble in toluene (Bai and Bai, 2005). Although resulting from biogenic and geological processes, petroleum hydrocarbons become severe pollutants when dispersed in the environment. Dispersal and spills can occur during exploration activities, extraction, refinery activities, transport and storage (Alvarez and Vogel, 1991; Das and Chandran, 2011). According to Varjani *et al.* (2017), toxic effects on human health can be resumed in: acute effects; chronic effects; genotoxicity; carcinogenicity; teratogenicity and immunotoxicity. Nevertheless, a Health, Safety and Environmental (HSE) culture has not been fully shared by the companies at a global level (Kongsvik *et al.*, 2016).

Conventional remediation *in-situ* technologies have reported poor results when applied to petroleum, as heavy fractions cannot be volatilised, and processes based on vitrification are very expensive. On the other hand, conventional *ex-situ* technologies (including e.g. excavation, landfilling and washing) resulted in higher efficiency but are very expensive and slow-acting (Das and Chandran, 2011; Asemoloye *et al.*, 2017a; Cole, 2018). Conventional treatments often need chemicals in operational steps. The incomplete degradation of petroleum compounds can moreover lead to other toxic products. Finally, conventional treatments strikingly alter the soil surface and sub-surface structure, nutrients, and furtherly reduce/alter the

microbial population (already compromised by petroleum pollution) (Block *et al.*, 1991; Spini *et al.*, 2018). Looking for a sustainable alternative, the scientific literature has devoted in the past few decades to exploration and optimisation of biological treatments (bioremediation) (Rosenberg and Ron, 1996; Gadd, 2001). Far beyond a primitive concept of “no-deal” (that is passive auto-remediation by ecosystems), biological treatments rely on the exploitation of microbial metabolism and co-metabolism. Metagenomic analysis exhibited the co-metabolism of polycyclic aromatic hydrocarbons by bacterial community from estuarine sediment, as bacteria and fungi can use petroleum molecules as a source of carbon (Cole, 2018). This makes bioremediation a “green” strategy, simple to design and a cost-effective both from an economic and energetic point of view. Finding a single strain able to degrade all the different hydrocarbons present in the crude oil could be very useful, but valid results have been collected with the use of consortia of bacteria and fungi, in which microbial synergy can attenuate and gradually remove them instead of accumulating in another form (Gan *et al.*, 2009). Moreover, pollutant-degrading bacteria are favoured in penetration by fungal action and consortia of bacteria-fungi are naturally formed in soil (April *et al.*, 2000; Banitz *et al.*, 2013). Although research has mainly focused on bacteria, the use of fungi (mycoremediation) has recently received considerable attention because of their ability to synthesise different cassettes of unspecific enzymes (Aranda, 2016; Deshmukh *et al.*, 2016). Some of them, evolved in nature for lignin decay, as observed in *Basidiomycota*, are often involved in the degradation of a wide range of recalcitrant pollutants such as hydrocarbons, chlorophenols, and pesticides (Young *et al.*, 2015; Deshmukh *et al.*, 2016; Hasan and Al-Jawhari, 2019). Mycoremediation of hydrocarbons must be tailored to the site-specific conditions and

environmental parameters which could interact with the process, including the autochthonous microbial population already present in the site. Therefore, treatability studies are to be considered as a function of preliminary site characterisation (Boopathy, 2000; Srivastava *et al.*, 2014) This approach offers several advantages: reduce alteration in autochthonous microbial coenoses; lower cost; permanent waste elimination; possibility to couple with other physical/chemical treatment. The speed and the effectiveness of fungal remediation mainly depend on environmental conditions and the number and behaviour of fungal species involved. It also depends on the chemical nature of the substrate and on the type, source, concentration and bioavailability of hydrocarbon contaminants (Stroud *et al.*, 2007). Finding a single strain able to degrade all the different hydrocarbons present in the crude oil could be very useful, but valid results have been collected with the use of consortia of bacteria and fungi, in which microbial synergy can attenuate and gradually remove them instead of accumulating in another form (Gan *et al.*, 2009; Liu *et al.*, 2017). Moreover, pollutant-degrading bacteria are favoured in penetration by fungal action and consortia of bacteria-fungi are naturally formed in soil (April *et al.*, 2000; Banitz *et al.*, 2013).

The wide majority of the bioremediation studies have focused on aromatic hydrocarbons, especially on Polycyclic Aromatic Hydrocarbons (PAHs) because they are usually the most harmful, being toxic, mutagenic and carcinogenic (Clemente *et al.*, 2001; Cerniglia *et al.*, 2001; Kaushik and Haritash, 2006). However, the aliphatics are also dangerous because of their significant presence in the environment, especially due to their abundance in the crude oil mixture and its derivate.

The present paper is a review of the main results that have been revealed up to now on the ability of fungi in degrading aliphatic hydrocarbon.

The work focuses on the biochemical processes and mechanisms involved in the degradation, analysing the differences observed among fungal groups mainly belonging to the phyla *Ascomycota* and *Basidiomycota*. The application of fungi in bioremediation in field-scale experiences is also discussed evaluating the importance of environmental parameters.

2. Fungal degradation mechanisms of aliphatic hydrocarbons and enzymes involved

One of the main mechanisms by which hydrocarbon pollutants can be removed from the environment is the biodegradation activities of natural fungal and bacterial populations. Generally, the degradation process in fungi mainly follows the aerobic (in the presence of oxygen) mode; the anaerobic one (without oxygen) is expressed mainly by yeasts but also is recorded in filamentous fungi as reported i.e. by Rouches *et al.* (2016) and Aydin *et al.* (2017).

Hydrocarbons, as the most organic pollutants, are degraded by fungi mainly under aerobic conditions; in fact, fungi act through enzymes which oxidise hydrocarbons to form water and non-toxic or less toxic residues. Hydrocarbon compounds differ in their susceptibility to microbial attack and they have generally been ranked in the following decreasing order: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes > high-molecular-weight aromatics and polycyclic aromatic compounds (Chandra *et al.*, 2013, Varjani *et al.*, 2015). Among the aliphatics, the position of the double bond is another important factor affecting the degradability; the alkenes with the double bond in the terminal position, for example, are more easily degradable than alkenes with an internal double bond (Morgan and Watkinson, 1994). Moreover, the cyclic aliphatics are the most resistant

compounds to fungal attack because the primary step is hindered due to the absence of an exposed terminal group (Varjani and Upasani, 2017). Nevertheless, Dallinger *et al.* (2016) detected the transformation pathway of cyclohexane by *Candida maltosa* and *Trichosporon* spp., depending on the concentration of the pollutant.

The metabolic pathways observed in fungi, require the substrate oxidation in microsomes mediated by cytochrome P450 (CYP) monooxygenases (EC:1.14.13.12) containing alkane-oxygenase enzymes (Durairaj *et al.*, 2015, Asemoloye *et al.*, 2019). These enzymes belong to the heme-protein superfamily and are involved in diverse biological processes including the adaptation to environmental stresses and new niches, the production of toxins and the metabolisation of different types of endogenous and xenobiotic compounds, contributing to their fitness (Bernhardt, 2006; van Beilen and Funhoff, 2007; Kelly *et al.*, 2009; Moktali *et al.*, 2012). The number of CYP genes varies depending on the fungal species lifestyle. Yeast and yeast-like fungi possess relatively few CYPs (3 in *Saccharomyces cerevisiae*, 6 in *Cryptococcus neoformans*, and 10 in *C. albicans*), while filamentous fungi tend to have more CYP genes, especially plant pathogens. For example, *Magnaporthe oryzae* has 107 genes and *Cryphonectria parasitica* 121 (Park *et al.*, 2008; Chen *et al.*, 2014). Indeed, Shin *et al.* (2017) suggest that CYP enzyme reactions are closely involved in fungal pathogenesis. However, compared to plants and animals, few fungal CYPs have been functionally characterised (Shin *et al.*, 2018).

CYP monooxygenases are, in general, the terminal oxidases in electron transfer chains coupled to NADPH reductases. These enzymes supply the electrons for the insertion of one atom of oxygen into the aliphatic chain, while the other oxygen atom is reduced to water (Chen *et al.*, 2014).

Higher eukaryotes generally possess several different CYP families consisting of a large number of individual P450 forms that may contribute as a set of isoforms to the metabolic conversion of a substrate (Kelly and Kelly, 2013; Lamb and Waterman, 2013). Different reactions are catalysed by cytochrome P450 isoforms; a very common one is a monooxygenase reaction that may lead to the formation of epoxides, the oxidation of alcohols and the hydroxylation of aliphatic or aromatic compounds. Several yeasts species such as *Yarrowia lipolytica*, *Candida albicans*, *C. tropicalis*, *C. maltosa*, *C. apicola* and *Debaryomyces hansenii* can degrade hydrocarbons by exploiting the action of the multiple microsomal forms of cytochrome P450 (Scheuer *et al.*, 1998). The first step of aliphatic degradation involves an hydroxylation, introducing an oxygen atom inside the terminal methyl group to form an alcohol (Sěpič *et al.*, 1995; Koma *et al.*, 2001; van Hamme *et al.*, 2003; Meng *et al.*, 2017). Subsequently, the alcohol is oxidised to the corresponding aldehyde and then to the corresponding fatty acid. The resulting fatty acid is incorporated within the central catabolic pathways via *p*-oxidation, involving the initial activation of the fatty acid to form an acyl-CoA ester. The latter is often further catabolised in the citric acid cycle jointly with the production of electrons in the electron transport chains (Prenafeta-Boldú *et al.*, 2019).

This is the most frequently encountered mechanism, but two variants have been described by Boulton and Ratledge (1984): diterminal and subterminal oxidation (Li and Liu, 2002; Das and Chandran, 2011). In the diterminal pathway, the molecule is oxidised to a dicarboxylic acid and processed by β -oxidation (Abbasian *et al.*, 2015). In subterminal oxidation, the aliphatic hydrocarbon is oxidised to a secondary alcohol and then to corresponding ketone and ester (Prenafeta-Boldú *et al.*, 2019). Moreover, unsaturated and branched-chain aliphatics, as well as cyclic alkanes, can be

oxidised by fungi via several routes yielding epoxides, alcohols, diols, and carboxylic acid units (Morgan and Watkinson, 1994; Sutherland *et al.*, 2004) (Fig.1). However, the literature concerning the degradation of these compounds is very incomplete. To compare the different degradation processes between aliphatics and PAHs, Fig. 2 shows the degradation pathway of a generic PAH by fungi.

Despite the aliphatic degradation pathways in fungi, previously discussed, is quite well known, there is still less information about the enzymes involved in these processes and, consequently, about their encoding genes. However, some possible enzymatic mechanisms have been proposed:

- i. the bacterial methane monooxygenase-like enzymes which catabolise the degradation of C₁-C₄ alkanes (from methane to butane) (Prenafeta-Boldú *et al.*, 2019);
- ii. the non-heme iron oxygenases enzymes, which catabolise the degradation of C₅-C₁₆ alkanes (from pentane to hexadecane) (Ryle and Hausinger, 2002);
- iii. the essentially unknown enzyme systems which catabolise the degradation of C₁₇ and longer alkanes (Singh, 2006).

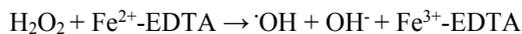
The sequencing of the genome of hydrocarbon-degrading organisms has allowed the identification of different genes involved in the metabolism of hydrocarbons (Desai *et al.*, 2010). However, the number of sequenced genomes of fungal species is lower than that of bacteria and most works principally focused on the identification of genes for the degradation of PAHs (Morales *et al.*, 2017). According to the literature, the degradation of aliphatic hydrocarbons by *Basidiomycota* (macrofungi) is limited to saprotrophic or saprotroph-behaving necrotrophic taxa associated with lignocellulosic

substrates, i.e. wood decay fungi. Moreover, growing *P. strigosozonata* on an oil-containing medium, Young *et al.* (2015) found a significant upregulation of genes related to three enzymes with potential roles in xenobiotic biotransformation. In fact, these genes have been related to B-class CYP mono-oxygenase, carboxylesterase (type B) and dienelactone hydrolase. Unlike the degradation of aromatic compounds, the degradation of aliphatic hydrocarbons apparently does not rely on the same enzymatic complex known to be involved in lignolysis, i.e Mn peroxidases (MnP), lignin peroxidases (LiP), dye-degrading peroxidases (DyP), versatile peroxidases (VP), and laccases, just to cite the most important ones. Although Khindaria *et al.* (1995) had proposed a mechanism involving LiP in the aerobic degradation of trichloroethylene (TCE) by *Phanerochaete chrysosporium*, Yadav *et al.* (2000) suggested that the complete mineralisation of TCE by the same species was unaffected by the presence or the lack of LiP and MnP. Marco-Urrea *et al.* (2008a) investigated the TCE degradation by *Trametes versicolor*, *Irpex lacteus*, and *Ganoderma lucidum*. These authors found that TCE stimulated the secretion of laccases; however, TCE was degraded even in the absence of such enzyme. In contrast, CYP was suggested to be an indispensable enzyme for the TCE degradation, since inhibition of the enzyme activity by 1-aminobenzotriazole severely hampered the degradation (Marco-Urrea *et al.*, 2006, 2008b). In the first investigation of the aerobic degradation of perchloroethylene (PCE) by *T. versicolor*, Marco-Urrea *et al.* (2006) also demonstrated the final production of trichloroacetic acid (Fig. 3), thus showing that degradation via CYP led to the formation of halogenated organic products, instead of complete mineralisation to CO₂ (Marco-Urrea *et al.*, 2009).

Despite the above findings, oxidation by cytochrome P450 is not the only pathway available to wood-decay fungi for the degradation of halogenated aliphatic hydrocarbons. Although the role proposed for LiP has later been ruled out by other authors, the pioneering intuition by Khindaria *et al.* (1995) about a major role of radical oxygen species (ROS), was further sustained by Marco-Urrea *et al.* (2009). The latter authors proved the involvement of extracellular hydroxyl radical ($\cdot\text{OH}$), produced via the quinone redox cycle, in the degradation of TCE, PCE and TCB (1,2,4- and 1,3,5-trichlorobenzene) by *T. versicolor*. In nature, quinones occur in cells and are also produced in plants by the enzymatic de-polymerisation of lignin by wood-decay (white-rot) fungi. Quinones are easily converted to hydroquinones by reversible reduction, as the extensive conjugation makes their stability very similar. Consequently, the quinone/ hydroquinone couple provides a versatile redox system even in an extracellular environment (Bianchetti and Rosnati, 1978). The quinone redox cycle is involved in several reactions which release ROS, i.e. the superoxide anion radical ($\text{O}_2^{\cdot-}$), H_2O_2 and the hydroxyl radical ($\cdot\text{OH}$). The production of hydroxyl radicals by the white-rot species *Pleurotus eryngii* via the quinone redox-cycle was first reported by Guillen *et al.* (1996, 1997). Among ROS, the hydroxyl radical is considered the most active oxidant in fungal systems due to its high redox potential (Backa *et al.*, 1993; Lawton and Robertson, 1999). Consequently, ROS radicals can be involved in the oxidation of both aliphatic and aromatic hydrocarbons, whether they are produced from enzymatic reactions or from the quinone redox cycle.

According to Gómez-Toribio *et al.* (2009a,b), the production of hydroxyl radicals by quinones involves reactions which can be summarised as follow:

- i. reduction of quinones to hydroquinones by cell bound de-hydrogenases;
- ii. oxidation of hydroquinones to semiquinone radicals by laccases;
- iii. auto-oxidation of semiquinones catalysed by Fe³⁺-EDTA, that is reduced to Fe²⁺ (this step produces the superoxide anion radical);
- iv. dismutation of the superoxide anion radical to produce H₂O₂ and O₂;
- v. decomposition of H₂O₂ and release of hydroxyl radicals by the comprehensive Fenton reaction:



The findings discussed above confirm the synergy and positive feedback generally occurring between the pathways involved in the degradation of aromatic and aliphatic components of lignin. In this light, wood-decay fungi, namely selective white-rot species, have been proved to be effective degraders of complex hydrocarbon matrixes.

Most fungal taxa are aerobic or facultatively anaerobic, whereas a small fraction only has been reported to be obligate anaerobic. Aerobic conditions generally provide a faster and more efficient degradation. Aerobic degradation is favored by the formation of thin biofilm layers onto the hydrocarbon matrix that prevents the complete segregation of the organism from the aerobic environment. As the term “aerobic” strictly involves the availability of atmospheric oxygen, several oxidative processes are referred

to both aerobic and anaerobic conditions, exploiting other substrates than O₂. More specifically, that is the case of:

- i. peroxidases, whose substrate is H₂O₂ (Lundell *et al.*, 2010);
- ii. cytochromes P450nor, whose substrate are NO₃⁻ and NO₂⁻ (Shoun *et al.*, 1991; Shoun *et al.*, 1992; Nelson *et al.*, 1993);

Based on above, the distinction between aerobic and anaerobic degradation pathways in fungal degradation is often subtle. This is why at least up to '90s the degradative process was assumed to be initiated in aerobic conditions only, being the role of oxygenases indispensable (Morgan and Watkinson, 1994). Nevertheless, fungi have developed mechanisms able to mineralise hydrocarbons under extreme conditions of oxygen limitation/absence such as in aquifers, sludges or mangroves dominated habitats (Santos *et al.*, 2011). The aliphatics degradation pathways in anaerobic conditions in bacteria are known in detail, such as for alkenes (Mbadinga *et al.*, 2011). This is not the same for fungi where the pathways of anaerobic aliphatics degradation are substantially unknown. Anyway, it has been reported that some strains can convert aromatics to benzoyl-CoA by using reductase enzymes (Hosoda *et al.*, 2005).

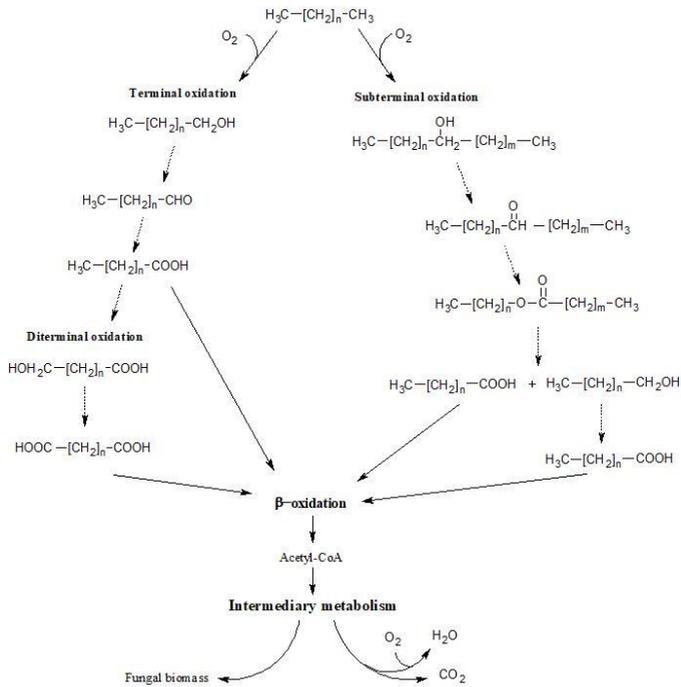


Fig. 1: Aerobic degradation of aliphatic hydrocarbons by fungi. In the figure is reported the degradation pathway of n -alkane (modified from Prenafeta-Boldú *et al.*, 2019).

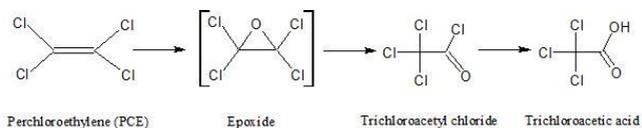


Fig. 2: Pathway for PCE degradation (Marco-Urrea *et al.*, 2006)

3. Aliphatic hydrocarbon consumption by fungi and the influence of environmental conditions.

3.1. Aliphatic hydrocarbon consumption by fungi

Scientific literature reports a huge number of fungal strains isolated from different hydrocarbon polluted sites. A high percentage of them showed to be able to grow on hydrocarbon complex mixture as the sole carbon source demonstrating to have adopted strategy to overcome the polluted environments (Chaillan *et al.*, 2004; Singh, 2006). The isolates mainly belong to the phylum *Ascomycota* - mainly isolated as anamorphs (Prenafeta-Boldù *et al.*, 2019), less to *Basidiomycota* and very rarely to other phyla, e.g. *Mucoromycota*, such as, *Rhizopus* spp., *Mucor* spp. and *Cunninghamella echinulata*. (Alarcón *et al.*, 2008; Mancera-López *et al.*, 2008; Hatami *et al.*, 2018; Prenafeta-Boldú *et al.*, 2019). As expected, most of fungi able to utilise aliphatic hydrocarbons are found among the saprotrophs, that is they are independent from a living host. To date, data on mycorrhizal fungi or obliged plant pathogens have been not available. In *Ascomycota* one of the most famous species is the yeast *Candida lipolytica* whose substrata of isolation (e.g. spoiled margarine, olive pulp, steep water or other sources, sausage, kerosene, crude oil) bear a relationship to its most pronounced degradation abilities. In particular, *C. lipolytica* (as *Yarrowia lipolytica*) was observed to

efficiently degrade triglycerides, alkanes and fatty acids, as reported by Hassanshahian *et al.* (2012). Also the spoilage yeast species of *Pichia* or yeast-like *Geotrichum* extremely common in soil or rotting plants and food (Domsch *et al.*, 2007; Passoth *et al.*, 2006) has been also isolated from hydrocarbon contaminated water or soil (Boguslawska-Wąs and Dąbrowski, 2001). Efficacy in degrading alkanes by yeasts was measured by Gargouri *et al.* (2015); isolates from the industrial refinery wastewater were able to use the *n*-alkanes C₁₁ to C₂₆ present in a petroleum sample within 12 days.

Aspergillus, *Penicillium*, *Cephalosporium* and *Gliomastix* are other important soil-inhabiting saprotrophic genera which are frequently reported as potential strong degraders of aliphatics (Chaillan *et al.*, 2004; D'Annibale *et al.*, 2006; Tosi *et al.*, 2010; Maddela *et al.*, 2016). *Penicillium* strains isolated from used motor oil were able into degrade the *n*-C₁₅ to *n*-C₂₃ alkenes present in it. In addition, Govarthanan *et al.* (2017) demonstrated that a strain of *Penicillium* sp. (CHY-2), isolated from an Antarctic soil, can degrade up to 49.0% decane and 33.0% dodecane at 20 °C and that the addition of glucose (5gL⁻¹) and Tween-80 (5gL⁻¹) improve decane degradation at 20 °C by about 1,8-fold and 1,61-fold, respectively. Covino *et al.* (2015) showed that some ascomycetes (strains of *Fusarium*, *Pseudallescheria*, *Penicillium* and *Aspergillus*) degraded up to 79% aliphatic hydrocarbons in contaminated soils. Also black fungi, mainly belonging to the genus *Exophiala*, were frequently isolated from polluted sites (Isola *et al.*, 2013).

Among *Basidiomycota* few taxa have been isolated from polluted environments, mainly yeast forms such as *Trichosporon mucoides*, *Rhodotorula mucilaginosa* (Gargouri *et al.*, 2015) and *Rhodospiridium* sp. (Mbachu *et al.*, 2016). Nevertheless, interesting results on effective

degradation have been mainly detected in wood-decay fungi, mainly in genera *Pleurotus*, and *Phanerochaete* (strictly saprotrophic) as well as in *Trametes* (necrotroph showing a prolonged saprotrophic stage) (Bernicchia, 2005; Bernicchia and Gorjon, 2010).

To date, most studies about hydrocarbon degradation by wood-decay fungi have dealt with the aromatic fraction, as the chemical similarity with lignin structural units enables the enzyme system of several species to attack aromatics as well as other recalcitrant and structurally complex compounds (Martínková *et al.*, 2016; Kadri *et al.*, 2017). On the contrary, the aliphatics have been neglected. Bioremediation tests of polluted soil by *Basidiomycota*, are generally carried out in batches containing a large lignocellulosic fraction to stimulate the fungal growth. This condition is particularly important for wood-decay taxa that would not be otherwise expected to develop mycelia in soil (Colombo *et al.*, 1996). To enhance degradation efficiency and simulate natural conditions, tests reported in the literature have often assessed consortia containing also soil filamentous fungi and/or bacteria. Consortia are moreover aimed to complementarily attack all the total petroleum hydrocarbon (TPH) of the crude oil thanks to the co-metabolic competences. Unfortunately, a common pitfall in the literature about this topic is the lack of specific information about the TPH composition under examination, i.e. about the percentages of the aromatic and the aliphatic fractions (<https://www.atsdr.cdc.gov/toxprofiles/>). Pozdnyakova *et al.* (2008) tested a consortium of *Pleurotus ostreatus* and *Lentinus tigrinus* with microbes naturally occurring in a non-sterile soil mixed with crude oil. This pioneering study provided surprising results about the enhanced degrading properties of the consortium in comparison with the single partners. Namely, *P. ostreatus* alone degraded up to 6% of oils in two weeks versus 34.5% of microbes

alone, whereas the consortium reached 60.5%. Fungi were generally reported to minimally degrade aliphatic compounds as compared to bacteria. The bacteria efficiency was, however, enhanced by the fungal action as demonstrated by Zanaroli *et al.* (2010) who used a consortium made of *Trametes gibbosa* and several strains of bacteria for the decontamination of diesel polluted soils. According to the authors, the fungus is necessary to reduce the toxicity of diesel and consequently to allow for bacterial growth and colonization. Unlike most studies in literature, this work indicated that the fungus, rather than bacteria, was the major degrader of the diesel hydrocarbons. Although the study does not provide any analysis of the diesel composition, technical literature indicates about 64% of aliphatic hydrocarbons and 35% of aromatic hydrocarbons respectively (<https://www.atsdr.cdc.gov/toxprofiles/>). The results of a study by Lladó *et al.* (2012) are also in agreement, although with little differences, with the above data. The authors observed that inoculation of *Trametes versicolor* into a non-sterile soil polluted with heavy mineral oil (C₁₅-C₃₅ hydrocarbons) maximize the TPH degradation (up to about 40%). The fungus was suggested to play a key role in changing the microbial community structure and population density, namely by increasing hydrocarbon degraders up to 100%, as confirmed by PCR-DGGE analysis. Other authors suggested that *T. versicolor* provides microbes with an additional carbon source, e.g. through the supply of secondary metabolites (Vázquez *et al.*, 2000; Boer *et al.*, 2005). A consortium comprising *Pleurotus ostreatus* and *Bacillus licheniformis* was tested by Liu *et al.* (2017). The fungus alone was found to degrade up to 54% TPH, while the consortium reached 58%. The major role of *P. ostreatus* was attributed to its complex enzymatic apparatus which is specifically white-rot oriented. Such a degradation mode is also consistent with a residue containing

less aromatic than aliphatic hydrocarbons. In fact, aromatic compounds are the preferred substrates of white-rot fungi, e.g. of the genera *Pleurotus* and *Trametes* (Chiu *et al.*, 2009; Kulikova *et al.*, 2016). In contrast to these results, Kulikova *et al.* (2016) found that the degrading efficiencies of aliphatic and aromatic hydrocarbons in soil and peat were similar for *Steccherinum murashkinskyi*, *Trametes maxima*, and *P. ostreatus*. In fact, the residual hydrocarbons were 49%-19% and 43%-24%, respectively, after degradation. Regarding the complex relationship with the microbiome, this study suggested that the fungi may partially suppress the aboriginal microflora, consequently reducing hydrocarbon degradation. This finding conflicts with the hypothesis previously shared by most authors since the concomitant application of wood-decay fungi and microbes in Kulikova *et al.* (2016) tests resulted in less degradation of the aliphatic components than the control. Besides the controversial relationship with the soil microbiome, the degradation potential of wood-decay species axenically cultured in polluted soils has for decades. Colombo *et al.* (1996) reported up to 80%, 82% and 8% degradation of aliphatic hydrocarbons by *Pleurotus ostreatus*, *Trametes villosa* and *Coriolopsis rigida* (current name: *Coriolopsis floccosa*), respectively. Interestingly, this study provided a complete profile of the components of the examined crude oil. It was observed that all the aliphatic fractions (from C₁₅ to C₃₀) were degraded almost to the same extent. Such high degrading activity was considered as the result of the high (75-85%) colonization efficiency which, in turn, was favored by the axenic cultural condition. When wood-decay fungi grow in a wood-rich medium rather than on a soil matrix, they reach the highest degrading efficiency. Young *et al.* (2015) observed an average 98% degradation for C₁₀ and 48.6 % for C₁₄ alkanes in 180 days, testing *Irpex lacteus*, *Phlebia radiata*,

Pleurotus ostreatus, *Trametes versicolor*, *Trichaptum biforme* on wood-rich matrix. *Punctularia strigosozonata* degraded 99% of the initial C₁₀ alkane fraction but did not affect the C₁₄ fraction in 20 days.

3.2 Influence of environmental conditions

As for other hydrocarbons, aliphatic degradation is influenced by several factors as discussed by Brusseau (1998) and Cooney *et al.* (1985). Tuning properly culture conditions can increase, for example, the complete mineralization of TCE, PCE, and PCB as it was demonstrated by Marco-Urrea *et al.* (2009) on *Trametes versicolor*.

One of the most important factors, either in soil or in aquatic ecosystems, concerns the bioavailability of hydrocarbons to fungal attack (Barathi and Vasudevan, 2001). Most hydrocarbons form complexes with particulate matter which affect oil susceptibility to fungal degradation (Bossert and Bartha, 1984). In fact, the particulate matter can reduce the oil toxicity by absorption, but it has been demonstrated that humic substances, by absorbing hydrocarbons, can contribute to the formation of persistent residues (Leahy and Colwell, 1990). The migration and distribution of the oil depend on its physical and chemical nature; degradation has been shown to be highest for saturated compounds, followed by light aromatics, high-molecular-weight aromatics and polar compounds (Fusey and Oudot, 1984; Semple *et al.*, 2003). Hydrocarbons, thanks to the solubilization or emulsification activities of biosurfactants, can be more bioavailable to the fungal action (Barathi and Vasudevan, 2001; Singh *et al.*, 2017) Consistently, the production of biosurfactants to support the attack on hydrocarbons, has been reported in a few fungal species (Bhardwaj *et al.*, 2013) whereas it seems to be more common in bacteria (Yan *et al.*, 2012; Silva *et al.*, 2014).

With concern to fungi, it has been reported, for example, in *Candida bombicola* (Deshpande and Daniels, 1995; Cavalero and Cooper, 2003; Felse *et al.*, 2007), *C. lipolytica* (Rufino *et al.*, 2007; Sarubbo *et al.*, 2007), *C. ishiwadae* (Thanomsub *et al.*, 2004), *C. batistae* (Konishi *et al.*, 2008), *Aspergillus ustus* (Kiran *et al.*, 2009), *Ustilago maydis* (Cortes-Sánchez Alejandro, 2011) and *Trichosporon ashii* (Chandran and Das, 2014). According to Rufino *et al.* (2007) and Felse *et al.* (2007), the sophorolipids from *C. lipolytica* and *C. bombicola* are very promising for the cleaning of oil tanks, decontamination of polluted areas and microbial enhanced oil recovery. In the same way, biosurfactants produced by *Torulopsis bombicola* and *A. ustus* MSF3 were used for the release of bitumen from contaminated soils and for the degradation of hydrocarbons (Cooper and Paddock, 1984; Kiran *et al.*, 2009). Mannosylerythritol lipids from *Candida antarctica* also have potential applications for the removal and biodegradation of hydrocarbons in oil-contaminated soils (Kitamoto *et al.*, 2001).

Environmental parameters strongly affect the microbial attack on hydrocarbons, both in fungi and bacteria. Temperature is negatively correlated to the oil viscosity and positively correlated to the volatility of low molecular weight toxic alkanes (Foght *et al.*, 1996; Aislabie *et al.*, 2006). Fungi involved in hydrocarbons degradation are generally mesophilic organisms, that is they can grow in the range 10-40 °C, showing an optimum in the range 20-35 °C (Kirk *et al.*, 2008). This is consistent with Atlas (1975) who reported highest degradation rates in the range 30-40 °C in soil, 20-30 °C in freshwater and 15-20 °C in marine environments.

Another major factor in hydrocarbon biodegradation is the soil acidity because pH influences the enzymatic activities, the cell membrane transport and the catalytic reaction balance (Bonomo *et al.*, 2001). As hydrocarbons

degradation by fungi occurs mainly as an aerobic process, oxygen concentration is one of the most influential speed-limiting factors at least for the initial breakdown stages of hydrocarbon molecules. On the other hand the anaerobic degradation in soils has less ecological significance because it occurs only at negligible rates and especially in bacteria (Floodgate, 1984; Barathi and Vasudevan, 2001).

4. Application of fungi in bioremediation of aliphatic hydrocarbon

Fungal application for bioremediation can be *ex-situ* or *in-situ*. In either way, hydrocarbons-degrading fungal strains are selected on the basis of their metabolic pathways and eco-physiological characteristics. In an *in-situ* bioremediation process, fungi are usually exploited through bio-stimulation (a process involving nutrient addition to enhance the action of native soil fungi), as explained by Asemoloye *et al.* (2017a) who reported bio-stimulation of some rhizosphere fungi by a nutrient source called Spent Mushroom Compost (SMC). Application of fungi for *in-situ* soil remediation can also be implemented through the introduction of allochthonous strains that have been selected for hydrocarbon degradation (bio-augmentation). Several scientists have demonstrated the biostimulation and bioaugmentation applications of fungi in the remediation of many hydrocarbon polluted soils; however, these approaches are needed to be fully studied and planned for each polluted site, due to differences of pollutant type and concentration as well as of environmental conditions from site to site, along with the other factors discussed previously.

The synergistic bioremediation approach by plants and microorganisms has also been reported to reduce bioaccumulation of contaminants in plant tissues, as they are mostly degraded and mineralised

through combined actions (Wu *et al.*, 2006; Ramos *et al.*, 2009). This mechanism, if well implemented, can also enhance ecological stability and revegetation of thinning soils (Ukaegbu-Obi, K.M. and Mbakwem-Aniebo, 2015). Recently, the use of combined interactions between fungi and plants, either through augmentation or stimulation, has been documented for the degradation of hydrocarbon pollutants. The phytoremediation technology (the use of plants for soil remediation) faces the challenge of prolonged times and phyto-accumulation of toxic pollutants. It was observed that the plant's rhizosphere is colonized by several plant growth-promoting bacteria and fungi, which may also be valuable in supporting rhizo-degradation. The symbiotic association between plants and plant growth-promoting rhizosphere fungi in hydrocarbon-polluted sites can as well improve co-degradation of pollutants. Weyens *et al.* (2009) reported the importance of synergistic plant and fungal treatment of hydrocarbon-polluted soils. In our previous studies, we observed that the application of a SMC-fungal consortium to the rhizosphere of *Megathyrsus maximus* (Guinea grass) improved degradation efficiency, rate, and half-life of key hydrocarbon contaminants in crude oil and black oil-polluted soils (Asemoloye *et al.*, 2017a, 2017c).

Moreover, it has recently been proposed that fungal enzymes may be extracted and applied directly on the polluted site for the transformation of toxic hydrocarbons to no- or less toxic compounds (Alcalde *et al.*, 2006; Santos *et al.*, 2011). Fungal enzymes are isolated and subjected to modelling to be used as biocatalysts to reduce the industrial costs of soil remediation, the environmental impact and the generation of toxic by-products resulting from the incomplete degradation of hydrocarbon pollutants, in accordance with Alcalde *et al.* (2006). Enzyme application for soil remediation is much

less demanding than using the whole fungi, as enzymatic mechanisms can be improved in the laboratory to target specific pollutants with fewer competitions (Sutherland *et al.*, 2004). However, enzyme search and production are difficult and expensive, and yields are usually limited; moreover, the stability of enzymes in the field is scarce and needs to be optimized.

Sutherland *et al.* (2004) itemized the factors to be considered during search, production and application of enzymes in bioremediation. The enzyme must be able to target specific pollutants and to degrade them to non-toxic products. Moreover, enzymes must not depend on co-factors which may increase commercial costs and must be purified from soluble materials, such as those associated with some fermented liquors and un-lysed cells. The knowledge of the genes coding for the enzymes to be obtained in fungi is often necessary because their production can be improved. This target can be accomplished by using modern biotechnology tools. In addition, the enzyme shelf-life and stability must be pre-determined before attempting site applications.

Fungi are unique organisms and they differ in their modes of action. Therefore, a great deal of efforts is needed to search for fungal enzymes to be used for the bioremediation of particular polluted soils. Many microbial enzyme-based products, such as the popular Oil Spill Eater II (OSEI Corp., Dallas TX), have been promoted for bioremediation nowadays. The world's sales of these products, including microorganisms, nutrients and blends for manufacturers in the US, was estimated to be about the U.S. \$153.87 million in 2006 (<http://www.bccresearch.com/report/BIO019A.html>). As previously described, many common enzymes can be isolated from different fungi.

Fungal laccases, for example, have been well exploited for PAHs removal from polluted soils (Alcalde *et al.*, 2006).

Today, advancements in fungal biotechnology have made it possible to genetically modify and improve the efficiency of fungi as to their use in soil remediation (Behjati and Tarpey, 2013). Many molecular tools, such as Transcriptome analysis, Metagenomics, Metatranscriptomics, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), can be used to detect hydrocarbon degrading genes in native fungal strains isolated from different hydrocarbon polluted sites. It is also possible to directly detect key degrading fungal taxa and genes from the environment/polluted soil samples through deep sequencing techniques (NGS, Next Generation Sequencing) (Behjati and Tarpey, 2013). The NGS has been used in several molecular studies and currently, there are two common NGS platforms used in environmental sequencing: Illumina and Ion Torrent (Salipante *et al.*, 2014). They are easier for identifying members of the fungal community using longer sequences however, shorter gene regions have been successfully studied using other technologies including the Ion Torrent platform (GemL *et al.*, 2014). One example of how NGS has inform bioremediation remedies was when metagenomic and amplicon-based approaches were integrated in a study performed on microbial mats treating wastewater from hydraulic fracturing (Akyon *et al.*, 2015). Hauptmann *et al.* (2017), Czaplicki and Gunsch (2016) and Chistoserdova (2017) investigated various microbial metagenomes and isolated potential microbial genes for the degradation and resistance to contaminants. Function based metagenomic analysis involving the isolation of DNA from environmental samples has been used to study the functions of encoded proteins (Lam *et al.*, 2015). Similar environmental studies used metagenomic and amplicon-based approaches to find a level of

ethylenediaminetetraacetic acid (EDTA) that enabled bioaugmented bacteria to sustain effective hydrocarbon biodegradation in the microbial community (Al Kharusi *et al.*, 2016) and to track how bioaugmentation affects the *in-situ* community to enhance degradation (Festa *et al.*, 2016). Amplicon gene sequencing of conserved marker genes was applied by Bell *et al.* (2014) to identify bacterial and fungal strains in hydrocarbon polluted soil, functional roles of soil fungi in hydrocarbon polluted sites were detected through metagenome short-gun sequence (Aßhauer *et al.*, 2015), the expression patterns of key oil degrading microbes in polluted soil was reported by He *et al.* (2010) and Alberti *et al.* (2014) using metatranscriptome analysis. The use of pyrosequencing has been reported for monitoring fungal responses to environmental contaminants (Kato *et al.*, 2015; Barbi *et al.*, 2014). These advanced tools can also allow scientists to engineer enzyme production in desired host fungi. For example, Bulter *et al.* (2003) reported the expression of *Myceliophthora thermophila* laccase genes after their cloning in the yeast *Saccharomyces cerevisiae*. However, this aspect is still facing some challenges such as legislation restrictions, public concerns and ecological impacts of recombinant strains on wild-type strains.

Alcalde *et al.* (2006) and Asemoloye *et al.* (2017b, 2018) reported that advancements and applications of Proteomics, Protein engineering and Metagenomics, especially with the applications of Fosmid and Cosmid shotgun metagenomic libraries, could immensely reduce the costs of soil remediation and minimize the use of chemicals. Molecular tools for enzyme recovery and application of biocatalysts can also help in solving the problems/concerns on the use of Genetically Modified Organisms (GMOs).

5. Conclusions and future perspectives

Hydrocarbon pollution has become a major environmental challenge in many crude oil exploiting host communities of the world. The reclamation of affected soils and waters requires heavy expenditure using many conventional technologies; however, 'Bioremediation technology' unlike others offers an environmentally friendly and cost-effective approach for the transformation of toxic compounds into less or non-hazardous products while saving chemicals, energy, and time. This technology depends majorly on the type and nature of hydrocarbon pollutants, the microbial communities (native or introduced) as well as the environmental conditions. Application of bioremediation technology *in-situ*, therefore, requires deeper scientific understanding as well as in-depth researches for the development of inexpensive, simple/rapid standardized toxicity bioassays for detection of hydrocarbon pollutants in soils.

To date, the hydrocarbon-degrading pathways by fungi have been studied extensively, above all about aromatics, but there is still a dearth of knowledge on the fungal degradation of aliphatics. More in particular, intermediate products in the degradation pathways of aliphatics, have been already identified whereas the extracellular enzymes catalysing the initial steps have been only proposed. This again has led to less understanding of how fungi can be exploited for the development of strategies feasible for remediation/reclamation of polluted environments, especially with aliphatic hydrocarbons.

In our opinion, there are different strategies that can be implemented to make fungal remediation processes more efficient, here briefly outlined:

- i. investigation of fungal CYPs to improve the comprehension of fungal physiology to exploit their catalytic functions;
- ii. the quest and applications of novel fungal enzymes, which could target key hydrocarbon contaminants;
- iii. further exploration of aerobic and anaerobic biodegradation pathways of aliphatic hydrocarbons and genes involved in the pathways;
- iv. application of supplements/bio-stimulants to the polluted sites to enhance fungal survival and response for speedy degradation kinetics;
- v. monitoring and manipulation of possible environmental conditions to favour fungal action on-site;
- vi. an integrated approach, which combines different mechanisms such as physical, chemical and biological approaches with special attention to the ecological implications of such integrated measures;
- vii. development and testing of a combined approach between bioremediation and phytoremediation to improve the degradation rates;
- viii. adequate implementation of regulatory policies for the pre-treatments of hydrocarbon-based wastes with fungal mechanisms before and after discharge;
- ix. the “control at the source”, by the strategic implementation of mycoremediation mechanisms which can significantly reduce harmful hydrocarbon levels by mitigation and remediation.

Over the years, applications of genetic engineering for the improvement degradative pathways in fungi have also been encouraged due to advancements in biotechnology. It could be possible to improve survival, substrate specificity and complete metabolic pathways for fungi through the use of biotechnology. The advent of molecular tools such as Metagenomics, Meta-transcriptomics, CRISPR-Cas system, have now made it possible to reconstruct different novel fungal genetic and metabolic pathways in a single host cell for the degradation of highly recalcitrant hydrocarbons. However, it is necessary to exclude any side effect and ethical misconduct before applying such GMOs into the environment.

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Paper II. Hydrocarbon degradation and enzyme activities of *Aspergillus oryzae* and *Mucor irregularis* isolated from nigerian crude oil-polluted sites

Hydrocarbon Degradation and Enzyme Activities of *Aspergillus oryzae* and *Mucor irregularis* Isolated from Nigerian Crude Oil-Polluted Sites

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Abstract: Many free-living saprobic fungi are nature recruited organisms for the degradation of wastes, ranging from lignocellulose biomass to organic/inorganic chemicals, aided by their production of enzymes. In this study, fungal strains were isolated from contaminated crude-oil fields in Nigeria. The dominant fungi were selected from each site and identified as *Aspergillus oryzae* and *Mucor irregularis* based on morphological and molecular characterisation, with site percentage incidences of 56.67% and 66.70%, respectively. Selected strains response/tolerance to complex hydrocarbon (used engine oil) was studied by growing them on Bushnell Haas (BH) mineral agar supplemented with the hydrocarbon at different concentrations, i.e., 5%, 10%, 15%, and 20%, with a control having dextrose. Hydrocarbon degradation potentials of these fungi were confirmed in BH broth culture filtrates pre-supplemented with 1% engine oil after 15 days of incubation using GC/MS. In addition, the presence of putative enzymes, laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP) was confirmed in culture filtrates using appropriate substrates. The analysed fungi grew in hydrocarbon supplemented medium with no other carbon source and exhibited 39.40% and 45.85% dose inhibition response (DIR) respectively at 20% hydrocarbon concentration. An enzyme activity test revealed that these two fungi produced more Lac than MnP and LiP. It was also observed through the GC/MS analyses that while *A. oryzae* acted on all hydrocarbon components in the used engine oil, *M. irregularis* only degraded the long-chain hydrocarbons and BTEX. This study confirms that *A. oryzae* and *M. irregularis* have the potential to be exploited in the bio-treatment and removal of hydrocarbons from polluted soils.

Keywords: pollutants; dose inhibition response; extracellular enzymes; fungi; hydrocarbon degradation; tolerance; bio-treatment

1. Introduction

An increase in anthropogenic activities has affected the natural environment in many ways. Studies have been reported on the occurrence of several pollutants and their various products in different environments [1–3]. In particular, the extraction, refinement, and transportation of petroleum oil and products are of critical concerns throughout the world in terms of environmental contamination [4–6]. Over 1.5 billion tons of petroleum oil is transported yearly, and despite prevention strategies, some significant amount still spills into the environment due to either operational issues or accidents. Petroleum hydrocarbons are generally characterised by many carbon bonds, which could bind to other compounds to form exceptional multi-complex structures, such as aliphatic alkanes/alkenes, chlorinated hydrocarbons or polycyclic aromatic hydrocarbons (PAHs), potentially harmful to the environment [7,8].

Many hydrocarbon complexes can alter the soil or water conditions in nature. They can percolate through the ecosystems and accumulate both in animal and plant tissues, thus exerting various toxic effects including cancer induction, mutations, and malfunctioning of respiratory and central nervous systems [9–13]. For this reason, PAHs and some organic compounds like mono-aromatic hydrocarbons have been listed as priority pollutants by the United State Environmental Protection Agency [14,15]. In addition, hydrocarbon contaminants in soil have been

shown to affect the microbial communities negatively; gasoline for example have been shown to exert poisonous effect on different soil microorganisms [16]. Many other petroleum-based products like engine oil, diesel, or paraffin are non- or semi-soluble in water, causing significant water contamination and less bioavailability for microbial degradation [17]. Moreover, used engine oil contains several mixtures of hydrocarbons, engine additives and toxic metals like Al, Cr, Cu, Fe, Ni, Si, and Pb [18]. Remediation of soils polluted with hydrocarbons has attracted much attention over the last few decades. A variety of methods such as chemical/electrokinetic separation, photo-oxidation, extraction/thermal treatments, and soil flushing have been suggested to salvage the environment from the impact of petroleum spill and hydrocarbon contamination [19–21]. However, bioremediation is becoming a more attractive among other strategies used for pollution containment, being non-evasive, low cost, and environment-friendly [22–24]. More specifically, microbial degradation, among other available bioremediation technologies, has been adopted mainly for treatment of hydrocarbon impacted soils [25–27]. Many filamentous fungi have been reported as bioremediation agents due to their verse mycelial networks and enzyme secreting activities. Most rot fungi produce high redox potential enzymes such as manganese peroxidase (MnP), laccases (Lac), and lignin peroxidases (LiP) for the oxidation of lignin. These enzymes are not generally substratespecific as they can oxidise a wide range of xenobiotics, including pesticides, plastics, and hydrocarbons [26,27].

A continuous search for naturally existing microbes with bioremediation potentials, especially for most concerned pollutants like the persistent organics, has been performed in the last few decades.

Mycelial degradation mechanisms on many complex hydrocarbon pollutants have been clearly described in a large number of studies [17,18,25,27]. In this work, culturable filamentous fungal strains were isolated from two crude oil impacted sites in Niger Delta area, Nigeria. It was hypothesised that fungi inhabiting crude oil contaminated soils over a long time could have developed the ability to degrade/mineralise the hydrocarbons due to their survival in such environment. Dominant strains from the investigated sites were characterised for enzyme secreting activities and hydrocarbon degradation.

2. Materials and Methods

2.1. Study Sites and Sampling

Two sites that have been frequently exposed to oil spillage in Niger Delta area of Nigeria were selected for this study. These investigated sites included Yorla-10 (latitude 4°39'45.34" N and longitude 7°26'11.23" E) and Effurun (latitude 5°34'49.26" N, longitude 5°46'56.11" E) communities as shown in Map Supplementary File Figure S1. Yorla-10 (Yorla oil field 10 location) is included in a cluster of oil wells at Kwawa community located in Khana Local Government Area of Rivers State, while Effurun community is located in Uvwie Local Government Area of Delta State, Nigeria. Soil samples (100 g each) were collected from 30 sampling points on each site at a depth of 5–20 cm from soil surfaces. The samples were kept in sterile cryovial tubes and brought to laboratory for fungal isolation. Physicochemical properties of these polluted soil samples were analyzed using standard analytical procedures [28] as presented in the supplementary file (Tables S1 and S2). Used engine oil was obtained from a private Italian company in this study and treated as complex hydrocarbon mixture, this

mixture consisted of short and long-chain aliphatics/aromatics as well as polycyclic aromatic hydrocarbons [7]. The composition of this oil was defined using Thermo Scientific DSQII single-quadrupole gas chromatography coupled with a mass spectrophotometry (GC/MS) system.

2.2. *Fungal Strains*

Fungal strains were isolated from each soil sample in Petri dishes, on sterile potato dextrose agar (PDA) medium supplemented with ampicillin (100 mg/L) to suppress bacteria interference. The soil samples were subjected to serial dilution at 10^{-6} using sterile water, inoculated on prepared PDA, and then incubated at 30 °C, in the darkness, for three days to allow fungal growth. Pure cultures were made from primary PDA plates by carefully picking and inoculating each colony into a new plate. Isolated strains were grouped based on plate morphology, while the number of samples from which a particular strain was isolated was used to calculate its percentage incidence in that site [29–31].

$$\text{Percentage Incidence} = \frac{\text{Number of samples having a particular fungus}}{\text{Total number of samples analyzed}} \times 100$$

The fungal strain having the highest incidence was regarded as the dominant strain and was selected from each site for further studies.

2.3. *Morphology and Molecular Identification of Dominant Strains* The living fungal cultures of selected dominant fungi were deposited in the Laboratory of Mycology, at the Department of Earth and Environmental Sciences, University of Pavia, Italy and LP Culture Collection (personal culture collection held in the laboratory of Prof. Lorenzo Pecoraro), at the

School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, China. The strains were grown on different media, including Czapek yeast extract agar (CYA), Czapek Dox solution agar (CZA), Malt extract agar (MEA), and Potato dextrose agar (PDA), with incubation temperature of 25 °C. The selected strains were characterized based on morphological and molecular methods. For morphological characterization, conidial/mycelial color, reverse plate color, colony diameter, seriation, vesicle, presence/absence of cleistothecial wall, presence/absence of Hulle cells and sclerotia, and zygospore/ascospore/conidia structures were observed, following keys for morphological identification [32]. Molecular identification of the selected strains was made using PCR amplification of the internal transcribed spacer (ITS) region. Fungal genomic DNA was extracted using the hexadecyl trimethyl ammonium bromide (cTAB) extraction buffer made up of 50 mM Tris Buffer pH 8.0, 100 mM EDTA, 150 mM NaCl, and 1% mercaptoethanol. Fungal mycelia were transferred into 2.0 mL Eppendorf tubes (400 mg each) and stored at 80 °C overnight, then kept in ice for 15 min to allow cell thaw. The genomic DNA extraction was carried out as described by Möller *et al.* [33], modified by Asemoloye *et al.* [23,24]. DNA concentration was checked using a nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at light absorbance of 260 and 280 nm respectively, while DNA quality was checked in 1% agarose gel electrophoresis.

The PCR ITS amplification was carried out using the universal primer pair pITS4-F (5'-TCCGTAGGTGAACCTGCCG-3') and pITS1-R (5'-TCCTCCGCTTATTGATATGC-3') according to White *et al.* [34]. 2 µL of genomic DNA (100–500 ng) was added to the PCR mixtures containing 25 µL T5 Tsingke colony PCR master mix, 1.5 µL of each primer (5 µg), and

20 µL of deionized water. The thermal cycler was set at PCR condition of 98 °C/3 min initial denaturation step followed by 35 cycles of 98 °C/10 secs, annealing temperature of 55 °C/10 secs, extension temperature of 72 °C/15 sec followed by final extension temperature of 72 °C for 2 min. Products obtained were checked on gel electrophoresis, purified using QIAquick PCR purification kit (Qiagen) following the manufacturers prescriptions and sent to Genewiz, Tianjin, China for sequencing.

Sequences were edited to remove vector sequences and to ensure correct orientation and assembled using Sequencher 4.1 for MacOSX (Genes Codes, Ann Arbor, MI, USA). Sequence analysis was conducted with BLAST searches against the National Center for Biotechnology Information (NCBI) sequence database (GenBank; <http://www.ncbi.nlm.nih.gov/BLAST/index.html>) to

determine the closest sequence matches that enabled taxonomic identification. Fungal DNA sequences amplified from strains (B-Yorla10 and C-Effurun) isolated from oil-contaminated soil in Nigeria were submitted to GenBank under accessions MW114835–MW114836. DNA Sequences were aligned using Clustal X 2.1 [35]. Phylogenetic analysis was performed using MEGA 7.0 [36], and neighbour-joining tree was constructed by Kimura 2-parameter distances, with bootstrapping of 1000 replicates. *Umbelopsis nana* was used as outgroup to root the *Mucor* tree, while the *Aspergillus* fungi tree was rooted with *Aspergillus peyronelii* [37–40].

2.4. Hydrocarbon Tolerance Test

The ability of selected fungi to respond and tolerate the used engine oil was studied by growing them on BH agar, a synthetic growth medium containing only minerals without any carbon source. Composition of this medium

included magnesium sulphate (0.20 g/L), calcium chloride (0.20 g/L), monopotassium phosphate (1.0 g/L), dipotassium phosphate (1.0 g/L), ammonium nitrate (1.0 g/L), ferric chloride (0.05 g/L) at pH 7.0 ± 0.2 . BH agar medium was supplemented with used engine oil as the only carbon source at 5, 10, 15 and 20% concentration, while a plate amended with dextrose (10 g/L) was used as control. The selected fungal strains were inoculated on 90×15 mm Petri plates containing the mixture mentioned above in six replications, while their hydrocarbon tolerance capacity was studied through mycelial growth measurements every 24 h for eight days. Data obtained were used to calculate fungal dose inhibition response (DIR) to hydrocarbons [41,42].

$$\text{DIR} = \frac{\text{Growth rate on hydrocarbon plate}}{\text{Growth rate on control plate}} \times 100$$

2.5. Enzyme Activities of the Selected Dominant Fungi

The analysed enzymes included lignin peroxidase (LiP) manganese peroxidase (MnP), and laccase (Lac), which were selected due to their largely recognized redox-potential activities on a wide range of hydrocarbon compounds. The latter enzymes belong to the natural ligninolytic oxidative system that has been reported in many fungi associated with degradation of several pollutants [27,43–45]. Colorimetric enzyme assay was performed by growing the fungi on Petri plates containing 15 mL of sterile malt extract agar (MEA) medium. MEA was prepared in three groups; a group with no chemical substrate, another group was supplemented with 0.05% guaiacol and 1 mM CuSO_4 as enzyme substrates while the last group was supplemented with 0.5 mM 2,2-

Azino-bis-3-benzthiazoline-6-sulfonic acid (ABTS) and 1 mM CuSO₄ [46,47].

A pilot enzyme activity study was conducted to characterize enzymes that are secreted by these fungi; fungal inoculum was prepared by cutting out a mycelial disc (50 mm diameter) from the outer edge of an actively growing fungus and transferred aseptically into a glass tube containing 10 mL sterile water with broken glasses. The mixture was vortex for 1 min and kept at -4 °C until further use. Bushnell Haas (BH) broth was prepared according to manufacturer's prescription (Sigma Aldrich, St. Louis, MO, USA) and supplemented with 0 (control), 2.5, and 5% of used engine oil in conical flasks. The flasks were tightly corked and sterilized at 121 °C. 1 mL of the mycelium-water mixture was transferred into different BH flasks with corresponding engine oil concentrations and kept in a shaker incubator at 120 rpm, 30 °C. Aliquots of 300 µL were taken from each flask for enzyme activity tests after 3, 12, and 24 days of incubation. This experiment was later repeated by adjusting the oil concentrations to 0.5%, 1.0%, and 1.5% of the BH medium with control (no oil supplemented). This time the enzyme activity tests were carried out at day 3, 6, and 9 days of incubation. Activities of secreted LiP in each culture aliquot were studied based on the evolution of hydrogen peroxide in the presence of veratryl alcohol and sodium tartrate serving as substrates [43–45]. For this analysis, a reaction volume containing 300 µL veratryl alcohol (2 mM), 300 µL sodium tartrate (100 mM at pH 4.5), and 40 µL H₂O₂ (0.4 mM) was mixed with 300 µL of the culture aliquots. Absorbance was measured at 460 nm (A₄₆₀) in a 10 min interval using a UV visible spectrophotometer (Perkin Elmer LAMDA 25). LiP activities was calculated and reported in U/mL. Manganese-dependent peroxidase activity was determined through the evolution of hydrogen peroxide in the presence

of magnesium sulphate and sodium tartrate as substrates, while phenol served as an indicator to monitor the reaction changes with time. Specifically, 40 μL magnesium sulphate (1 mM), 300 μL sodium tartrate (100 mM at pH 4.5), 0.01% phenol and H_2O_2 (60 μL) were mixed for reaction with the culture extracts (300 μL) according to the protocol followed by Paszcymski *et al.* [47] and Ameen *et al.* [44]. The decline in absorbance measured at 460 nm (A460) in a 10 min interval, and MnP activity was calculated and recorded in U/mL. In addition, Lac activity was determined according to the method of Novotny *et al.* [48] and Juhasz *et al.* [49] by measuring the oxidation of ABTS. 300 μL of sodium tartrate (100 mM at pH 4.5), and 300 μL ABTS (1 mM) were mixed with 300 μL of the culture extracts, and the decline in absorbance was measured at 490 nm (A490) in 5 min period intervals at 30 °C. Lac activity was calculated and recorded in U/mL (1 enzyme unit (U) = 1 $\mu\text{mol}/\text{min}$, where μmol refers to the amount of substrate converted) (<https://www.physiologyweb.com>).

2.6. Hydrocarbon Degradation Analysis

The ability of selected fungal strains to degrade used engine oil was studied in BH broth. 20 mL of this medium was supplemented with the used engine oil at 1% concentration (v/v) in conical flasks and inoculated with each fungus, while flasks with no fungus inoculated was treated as control. Mycelial plugs (5 mm diameter) were taken from 4-day old pure fungal cultures and transferred into the 20 mL BH-Hydrocarbon mixture kept in a shaker incubator at 80 rpm and 30 °C. The control was analyzed for hydrocarbon content using GC-MS at time (T0) and compared with medium treated with each fungus at day-15 of incubation. To determine the amount of hydrocarbon degraded, 1 mL aliquot solution was drawn from each

treatment and analyzed for hydrocarbon content at the inoculum moment and after 15 days of incubation. The 1 mL aliquot solution took was dissolved in 1 mL of dichloromethane (CH_2Cl_2), and vortexed vigorously for 1 min and then left on rack for 5 min to let the organic layer separate. The organic layer was carefully pipetted out into another tube, centrifuged for 3 min at 3000 rpm and then analyzed by gas chromatography coupled with mass spectrophotometry (Thermos Scientific DSQII single-quadrupole GC/MS system, Austin, TX, USA).

The GC/MS conditions were in split mode with injection at 250 °C, oven temperature was 70 °C for 1 min, 70–120 °C at 5 °C/min, 120–260 °C at 8 °C/min, and held at 260 °C for 5 min. A Restek Rxi- 5Sil MS 30 m × 0.25 mm × 0.25 μm film thickness capillary column was used with helium as the carrier gas at a constant flow rate of 1.0 mL/min. The transfer line temperature was 270 °C and the ion source temperature was 250 °C. Electron ionization mode was used with 70 eV, and the ions were registered in full scan mode in a mass range of m/z 35–800 amu. Chromatogram acquisition, detection of mass spectral peaks, and waveform processing were performed using Xcalibur MS Software Version 2.1 (Thermo Scientific Inc., Waltham, MA, USA). The assignment of chemical structures to chromatographic peaks was done based on comparison with the databases for the GC/MS National Institute of Standard and Technology (NIST) Mass Spectral Library (NIST 08) and Wiley Registry of Mass Spectral Data (8th Edition). The percentage content of each component was directly computed from the peak areas in the GC/MS chromatogram.

3. Results

3.1. Identification of Fungi Isolated from Crude Oil Polluted Sites Chemical analyses for the two aged crude oil polluted soils used for fungal isolation in this study confirmed elevated concentration of hydrocarbons and toxic metals as presented in Tables S1 and S2. Fourteen fungal strains were isolated from polluted soils and subjected to percentage incidence test to determine the dominant strains (Table 1). In the first polluted site (Yorla-10), fungal strain B appeared in 20 out of 30 samples, thus being the dominant fungus with 66.67% incidence. The second crude oil polluted site (Effurun) on the other hand had strain C as the dominant fungus, appearing in 17 out of 30 samples collected, with 56.67% incidence.

Table 1. Incidence and dominant fungal strains isolated from crude oil polluted sites.

| Strain Code | B-Yorla 10 | | C-Effurun | |
|-------------|------------------|-------------|------------------|-------------|
| | Incidence (N=30) | % Incidence | Incidence (N=30) | % Incidence |
| A | 9 | 30 | 1 | 3.33 |
| B | 20 | 66.67 | 13 | 43.33 |
| C | 13 | 43.33 | 17 | 56.67 |
| D | 0 | 0.00 | 0 | 0.00 |
| E | 5 | 16.67 | 11 | 36.67 |
| F | 3 | 10.00 | 2 | 6.67 |
| G | 0 | 0.00 | 3 | 13.33 |
| H | 11 | 36.67 | 0 | 0.00 |
| I | 1 | 3.33 | 1 | 3.33 |
| J | 5 | 16.66 | 11 | 36.67 |
| K | 2 | 6.67 | 3 | 10.00 |
| N1 | 9 | 30.00 | 0 | 0.00 |
| N2 | 6 | 20.00 | 5 | 16.67 |
| N3 | 11 | 36.67 | 0 | 0.00 |

The response and tolerance of these dominant fungi to complex hydrocarbon mixture (used engine oil) is presented in Table 2. It was observed that the two selected fungi survived the hydrocarbon mixture as high as 20% concentration in growth medium. Their ability to tolerate hydrocarbon presence was calculated based on dose inhibition response (DIR). Strain B had DIR of 75.52, 59.96, 51.24 and, 45.85 DIR on medium supplemented with 5, 10, 15, and 20% hydrocarbon respectively, while strain C had DIR of 53.97, 46.69, 39.07, and 39.40 respectively. The studied fungi were identified, based on morphological and molecular methods, as *Mucor irregularis* (strain B- Yorla10) and *Aspergillus oryzae* (C-Effurun).

Table 2. Hydrocarbon tolerance and dose inhibition by two selected fungal strains from crude oil polluted sites.

| Fungal strain | Oil concentration | Growth rate (cm/day) | DIR (%) |
|---------------|-------------------|----------------------|---------|
| B | 0% | 4.82 | 100.00 |
| B | 5% | 3.64 | 75.52 |
| B | 10% | 2.89 | 59.96 |
| B | 15% | 2.47 | 51.24 |
| B | 20% | 2.21 | 45.85 |
| C | 0% | 3.02 | 100.00 |
| C | 5% | 1.63 | 53.97 |
| C | 10% | 1.41 | 46.69 |
| C | 15% | 1.18 | 39.07 |
| C | 20% | 1.19 | 39.40 |

Phylogenetic analysis clarified the relationships of fungi isolated from crude oil-contaminated soil within *Mucor* and *Aspergillus*. Sequences

retrieved from the Nigerian sites analyzed strains could be aligned with sequences from fungi isolated from a variety of soils, animal faeces, water and oil-contaminated surface soils [37–39]. The neighbour-joining tree of *Mucor* fungi revealed that the sequence obtained from isolated *Mucor* strain B-Yorla10 in this study was closely related to KY474527-*Mucoromycotina* sp. previously found in the roots of *Drynaria quercifolia* in Philippines and *Mucor irregularis* found from rice (Figure 1). The phylogenetic tree from the *Aspergillus* dataset showed that the sequence amplified from strain C-Effurun clustered into a single well-supported clade (Figure 2) including endophytic fungi (MT071405) previously found in the kernels of *Coix lachrymal-jobi* cultivars (the best BLAST match, with 99% identity) and rhizosphere soil of *Lycium barbarum* in China.

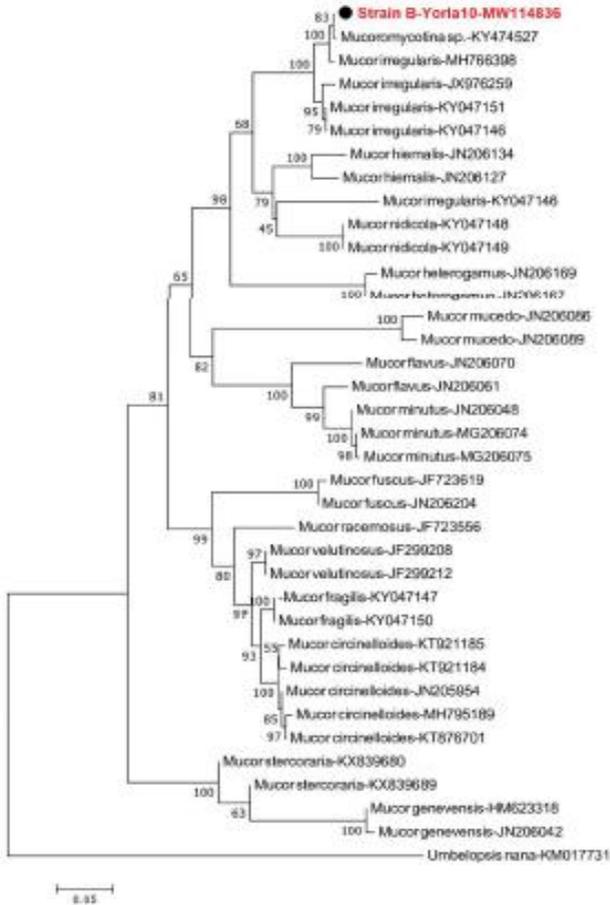


Figure 1. Neighbour-joining phylogenetic tree showing the relationship between the *Mucor* sequence from strain B-Yorla10 isolated from oil-contaminated soil in this study and selected database relatives. Kimura 2-parameter distances were used. Bootstrap values are based on percentages of 1000 replicates. The tree was rooted with *Umbelopsis nana* as outgroup.

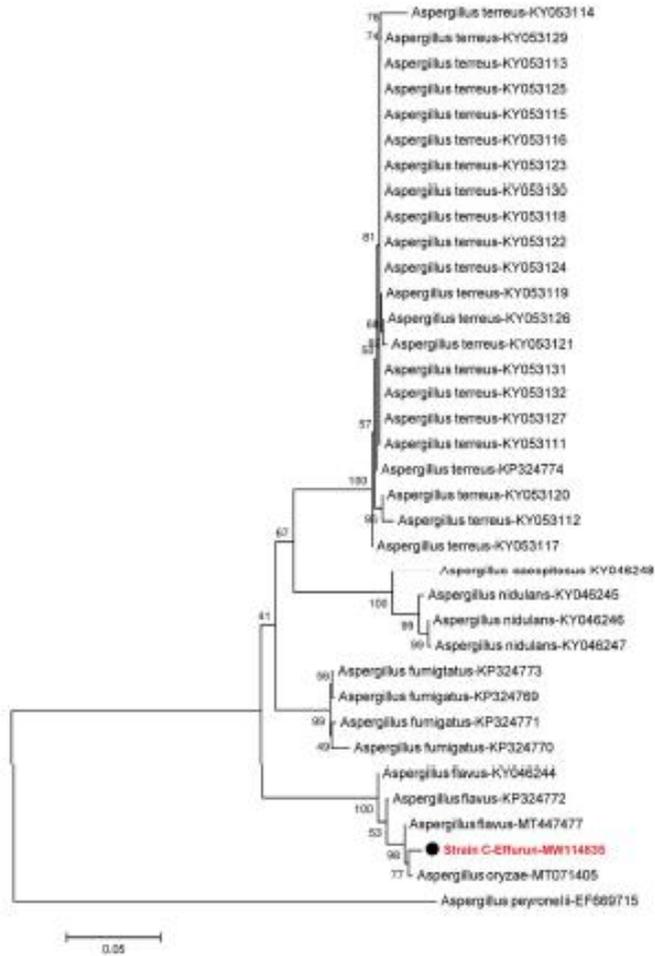


Figure 2. Neighbour-joining phylogenetic tree showing the relationship between the *Aspergillus* sequence from strain C-Effurun isolated from oil-contaminated soil in this study and selected database relatives. Kimura 2-parameter distances were used. Bootstrap values were based on percentages of 1000 replicates. *Aspergillus peyronelii* (EF669715.1) was used as the outgroup.

3.2. Assay and Activities of Enzymes Secreted by Selected Fungal Strains

In this study, it was demonstrated that the two isolated fungal strains, *Mucor irregularis* (B-Yorla10) and *Aspergillus oryzae* (C-Effurun) possess the ability to produce ligninolytic enzymes (LiP, MnP, and Lac). The plate assay showed enzyme secretions by the two strains. Indeed, the analyzed mycelia formed significant reddish-brown color on plates supplemented with different substrates as compared to control (Figure 3). Results from preliminary enzyme activity test for these strains is presented in supplementary Table S3. Generally, the two fungi showed enzyme activities in medium not supplemented with oil (control) but in low amount (Figures 4 and 5). It was also observed that the studied strains produced enzymes in response to oil concentrations in their growth medium and, in any case, they both showed more production of Lac as compared to LiP and MnP as presented in Figures 4 and 5. *M. irregularis* (B-Yorla10) at day 13 of incubation showed highest Lac activities of 15.00, 13.90, 11.07 U/mL in BH medium supplemented with 1.5%, 1.0%, and 0.5% engine oil respectively (Figure 4). *A. oryzae* (C-Effurun) showed highest Lac activities of 36.0 and 27.37 U/mL at day-9 and day-6 respectively, while its least Lac activity of 2.11 U/mL was recorded on day-3. Strain C-Effurun showed highest MnP activities of 12.40 and 11.38 U/mL in medium supplemented with 1.5 and 1.0% oil respectively (Figure 5). Both fungi showed some peroxidase activities; strain B-Yorla10 showed highest expression of 2.70 U/mL of LiP in medium supplemented with 0.5 and 1% oil respectively at day-3, while strain C-Effurun showed highest expression of 3.73 U/mL of LiP in medium supplemented with 1% oil also at day-3 (Figures 4 and 5). Generally, both strains grew in BH medium with no oil supplement (control), but they

showed better enzyme activity in media supplemented with oil as the only carbon source.

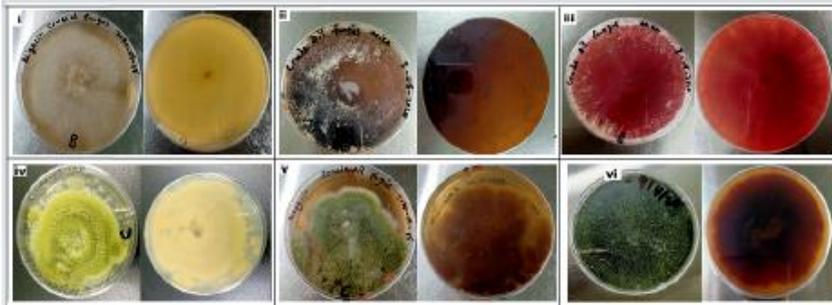


Figure 3. Plate assay for colorimetric screening of ligninolytic enzymes produced by the isolated fungi. (i) Control—*Mucor irregularis* strain B-Yorla10 growing on MEA without chemicals (ii) *Mucor irregularis* strain B-Yorla10 showing reddish brown coloration on reaction with MEA supplemented with CuSO₄ and Guaiacol (iii) *Mucor irregularis* strain B-Yorla10 showing reddish brown coloration on reaction with MEA supplemented with CuSO₄ and ABTS (iv) Control—*Aspergillus oryzae* strain C-Effurun growing on MEA without chemicals (v) *Aspergillus oryzae* strain C-Effurun showing reddish brown coloration on reaction with MEA supplemented with CuSO₄ and Guaiacol (vi) *Aspergillus oryzae* strain C-Effurun showing reddish brown coloration on reaction with MEA supplemented with CuSO₄ and ABTS.

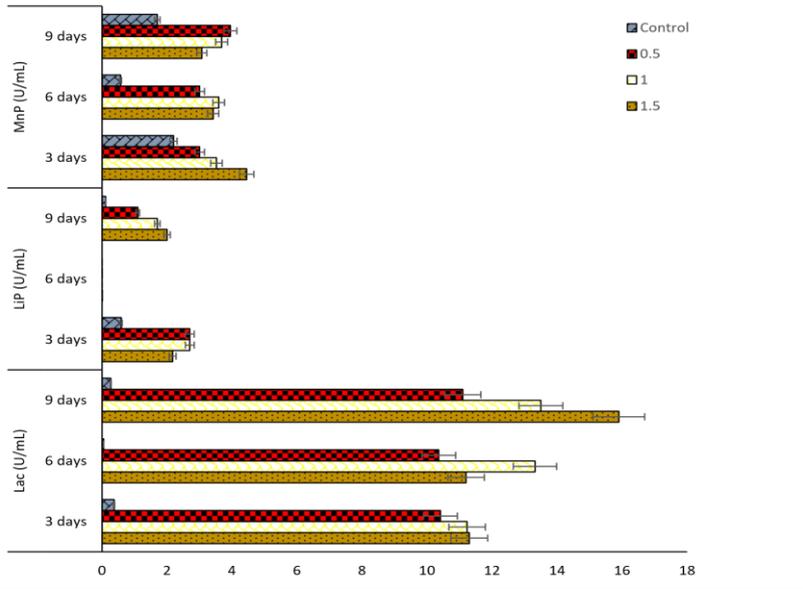


Figure 4. Activities of extracellular enzymes produced by *Mucor irregularis* B-Yorla10 isolated from a crude oil polluted site in Nigeria.

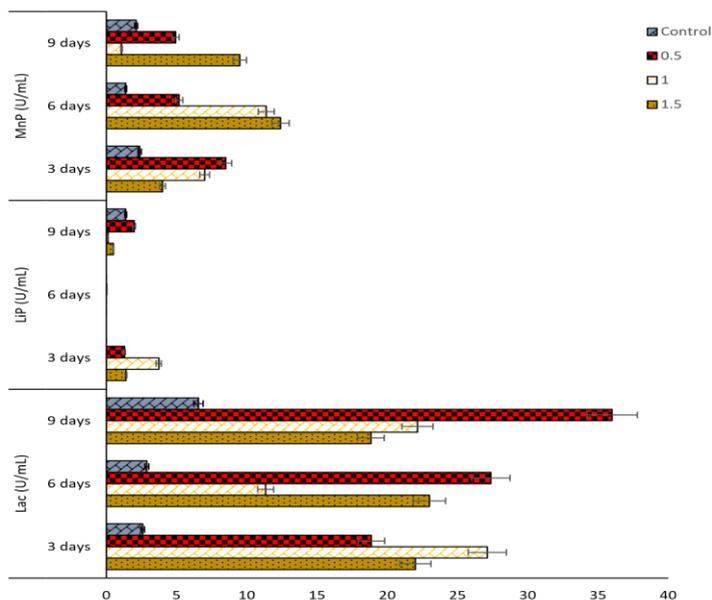


Figure 5. Activities of extracellular enzymes produced by *Aspergillus oryzae* C-Effurun isolated from a crude oil polluted site in Nigeria.

3.3. Hydrocarbon Degradation

The composition of engine oil used in this study was analysed by GC/MS (Figure 6). The oil included aliphatic components (e.g., C_1 – C_{20} and C_{20} – C_{50}), aromatics (e.g., benzene, toluene, ethylbenzene, and xylene isomers (BTEX), Alkyl benzenes, Alkyl indenenes, Alkyl tetralines, Alkyl biphenyls, polycyclic aromatics (PAHs)), and other compounds, such as methyl esters. The most abundant compounds in the used engine oil were the BTEX, C_{20} – C_{50} aliphatics, and PAHs together making 67% of the total mixture (Figure 6). It was observed that both fungi modified the hydrocarbon composition of the used engine oil in terms of as presented

in Figure 7. The chromatogram of oil composition for the control and treatments is presented in Supplementary File Figure S2. It was observed after a 15-day incubation that *A. oryzae* (C-Effurun) significantly reduced several components of the used engine oil as compared to *Mucor irregularis* B-Yorla10. More specifically, *A. oryzae* (C-Effurun) reduced all the hydrocarbons in the used engine oil, while the strain *Mucor irregularis* B-Yorla10 only showed a significant degradation activity of long-chain alkanes and BTEX. However, the degradation of long-chain alkanes by the latter fungus resulted in increased short chain alkanes in the treatment as compared to the control.

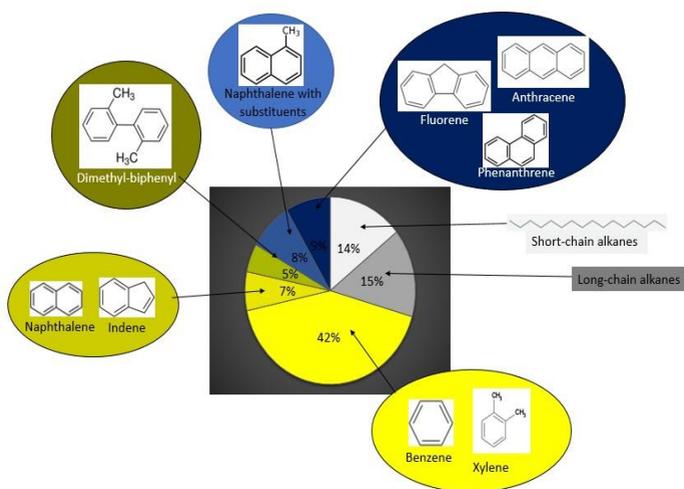


Figure 6. Composition of the used engine oil as revealed through GC/MS analysis.

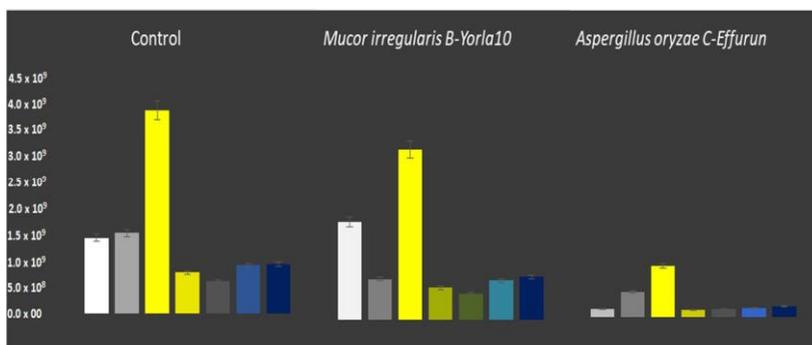


Figure 7. Degradation of hydrocarbon fractions in used engine oil by selected filamentous fungi. Degradation of used engine oil by *Mucor irregularis* and *Aspergillus oryzae*. The compounds were grouped in classes of Benzene (yellow), Naphthalene and Indene (dark-yellow), Dimethyl-biphenyl (brown), Naphthalene and substituents (blue), the group of Anthracene, Fluorene and Phenanthrene (dark-blue), Short-chain alkanes (white) and Long chain alkanes (grey).

4. Discussion

In this study, the two most frequently occurring fungi *Aspergillus oryzae* and *Mucor irregularis* were isolated from aged crude oil-polluted sites in Niger Delta Area of Nigeria. The isolated *Mucor* strain (B-Yorla10) was highly phylogenetically close to *Mucoromycotina* sp. discovered by Aban *et al.* [50,51] as a symbiotic fungus from the root of epiphytic fern, *Drynaria quercifolia* in Philippines being the dominant species with high colonisation rate [50]. In another study performed later by the same authors, this fungal strain isolated from fern showed significant capability in protecting rice seedlings against drought [51]. *M. irregularis* found from rice by Cai. W. in an unpublished study also revealed a close relationship with our isolated

Mucor strain. This result proves the wide ecological distribution of *Mucor* in soil and as plant associate, and may indicate the potentiality of *Mucor* to be applied in the field of environmental protection and agricultural industry. Chukwura *et al.* reported hydrocarbon degrading potentials of *M. racemosus* isolated from soil samples collected from a mechanic shop at Awka, Anambra State, Nigeria [52]. Our result represents a new record of the hydrocarbon degrading potential of *M. irregularis* isolated from oil-polluted soils in Nigeria.

The result of phylogenetic analysis showed that strain C-Effurun was similar to *Aspergillus oryzae* isolated as endophytic fungus from kernels of *Coix lachrymal-jobi* cultivars by Li *et al.* in China [53]. The latter study demonstrated that *A. oryzae* could produce a variety of enzymes including proteinases, amylases, glycases, cellulases and phytases, which promoted the degradation of the unabsorbable crude fiber and phytin [53]. The high enzyme-producing activity of *A. oryzae* was confirmed in our study where the analysed strain showed capabilities to produce laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). These results enriched our knowledge on the enzyme resource pool secreted by *A. oryzae*, suggesting that studied fungus could be regarded as a potential candidate for exploitation in microbial chemistry and hydrocarbon degradation.

In this study, it was found that both fungi could tolerate varying concentrations of used engine oil and utilise hydrocarbons as sole carbon source. While *M. irregularis* grew faster on solid BH medium supplemented with 20% used engine oil, *A. oryzae* tend to grow slower. On the contrary, the hydrocarbon degradation results showed that *A. oryzae* had more degradative potential as compared to *M. irregularis* which was confirmed by its higher enzymes production. Our findings confirm that fungal tolerance to

complex hydrocarbons is not a function of their degradation ability, and also that the enzyme secreting ability in fungi enhance hydrocarbon degradation faster than tolerance [23,25]. Several previous studies have shown the degradative capabilities of *Aspergillus* and *Mucor* species [54–56]. For example, Barnes *et al.* [57] reported 12 *Aspergillus* species able to degrade crude oil and derivatives, including *Aspergillus flavus*, *A. sydowii*, and *A. versicolor*. According to Harms *et al.* [58] and Banerjee *et al.* [59], many fungal species such as *Aspergillus niger*, *A. flavus*, and *RhizoMucor variabilis* can transform complex hydrocarbons to nontoxic compounds through different catabolic pathways. This is also in accordance with our observation that there was an increase in short-chain aliphatic hydrocarbons in medium used to grow *M. irregularis* as compared to their initial concentration in the used engine oil. This study showed that the investigated crude oil polluted sites were colonised by fungi having adapted mechanisms to degrade and mineralise hydrocarbons as sole carbon source. Our results are in accordance with a previous research where *A. terreus* isolated from hydrocarbon oil contaminated mangrove sediments from Red Sea coast of Saudi Arabia was found to accumulate significantly higher biomass, produce extracellular enzymes, and degrade 28– 56% hydrocarbons [60].

Some previous studies carried out on degradation of specific PAHs by *Aspergillus* fungi showed significant degradation of anthracene and naphthalene [61]. *A. terreus* isolated from a polycyclic aromatic hydrocarbons polluted soil degraded and metabolised Pyrene and benzo(a)pyrene [62–64], while *A. niger* was used for bioremediation of anthracene by AI-Jawhari [65]. In the latter work, degradation rate of benzo[a]pyrene in co-culture of *A. niger* and *Penicillium funiculosum* medium doubles that of medium without *A. niger*. AI-Jawhari [66] found

95% maximum crude oil bioremediation by mixed culture of *A. niger* and *A. fumigatus* after 28 days. Mahmoud *et al.* [67] found that *A. terreus* isolated from kerosene-polluted soil had a potential for both lipase production and crude oil degradation. The authors also suggested the use of hydrocarbon-polluted soils for fungal isolation in further studies seeking for highly lipase-producing and crude oil-removing fungi [68].

Although this study demonstrated that *A. oryzae* degraded more hydrocarbons than *M. irregularis*, the latter fungal species was found to act more specifically on long-chain hydrocarbons, thus resulting in increased short chain hydrocarbon concentrations after 15-day incubation as compared to the initial concentration in the used engine oil. In a previous study, *Mucor racemosus* was found to degrade about 50% of benzo[a]pyrene when subjected to metabolism evaluation using HPLC-DAD-MS technique [69,70]. Fungi in the *Mucoromycotina* have been continuously listed along with *Ascomycota* and *Basidiomycota* as major fungal *taxa* that are commonly involved in the biodegradation of oil [56,71–76]. *Mucor mucedo* was specifically characterised for exopolymeric substances produced during PAH degradation process in a work performed by Jia *et al.* [77]. Srinivasan and Viraraghavan [78,79] confirmed the application of *Mucor rouxii* and *Absidia coerulea* for biomaterial production in chitosan and walnut shell media for the removal of oil from aqueous solutions.

Enzymes produced by many filamentous fungi have been demonstrated to aid the degradation/mineralisation of hydrocarbons. For instance, biodegradation of diesel oil hydrocarbons was enhanced with enzyme preparations from *M. circinelloides* [80]. Biodegradation of slop oil by endophytic *Bacillus cereus* EN18 was enhanced by adding lipase from *RhizoMucor miehei* [81,82]. Asemoloye *et al.* [23,24] found the presence of

laccase and peroxidase genes in *A. niger* and correlated their expressions with hydrocarbon degradation of Nigeria Bonny light crude oil. Similarly, it was earlier reported by Ramirez *et al.* [83] that laccases and peroxidases were involved in fungal environmental response to different concentrations of carbon, nitrogen and other pollutants, such as toxic metal ions, pesticides, and hydrocarbons. In the latter study, the authors explained that these fungal enzymes also serve as an abiotic stress signal in temperature shock, various lengths of day light, and increased xenobiotic pollution [84]. Laccase and peroxidase enzymes are produced by fungi as secondary metabolites. Therefore, these enzymes production may be affected by available carbon or nitrogen concentrations in the substrates where the fungi grow [85–87]. This is in agreement with our observation that the concentration and chemical nature of engine oil supplemented in culture medium as the only carbon source significantly affected the fungal growth and their enzyme secretion capacity. It was also observed that the two analysed fungi produced more laccase than peroxidases. Laccase is a multicopper phenol enzyme that has been reported to take different roles in oxidation of several phenolic, aromatic amine compounds. This enzyme acts by oxidising several phenolic and aromatic compounds, using oxygen as terminal electron acceptors, as reported in different fungal species [87]. Laccase is becoming more popular in biotreatment of different recalcitrant materials and waste effluents from many industries. It has been well reported for many applications like the removal of aromatic phenolic compounds, deoxygenation of many hydrocarbon oils, treatment of waste waters, dye removals, and pulp bio-bleaching.

5. Conclusions

In this study, the dominant fungal strains isolated from two crude oil polluted sites were identified as *Aspergillus oryzae* and *Mucor irregularis*. The two fungi showed high tolerance to varying concentrations of a complex hydrocarbon mixture (used engine oil) and demonstrated hydrocarbon degradation abilities. The different enzyme expressions and activities showed by the analyzed fungi could be helpful for their survival in contaminated environments, by allowing them to utilize the hydrocarbons present in the substrate as nutrients. Therefore, these two strains are potential candidates for the remediation of hydrocarbon polluted soils. However, further studies are needed to understand, from genetic and biochemical points of view, the hydrocarbon degradation mechanisms of these fungi, and therefore enhance their degradation performance. This can be achieved through the use of the traditional recombinant DNA technology and advanced gene manipulation tools such as the CRIPR-Cas systems [88]. Efforts are ongoing in our laboratory to obtain more information on the chemical processes and pathways used by these fungi in degrading hydrocarbons. We are also aiming to characterize possible biosynthetic gene clusters/genes associated with laccase expression in these two fungi and attempt to further enhance their expressions in heterologous yeast hosts.

Supplementary Materials: The following are available online at www.mdpi.com/2076-2607/8/12/1912/s1. Table S1: Chemical properties of the contaminated soils. Table S2: Toxic metal composition of the contaminated soils. Table S3: Preliminary enzyme activities of *Aspergillus oryzae* and *Mucor irregularis* isolated from Nigerian crude

oil polluted sites. Figure S1: Location of crude oil polluted sites from which fungi were isolated Figure S2: Degradation of complex hydrocarbon mixture (engine oil) by selected filamentous fungi as determined by GC/MS analysis.

Author Contributions: M.D.A. conceived the study; the experiment was designed and supervised by S.T., L.P., S.G.J., M.A.M., and W.G.; the strains were isolated by M.D.A., while laboratory experiments and other analysis were performed by M.D.A., C.D., X.W., and S.X.; results were analyzed by L.P., M.D.A., C.D., X.W., and S.X.; M.D.A. prepared the original draft while all authors read, reviewed, and edited the manuscript and agreed to the published version of the manuscript.

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Chapter 2: Isolation and identification of fungal strains from hydrocarbon-contaminated substrates

Abstract

Autochthonous microbial strains were isolated from contaminated substrates from a gas production plant in Pakistan. Twenty-nine fungal strains belonging to eleven different genera and 50 bacterial strains have been isolated, many of which cannot be used for biosafety reasons (not belonging to group 1 of biohazard agents). The isolates have been identified morphologically (fungi) and molecularly (bacteria and fungi). Almost all the isolated strains were already known for their hydrocarbon-degrading properties, giving good reasons of hope for the results of subsequent experiments.

2.1 Introduction

2.1.1 Methods to isolate strains from contaminated substrates

As already highlighted in the Introduction chapter (paragraph 1.4.2), an effective method to isolate strains with hydrocarbon-degrading abilities is to search them in a contaminated environment. It is now recognised that indigenous microorganisms screened from polluted soils were often more effective to metabolize hydrocarbons than organisms obtained from elsewhere in bioremediation (Li *et al.*,

2008). The hydrocarbons in the environment have led to the development of microorganisms perfectly adapted to survive in hostile environments because hydrocarbon pollution modifies the composition of the microbiomes, influencing the structure and the metabolic activity of the microbial community (Liang *et al.*, 2014; Galazka *et al.*, 2018). The scientific literature reports a considerable number of papers about fungal and bacterial strains isolated from sites polluted by hydrocarbons. Marchand *et al.* (2017) isolated 781 bacterial strains and 279 fungal strains from soil samples obtained at a former petrochemical hydrocarbon biodegradation potential selecting, as promising strains, 95 bacterial and 160 fungal strains. The fungi belonged to three major taxonomic groups, 45 *Dothideomycetes*, 73 *Sordariomycetes*, and 42 *Mucoromycotina*. In comparison, bacteria belonged to five major taxonomic groups: 30 *Actinobacteria*, 23 *Bacilli*, 9 *Alphaproteobacteria*, 10 *Betaproteobacteria*, and 23 *Gammaproteobacteria*. Spini *et al.* (2018) isolated a total of 95 bacterial and 94 fungal strains from substrates enriched with PAHs, paraffines and benzene. On the whole, isolated bacteria were mainly ascribed to *Pseudomonas* genus, followed by *Sphingobacterium*, *Bacillus*, *Stenothrophomonas*, *Achromobacter* and *Serratia*. As for fungi, *Fusarium* was the most abundant genus, followed by *Trichoderma* and *Aspergillus*. Furthermore, more than 50 fungal strains were isolated from different aged PAH-contaminated soils sampled from four brownfields in the North of France. The

identifications revealed a large predominance of the Ascomycota phylum, and only one strain belongs to the Zygomycota phylum and the other to the Ascomycota phylum. Among these, *Penicillium canescens*, *Cladosporium cladosporioides*, *Fusarium solani*, and *Talaromyces helicus* degraded more than 30% of the initial 500 µg benzo[a]pyrene after 9 days of incubation (Fayeulle *et al.*, 2019). Bacteria and fungi were also isolated from eight different soil samples from different Kazakhstan regions contaminated with hydrocarbon plant site in Canada. The researchers then investigated their petroleum compounds. Thirty-two strains from 14 different genera were isolated, and 62% of them belonged to well-described hydrocarbon degraders like the bacteria *Rhodococcus*, *Acinetobacter* and *Pseudomonas*. Among fungi, *Fusarium*, *Candida*, and *Yarrowia* species were found (Mikolasch *et al.*, 2019).

Therefore, this work aimed to isolate and identify indigenous microbial strains from hydrocarbon-contaminated substrates of a Pakistan gas production plant.

2.2 Materials and Methods

2.2.1 Substrates used for the microorganism isolation

In October 2017, contaminated substrates with hydrocarbons of different nature were provided to the Mycology Laboratory of the University of Pavia to isolate fungal strains. These substrates came

from a gas plant in Pakistan owned by ENI. Simultaneously, in ENI laboratories, bacterial strains were isolated from the same samples. The type and the characteristics of the substrates are listed in Table 2.1. More information about the precise sampling location is not reported in this thesis as they are reserved data from ENI.

Table 2.1. Substrates for the isolation of microbial strains. Received from ENI S.p.A. and collected in Pakistan.

| Substrate | Type of substrate |
|------------------|---|
| Settled water | Water used to clean the tanks after production. A dark particulate is visible, given by the processing residues detached from the tank's surface thanks to the mechanical action exerted by the water lances used for cleaning. |
| Soil | Soil polluted by hydrocarbons from the area surrounding the site. |
| Water | Sample of clean "settled water" used for tank cleaning. |
| Slop | Pink-coloured liquid substrates. It is a mixture of light alkanes ($< C_{18}$) and light aromatic hydrocarbons. |
| Condensate-16 | It comprises two phases: a heptane-soluble phase mainly composed of medium-long chain alkanes and a black and tarry non-chromatographable fraction of difficult characterisation. |

2.2.2 Isolation of fungal strains from hydrocarbon-contaminated substrates

Autochthonous fungi were isolated following the Prenafeta-Boldú *et al.* (2001) procedure. Aliquots of each substrate received were distributed on Petri plates to screen the fungal populations naturally present in it. The liquid substrates (1 mL of Settled water, water, Slop and Condensate-16) were evenly plated on Potato dextrose agar (PDA), the ideal medium for the growth of fungal organisms, and, in order to prevent the growth of bacterial colonies, 1 mL of antibiotics mix, containing different antibiotics that can act on a wide range of bacterial strains, was distributed on each plate. All the medium recipes and the antibiotics recipe are reported in Supplementary Materials 1.

The strains were obtained from soil substrate following D'Annibale *et al.* (2006) protocol. 1 mL of soil dilution 10^{-4} and 10^{-5} , prepared in distilled sterile water, was distributed on Rose Bengal (RB) plates. RB was used as growth medium because it contains the dye Rose Bengal that retard the radial growth of filamentous fungi facilitating their isolation and counting (Banks *et al.*, 1985). All the plates were prepared in double copy and incubated at 26°C for periods ranging from 7 to 14 days. Pure fungal cultures were obtained by subsequent strains transfers to fresh PDA plates.

Despite the use of RB medium and serial dilutions to isolate strains from the soil, the process proved difficult due to the high number of strains present. In order to initially select only the fungal

able to grow on complex hydrocarbon substrates from the soil sample, another experiment was carried out. Petri plates containing minimum Water agar (WA) medium (without carbon source) and antibiotics mix were prepared, and 1 mL of soil dilution 10^{-4} and 10^{-5} was spread. An aliquot of 1 mL of Settled water, Condensate-16 and Slop, was also spread on separate AW plates. Settled water, Slop and Condensate-16 represented complex hydrocarbons substrates since they contain hydrocarbons residues from the particular place they were collected. All the plates were prepared in double copy and incubated at 26°C for 7 to 14 days. Pure fungal cultures were obtained by subsequent strains transfers to fresh PDA plates.

2.2.3 Isolation of bacterial strains from hydrocarbon-contaminated substrates

I present here the procedure of bacteria isolation, but this step was carried on ENI S.p.A. by Dr Giovanna Carpani. Bacteria were isolated through the enrichment culture technique (Obi *et al.*, 2016). The 1% of each contaminated substrate was inoculated in 1 L flasks, closed with Teflon-coated caps and aluminium rings to prevent hydrocarbon leakage, filled with 100 mL sterile mineral salts medium Büshnell-Hass (BH). Cultures were incubated in a rotary incubator at 90 revolutions per minute (rpm) and 26 ° C for two weeks. After this time, an aliquot of 1% was sub-cultured onto a fresh BH medium with 1% of Condensate-16 or Slop as hydrocarbon source to make an initial

selection of strains adapted to the presence of hydrocarbons. Incubation continued for additional 9 days under the same conditions previously described. A further 1% dilution step in BH was made and incubated under the same conditions for 6 days. Subsequently, serial dilutions of the enriched cultures were made in complete medium Luria Broth (LB), and 100 mL of each dilution was plated onto LB agar (LBA) plates. The plates were incubated at 26° C until the colonies were evident and transferable on new LBA plates.

2.2.4 Morphological and molecular identification of fungal strains

Each fungal morphotype was isolated as a pure culture on Malt Extract Agar (MEA) incubated at 26 °C for seven days or more based on when isolates sporulate. All the fungal strains were identified by their macro and microstructural morpho-dimensional examination under a light microscope (40x). The morphological characteristics observed include pigment production, colony aspect and reproductive structures. Morphological traits useful for taxonomical identification were compared with dichotomous keys for morphological identification (Klich, 2002; Kirk *et al.*, 2008; Seifert *et al.*, 2011). Strains not showing the reproductive structures after a prolonged incubation time were named *mycelia sterilia*.

The fungal genomic DNA was extracted using the NucleoSpin Plant II by Macherey-Nagel (Bethlehem, PA, USA), with one hour of incubation at 65 °C after the addition of RNase, and then subjected to

PCR amplification of the internal transcribed spacer (ITS) region of the ITS1-5.8S-ITS2 rDNA gene. The PCR reaction was performed on a Thermal Cycler Bio-Rad T100 in 20 μ L reaction mixture containing DREAM Taq Green PCR MasterMix (2 \times) reaction buffer (Thermo Fisher Scientific, Pittsburg, PA, USA), 2 μ L (5 μ M) of each primer, 2 μ L of DNA sample (10 ng), and 4 μ L of Nuclease Free water. The PCR program was as follows: denaturation by heating for 5 min at 95 $^{\circ}$ C, then 35 cycles of 30 s at 95 $^{\circ}$ C, 45 s at 50 $^{\circ}$ C, and 1 min at 72 $^{\circ}$ C, and a final elongation step for 10 min at 72 $^{\circ}$ C. The primers used were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). PCR products were purified with ExoSAP-IT (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The amplified and purified DNA was sent to BMR Genomics (Padova, Italy), and the sequences were compared with target sequences using BLAST online (<https://blast.ncbi.nlm.nih.gov/>) and Mycobank (<https://www.mycobank.org/>).

The extraction, amplification, and sequencing of bacterial DNA was carried out in ENI's laboratories. The extraction was performed using the FastDNATM SPIN Kit for Soil by MPbio (Illkirch-Graffenstaden, France). The extracted DNA was amplified through PCR reaction was performed on a Biometra Thermocycler T-Gradient ThermoBlock in 20 μ L reaction mixture containing 10 μ L PCR MasterMix (2 \times) reaction buffer (Promega, Madison, WI, USA), 0,2

μL (3 μM) of each primer, 1 μL of DNA sample (2 ng), and 8,6 μL of Nuclease Free water. The PCR program was as follows: denaturation by heating for 5 min at 95 °C, then 33 cycles of 30 s at 95 °C, 1 min at 53 °C, and 2 min at 72 °C, and a final elongation step for 5 min at 72 °C. The primers used were 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). PCR products were purified with ExoSAP-IT (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The DNA bacterial DNA was sequenced in Applied Biosystem 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained 5,5 μL of purified DNA, 0,5 μL of the used primers and 4 μL of BigDye sequencing buffer from BigDye™ Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The reactions were performed according to the manufacturer's protocol.

2.3 Results and Discussion

2.3.1 Selection of microbial strains based on the guidelines for biological risk in the workplace

The handling of biological agents is regulated at the global level by the World Health Organisation (WHO) and at the national level by the Titolo X, D.Lgs. 81/08 regarding biological risk in the workplace.

Due to differences in pathogenicity, microorganisms have been classified based on their risk for humans and animals. WHO (2004) grouped microorganisms according to their hazard in 4 groups:

- Risk Group 1 (no or very low individual and community risk).
A microorganism that is unlikely to cause human or animal disease;
- Risk Group 2 (moderate individual risk, low community risk).
A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment;
- Risk Group 3 (high individual risk, low community risk). A pathogen usually causes severe human or animal disease but does not ordinarily spread from one infected individual to another;
- Risk Group 4 (high individual and community risk). A pathogen that usually causes serious human or animal disease and can be readily transmitted from one individual to another, directly or indirectly.

For this reason, we needed to exclude from the list of potentially useful bacterial strains all of those selected not belonging to group 1 of biological risk agents. This is because ENI's laboratories follow a rigid security protocol, and the laboratories I worked in were not adapted to the requested security levels for manipulating these agents. The bacterial strains excluded were not tested or kept in our collations.

Concerning fungal strains, on the other hand, the mycology laboratory has less restrictive rules. We cannot work with species in group 3 or 4, but we can handle group 2 species with the requested precautions.

2.3.2 *Fungi isolated from contaminated substrates*

From ENI's substrates, 29 fungal strains belonging to 11 genera have been characterised as presented in tables 2.1-2.2. All identified strains are filamentous fungi, cosmopolitan, ubiquitous saprotrophs, belonging to the Phylum Ascomycota, except for L1, the yeast *Rhodotorula mucilaginosa* (Basidiomycota), and F3 *Rhizopus* sp. (Mucoromycota).

The first evidence emerging from these results is that the strains most isolate belong to the Ascomycota phylum. This tendency correlated well with other studies resting on fungal isolation from aged hydrocarbon-contaminated soils. Indeed Potin *et al.* (2004) isolated 19 Ascomycota, 1 Zygomycota and 1 Mastigomycota from an aged hydrocarbons-contaminated soil and Zafra *et al.* (2014) isolated 20 Ascomycota and 1 Zygomycota.

The second result is that the fungal strains isolated on minimum medium (AW) supplemented with hydrocarbon sources are less than the ones isolated on rich medium (PDA and RB). From AW and hydrocarbons were isolated 10 different strains belonging to 6 different genera (3 *Fusarium*, 2 *Alternaria*, 2 *Aspergillus*, 1

Penicillium, 1 *Curvularia*, and 1 *Coniochaeta*) while from PDA and RB the different strains were 19 belonging to 10 different genera (4 *Aspergillus*, 3 *Cladosporium*, 2 *Alternaria*, 2 *Penicillium*, 2 *Trichoderma*, 1 *Fusarium*, 1 *Chrysosporium*, 1 *Scedosporium*, 1 *Rhodotorula* and 1 *Rhizopus*). This is not surprising since AW is a very restrictive soil with no nutrient source. However, the purpose of this type of isolation was to pre-select the strains present in the soil from the area surrounding the gas plant and, therefore, certainly able of using hydrocarbons as their only carbon source.

In general, the most retrieved genera were *Aspergillus* (21%), *Alternaria* and *Fusarium* (14%), *Cladosporium* and *Penicillium* (14%) (Fig. 2.1). These genera were frequently reported in many papers as isolated from hydrocarbon-contaminated substrates. For example, *Aspergillus*, *Fusarium* and *Penicillium* species were found in soil samples contaminated with oil (Romero *et al.*, 2002; Chaillan *et al.*, 2004; Al Nasrawi, 2012; Reyes-César *et al.*, 2014). In other studies, *Penicillium*, *Cladosporium* and *Fusarium* were isolated from aged PAH-contaminated soils, while four *Penicillium* species were found in soil at a former gasworks site (Saraswathy and Hallberg, 2002; Fayeulle *et al.*, 2019).

Even many of the species isolated in this work were already isolated from contaminated environments. *Fusarium solani* was found in petrol station soil (Hong *et al.*, 2010; Fayeulle *et al.*, 2019) and in historically contaminated soils (Fayeulle *et al.*, 2019); *Cladosporium*

cladosporioides was isolated from soil of an aged gas manufacturing plant (Potin *et al.*, 2004) and a Mediterranean marine site chronically interested by oil spills (Bovio *et al.*, 2017). In the same work, they also reported a *Trichoderma harzianum*.

Particular attention should be paid to the fungus *Scedosporium apiospermum*. This species was isolated from crude-oil contaminated soil by Reyes-César *et al.* (2014) and from a biofilter operated with toluene by García-Peña *et al.* (2001). Nevertheless, it has been recognized as a group 2 agent: a potential etiologic agent of severe infections in immunocompromised and occasionally in immunocompetent patients (Guarro *et al.*, 2006). Its use should therefore be limited or otherwise subject to special cautions.

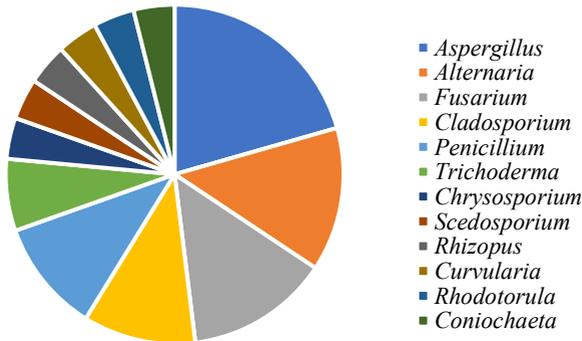


Figure 2.1. Isolated fungal strains grouped by genera.

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Table 2.1. Fungal strains isolated from the mix of substrates and their identification.

| Culture medium | Substrate | Strain | Species | |
|----------------------|-------------------|---------------|--|------------------------------------|
| PDA + antibiotics | Slop | F1 | <i>Alternaria alternata</i> | |
| | | F2 | <i>Fusarium</i> sp.1 | |
| | | F4 | M <i>Cladosporium cladosporioides</i> | |
| | Condensate- 16 | F5 | <i>Aspergillus</i> sp.1 | |
| | | F8 | <i>Chrysosporium</i> sp. | |
| | | F10 | M <i>Scedosporium apiospermum</i> | |
| | | F27 | <i>Penicillium</i> sp.1 | |
| | | F23 | <i>Aspergillus</i> sp.2 | |
| | | F25 | <i>Trichoderma</i> sp.1 | |
| | | F30 | <i>Aspergillus</i> sp.3 | |
| | | F31 | <i>Aspergillus</i> sp.4 | |
| | | F26 | M <i>Trichoderma harzianum</i> | |
| | | Settled Water | F6 | <i>Cladosporium sphaerospermum</i> |
| | | | F7 | <i>Alternaria</i> sp.2 |
| RB + antibiotics | Soils | F32 | <i>Cladosporium</i> sp.3 | |
| | | F34 | <i>Penicillium</i> sp.2 | |
| | | F35 | <i>Mycelia sterilia</i> | |
| | | L1 | M <i>Rhodotorula mucilaginosa</i> | |
| | | F3 | <i>Rhizopus</i> sp. | |

The **M** indicates the strains for which molecular identification has also been performed.

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Table 2.2. Fungal strains isolated from the mixed substrates and their identification.

| Culture medium | Substrate | Strain | Species | |
|---------------------|-------------------------|-------------|--|-------------------------|
| AW + antibiotics | Soil + Settled water | F22 | <i>Aspergillus</i> sp.5 | |
| | | F40 | <i>Fusarium</i> sp.2 | |
| | Soil + Condensate-16 | F13 | <i>Alternaria alternata</i> | |
| | | F15 | M <i>Penicillium oxalicum</i> | |
| | | F28 | M <i>Curvularia aerea</i> | |
| | | F39 | M <i>Fusarium solani</i> | |
| | | Soil + Slop | F21 | <i>Aspergillus</i> sp.6 |
| | | | F29 | <i>Alternaria</i> sp.4 |
| | F37 | | M <i>Coniochaeta</i> sp. | |
| | | F41 | M <i>Fusarium chlamyosporum</i> | |

The **M** indicates the strains for which molecular identification has also been performed.

2.3.3 Isolated bacterial strains from contaminated substrates

The bacterial strains isolated are 50 (Tab. 2.3), many more than the fungal strains, but, as for the fungi, they all belong to 7 genera (Fig. 2.2). This apparently suggests lower biodiversity of the bacterial community in these contaminated substrates than fungi. However, the isolation methods used are different, as the method used to isolate the bacteria also included the selection of strains capable of growing on hydrocarbons as a sole carbon source. The bacterial strains isolated are, therefore, all already potentially useful for bioremediation processes. Many of them, in fact, are already known for their degradation capabilities of complex substrates such as crude oil (Deng *et al.*, 2014; Prathyusha *et al.*, 2016), diesel (Garrido-Sanz *et al.*, 2019), petrochemical wastewater (Ahmadi *et al.*, 2017), pesticides and heavy metal-contaminated soils (Ng *et al.*, 2009).

A very noticeable result is that 44% of the isolated bacterial strains do not belong to group 1 of the biological agents and, therefore, were not used in this work and were not maintained in ENI's bacterial strains collection. They belong to *Achromobacter* (16%), *Klebsiella* (14%), *Stenotrophomonas* (10%) and *Salmonella* (4%).

Pseudomonas is the most represented genus among the isolates that can be used (46%), followed by *Gordonia* (8%) and *Leucobacter* (2%). *Pseudomonas* has often been isolated from contaminated environments, as reported by Barathi and Vasudevan (2001), Obayori *et al.* (2008) and Zhang *et al.* (2011). In particular, *Pseudomonas*

stutzeri, the most found strain, was isolated from coal mines in India (Singh and Tiwary, 2017), from high polluted soil near-surface impoundments of oil industries in Iran (Pourfadakari *et al.*, 2020) and other petroleum-contaminated soils (Kaczorek *et al.*, 2013; Grigoryeva *et al.*, 2014).

About *Gordonia*, the genus has been less studied than *Pseudomonas*. However, some researchers have isolated *Gordonia* species (and also *Pseudomonas*) from natural environments using waste car engine oil, base oil or the c-alkane fraction of base oil as the sole carbon (Kubota *et al.*, 2008; Silva *et al.*, 2019).

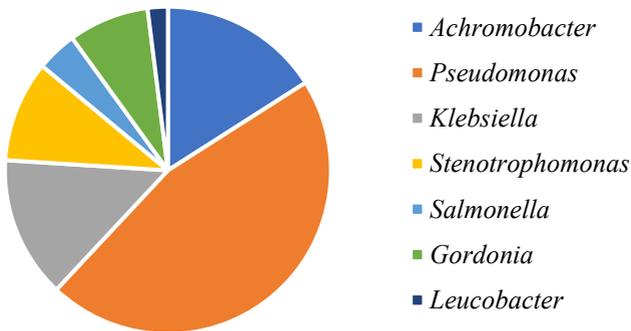


Figure 2.2. Isolated bacterial strains grouped by genera.

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Table 2.3. Isolated bacteria strains in ENI's laboratories.

| Strain | Species |
|---------------|--|
| Myc 1 | <i>Achromobacter pulmonis</i> ANB-1 * |
| Myc 7 | <i>Pseudomonas balearica</i> Z8 |
| Myc 9 | <i>Achromobacter xylosoxidans</i> MSAX3 * |
| Myc 10 | <i>Klebsiella pneumoniae</i> 50 * |
| Myc 14A | <i>Stenotrophomonas maltophilia</i> BCR * |
| Myc 14B | <i>Klebsiella pneumoniae</i> CCUG70747 * |
| Myc 20A | <i>Achromobacter pulmonis</i> ANB-1 * |
| Myc20B | <i>Achromobacter pulmonis</i> ANB-1 * |
| Myc24 | <i>Achromobacter</i> sp. NBTU-02 * |
| Myc25A | <i>Achromobacter pulmonis</i> ANB-1 * |
| Myc25B | <i>Achromobacter pulmonis</i> S-Mi16 * |
| Myc 27A | <i>Pseudomonas stutzeri</i> USW-CAP-1 |
| Myc27B | <i>Pseudomonas stutzeri</i> USW-CAP-2 |
| Myc37A | <i>Klebsiella pneumoniae</i> GSU 10-3 * |
| Myc37B | <i>Klebsiella pneumoniae</i> GSU 10-3 * |
| Myc 38 | <i>Stenotrophomonas</i> sp. MRC 2-1 |
| Myc40 | <i>Stenotrophomonas</i> sp. MRC 2-2 |
| Myc43 | <i>Klebsiella pneumoniae</i> GSU 10-3 * |
| Myc45 | <i>Achromobacter pulmonis</i> ANB-1 * |
| Myc46 | <i>Salmonella enterica</i> * |
| Myc48A | <i>Klebsiella variicola</i> RCZ5 * |
| Myc48B | <i>Stenotrophomonas maltophilia</i> CCC105 * |
| Myc70 | <i>Salmonella enterica</i> * |
| Myc74 | <i>Klebsiella</i> sp. HD4.5 * |
| Myc75 | <i>Stenotrophomonas maltophilia</i> CCC105 * |
| 1.5 | <i>Pseudomonas</i> sp. H16 |
| 1.19 | <i>Pseudomonas stutzeri</i> FM22 |

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| | |
|------|---|
| 1.29 | <i>Pseudomonas stutzeri</i> NCTC10451 |
| 2.1 | <i>Pseudomonas balearica</i> 56 |
| 3.1 | <i>Gordonia sp.</i> N-6-2 |
| 3.6 | <i>Pseudomonas sp.</i> 38M1 FL01 |
| 3.9 | <i>Gordonia sp.</i> hbs1 |
| 3.48 | <i>Pseudomonas plecoglossicida</i> MR70 |
| 4.8 | <i>Pseudomonas sp.</i> 09 |
| 4.26 | <i>Pseudomonas sp.</i> R2A2 |
| 4.27 | <i>Pseudomonas sp.</i> THAF7b |
| 5.1 | <i>Pseudomonas sp.</i> TW2-101 |
| 5.6 | <i>Pseudomonas sp.</i> ISA 15 |
| 5.10 | <i>Pseudomonas stutzeri</i> MN1 |
| 6.1 | <i>Pseudomonas sp.</i> RMR33 |
| 6.18 | <i>Pseudomonas sp.</i> ZGLJ8 |
| 6.35 | <i>Pseudomonas stutzeri</i> EGY-SCi2 |
| 7.2 | <i>Pseudomonas stutzeri</i> CUMB KMR-05 |
| 8.2 | <i>Leucobacter chromiireducens</i> Kh.C. M7 |
| 8.6 | <i>Gordonia sp.</i> N-6-2 |
| 8.10 | <i>Gordonia amicalis</i> |
| 8.11 | <i>Pseudomonas sp.</i> PrPco79 |
| 8.12 | <i>Pseudomonas taiwanensis</i> WTB6 |
| 8.19 | <i>Pseudomonas balearica</i> OT17 |
| 8.21 | <i>Pseudomonas sp.</i> SA03 |

The strains marked with * are the ones not belonging to Group 1 of biological risk agents.

Conclusions

In the first part of the work, 79 different microbial strains were isolated from a Pakistan gas production plant: 29 fungi and 50 bacteria. Among the bacterial strains, only slightly less than 50% of the isolates were maintained for the next steps because they are the only ones belonging to group 1 of biological agents. The most retrieved fungal genera have been *Aspergillus*, *Alternaria*, *Fusarium*, *Cladosporium*, and *Penicillium*, while *Pseudomonas* represented the higher percentage among bacteria.

What emerges from these initial results is that the contaminated substrates analysed host many microorganisms, even though they are challenging substrates to colonise as they are composed exclusively (or almost exclusively) of hydrocarbon mixtures.

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Chapter 3: Selection of potential fungal candidates to remediate hydrocarbon-contaminated substrates and detection of their enzymatic activities

Abstract

All the fungal strains isolated from the hydrocarbon-contaminated substrates were screened to select potentially useful fungal strains for bioremediation. To this end, the strains were inoculated on BHA supplemented with Condensate-16, Slop and BTZ (a heavy fuel oil low in sulphur). Their growth was compared to a control in pollutant-free plates. Eight fungal strains (F10, F13, F15, F26, F31, F37, F39 and F41) responded positively to the screening test and demonstrated to use of hydrocarbons for their metabolic activities. The selected fungal strains were then studied to understand their degradative abilities on hydrocarbon substrates. Colourimetric enzymatic tests were carried out to characterise the putative extracellular ligninolytic enzymes (LiPs, MnPs and Lacs) responsible for the hydrocarbon-degrading activities

3.1 Introduction

3.1.1 Hydrocarbon sources used in screening tests to select hydrocarbon-degrading fungi

One of the best and easiest method to screen fungal strains that utilize hydrocarbons as the sole carbon source is to inoculate them on a minimal or mineral medium (e.g. AW or BH) supplemented hydrocarbon source. In this way, it is possible to select only those able to use hydrocarbons for their metabolic activities. Many papers report similar experiments (Dos Santos *et al.*, 2008; Gargouri *et al.*, 2015). For example, Barnes *et al.* (2018) selected 10 fungal strains able to use crude oil as the sole carbon source by performing a screening test in which mineral medium and petrol were used as fungal culture medium.

Depending on which fungal degradation capacity is being sought, different hydrocarbon sources can be used. In most cases, the hydrocarbon source chosen is crude oil because it is one of the hazardous substrates on which the research is focused (Al Nasrawi, 2012; Garzoli *et al.*, 2015). However, petrol is often chosen because purified hydrocarbon products are more difficult to degrade than crude oil, and this is an effective method to select only those strains that have high degrading abilities (Barnes *et al.*, 2018). In other cases, specific hydrocarbon compounds (e.g. BTEX or PAHs) can be used to demonstrate the fungus's activity on that particular compound (Dos

Santos *et al.*, 2008). Even complex hydrocarbon mixtures, such as used motor oil or petroleum processing residues, are excellent substrates for isolating fungal strains with high degradative capacity and a broad spectrum of action (Husaini *et al.*, 2008; Chukwura *et al.*, 2016).

In this work, I have decided to use three complex hydrocarbon substrates to perform the fungal screening: Condensate-16, Slop and BTZ. Condensate-16 and Slop are two of the most complex and difficult to degrade substrates provided by ENI. They are composed of different hydrocarbon compounds: Condensate-16 is a mixture of long-chain alkanes and solid particulate (probably asphaltenes), while Slop is a mixture of light alkanes and light aromatic hydrocarbons. BTZ, instead, is heavy agricultural fuel oil, low in sulphur (< 1% w/v). These three substrates were chosen because they differ in their chemical composition, appearance and consistency. In this way, it was possible to select strains with a broader spectrum of action and more varied degradative capacities.

3.1.2 Fungal enzymes involved in hydrocarbon degradation

All fungi have innate efficiency in degrading petroleum hydrocarbons because they produce extracellular enzymes with extremely reduced substrate specificity. As already seen in Chapter 1, enzymes involved in the degradation of hydrocarbons are oxygenase, dehydrogenase and ligninolytic enzymes.

The fungal ligninolytic enzymes system contains three principal enzyme groups: lignin peroxidase (LiP), Mn-dependent peroxidase (MnP), phenoloxidase (laccase, tyrosinase), and H₂O₂ producing enzymes (Novotný *et al.*, 2004). They are extracellular and catalyze radical formation by oxidation to destabilize bonds in a molecule (Hofrichter *et al.*, 1999). Because of their low specificity, they are involved in the oxidation of a wide range of organic compounds (Mhuantong *et al.*, 2015; Zafra and Cortes-Espinosa, 2015; Aranda, 2016). Basidiomycetes have been extensively studied for their ability to produce ligninolytic enzymes over a wide range of environmental conditions (Jeon *et al.*, 2012; Si *et al.*, 2013; Liu *et al.*, 2014). Extracellular peroxidases (Per) of these fungi are responsible for the initial oxidation of the hydrocarbon (Acevedo *et al.*, 2011; Zhang *et al.*, 2015). LiP oxidize several hydrocarbons (especially PAHs) directly, while MnPs co-oxidize them indirectly through enzyme-mediated lignin peroxidation.

Studies on both Zygomycetes and Ascomycetes are much less common, but this does not mean that they cannot produce them (Asemoloye *et al.*, 2018). Except for MnP, whose existence is more limited to certain basidiomycetes families (*Agaricales*, *Corticiales*, *Polyporales*, *Hymenochaetales*), many filamentous fungi produce several Lacs and other types of Per (Bohlin *et al.*, 2006; Janusz *et al.*, 2013; Govarathanan *et al.*, 2017). For example, Asemoloye and collaborators (2018) demonstrated the LiP and Lac expression in

Penicillium purpurogenum and *Aspergillus flavus* and Cazares-Garcia *et al.* (2013) the production of Lac in *Trichoderma* species.

In addition to ligninolytic enzymes, Cytochrome P450 monooxygenase (CYP), epoxide hydrolases, lipases, proteases and dioxygenases have been extensively studied for their ability to degrade hydrocarbons (Bezalel *et al.*, 1997; Balaji *et al.*, 2014).

Although the strains isolated in this thesis belong mainly to the ascomycetes, I decided to focus the research on ligninolytic enzymes because they are the most involved in the degradation of hydrocarbons and still the most studied by mycoremediation researchers.

3.2 Materials and Methods

3.2.1 Screening of isolated fungal strains able to grow on hydrocarbons as the only carbon source

A screening of all the 28 isolated fungal strains was carried out to select those capable of using hydrocarbons as the only carbon source. For this purpose, a modified version of the Husaini *et al.* (2008) protocol was used.

The strains were inoculated in BHA plates and three different hydrocarbon substrates: Condensate 16 and Slop from ENI's samples and BTZ. The fungal strains were aseptically inoculated by taking a small portion of the colony grown for seven days on PDA.

The hydrocarbon sources were supplemented to the BHA plates at a concentration of 1% (v/v). One percent concentration was employed as this is reported by similar works presented in published papers (Husaini *et al.*, 2008; Grazoli *et al.*, 2015). Condensate-16 and BTZ substrates, due to their composition and density, were sterilised under UV light for 30 minutes, while Slop, more liquid, was filtered with 0,2 µm syringe filters. Plates containing only BHA were used as a control. The test was conducted in duplicate, and the plates maintained at 26 °C for one week. After this time, the plate coverage percentage by fungal mycelia growing on hydrocarbons compared to the coverage in plates without hydrocarbons was assessed. The screening test was considered positive for those strains that increased the plates coverage percentage by more than 26% compared to the control, on at least two hydrocarbon substrates.

3.2.2 Colorimetric screening for the detection of ligninolytic enzymatic activities

The presence of ligninolytic enzymes useful for the degradation of petroleum hydrocarbons was tested by colourimetric assays, based on methodology already available (Soden *et al.*, 2002; Kumar *et al.*, 2011; Yanto and Tachibana 2013; Batista-García *et al.*, 2017). MEA Petri dishes supplemented with 50 mg/L Remazol Brilliant Blue R (RBBR), 5 g/L 3,4,5-trihydroxybenzoic acid (gallic acid) and 0.2% (v/v) of 2-methoxyphenol (guaiacol). Fungal cultures in MEA without

the different substrates were used as controls. The strains selected from the screening test (paragraph 3.2.1) were aseptically inoculated by taking a small portion of the colony grown for 7 days on PDA.

The RBBR dye-decolourization test was used to determine whether the strains could degrade PAHs through ligninolytic enzymes' production (Batista-García *et al.*, 2017). RBBR is an anthracene derivative structurally similar to certain PAHs, and it is strongly decolourized by lignin-degrading fungi (Batista-García *et al.*, 2017).

Gallic acid was used to detect ligninolytic enzymes production (Batista-García *et al.*, 2017). The brown shaded colour production in the agar has been strongly correlated with the ability of fungi to oxidize gallic acid by ligninolytic enzymes (Shleev *et al.*, 2004).

Finally, guaiacol permits to verify the Lac and Per production. Guaiacol is a phenolic natural product, and the oxidation of guaiacol to its reddish-brown-coloured form in agar indicates Lac and Per activity in fungi (Kumar and Rapheal, 2011).

For each test type, each strain was inoculated onto the medium and incubated for seven days at 26 °C. The tests were conducted in duplicate. Petri dishes were visually examined daily to monitor the production of the halos (Lee *et al.*, 2010). The difference between the tested dishes and the control ones was estimated qualitatively, giving a mark from 0 (no colourimetric difference with the control) to +++ (colourimetric difference considerably higher than the control).

3.3 Results and Discussion

3.3.1 Screening of fungi for hydrocarbon degradation

After one week of incubation at 26 °C, the plates were visually examined. All the fungi inoculated on BHA and hydrocarbons showed more abundant growth than did those on BHA alone.

Although all the strains were isolated from contaminated substrates, the test showed that they grew differently on the various hydrocarbon substrates (Tab. 3.1). All the fungi displayed null or explorative growth on the BHA control plates; 22 strains were capable of growing on BTZ, 19 on Slop and only 11 on Condensate-16 (Fig. 3.1). BTZ seems to be the substrate that fungi exploit the most, which could be for its composition. Fuel oils, to which the BTZ belongs, are composed of complex and variable mixtures of aliphatic (alkanes, alkenes, cycloalkanes) and aromatic hydrocarbons (Laffon, 2014). Therefore, it is characterised by a greater variety of hydrocarbon compounds, which a more comprehensive range of fungi can use. On the contrary, Slop is mainly composed of light alkanes and aromatic, and Condensate-16 possesses asphaltenes and other hardly biodegradable compounds.

Another interesting result is that most of the strains can grow on all the hydrocarbon substrates tested (Fig. 2.2), meaning that the pollutants do not inhibit their growth and that the strains have the enzymatic capacity to exploit them as nutrient source. However, there were differences in the growth of the strain on the different

hydrocarbon mixtures. For example, *Alternaria alternata* F1 was able to grow on BTZ and Slop, but on BTZ, the increment in plate coverage was more than 51% than the control and only more than 26% on Slop. Similar cases can be observed in Table 3.1. This effect may be due to different metabolic patterns used by the strains, which may act better on one type of hydrocarbon than another. As already discussed in this chapter's introduction, fungi synthesize a different set of unspecific enzymes that may act on different hydrocarbon compounds depending on the type of the enzyme activated (Aranda, 2016; Deshmukh *et al.*, 2016).

The test was considered positive for those strains that increased the plates coverage percentage by more than 26% compared to the control on at least two hydrocarbon substrates. The strains that corresponded to these characteristics were F10, F13, F15, F26, F28, F37, F39 and F41, belonging to the genera *Scedosporium*, *Alternaria*, *Penicillium*, *Trichoderma*, *Coniochaeta* and *Fusarium*. Six of these eight strains were isolated from the contaminated soil sample on AW and Slop or Condensate-16 or Settled Water, demonstrating their effective adaptation to complex hydrocarbon substrates. More precisely, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Curvularia aerea* F28 and *Fusarium solani* F39 have been isolated using Condensate-16 as contaminated substrate, and this is the hydrocarbon substrate on which they better grew. The same is for *Coniochaeta* sp. F37 and *Fusarium chlamydosporum* F41 isolated

with Slop. Most of these species are already known for their hydrocarbon degradation abilities. *Alternaria alternata* is a great diesel degrader (Ameen *et al.*, 2016), *Penicillium oxalicum* efficiently act on anthracene (Aranda *et al.*, 2017), and *Fusarium solani* can degrade crude oil, alkanes, aromatic hydrocarbons and, in particular, benzo[*a*]pyrene (Colombo *et al.*, 1996; Al Nasrawi, 2012; Fayeulle *et al.*, 2019; Mikolasch *et al.*, 2019). Many authors have indicated *Trichoderma harzianum* as species able to degrade unsaturated hydrocarbons, BTEX, PAHs and resins (Chaîneau *et al.*, 1999; Romero *et al.*, 2020). *Scedosporium apiosperum* is also a species known for its degrading abilities; it acts on alkanes and benzenes (Morales *et al.*, 2017), PAHs, long chain-aliphatic hydrocarbons and complex mixtures (Claußen and Schmidt, 1999; Reyes-César *et al.*, 2014). No information is available at the moment about *Coniochaeta* sp., *Curvularia aeria* and *Fusarium chlamydosporum*.

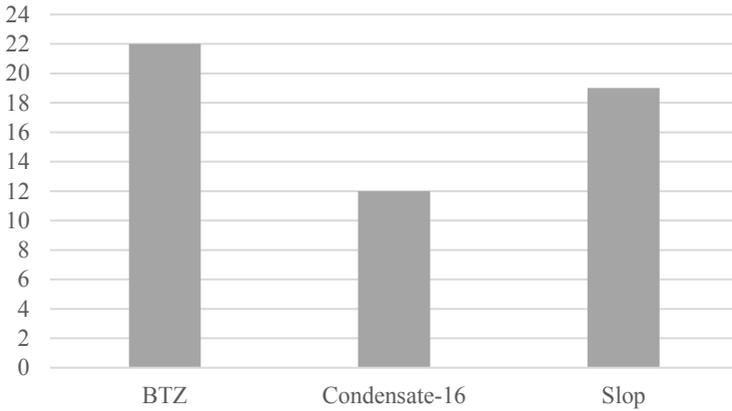


Figure 3.1. The number of strains able to degrade each substrate.

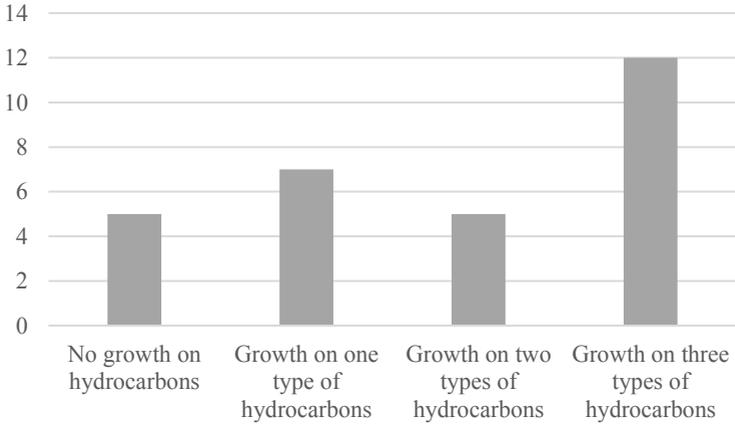


Figure 3.2. The number of strains grouped by the number of substrates on which they grew.

Chapter 3

Table 3.1. Results of the screening test. The table reports the fungal growth on BHA + 1% (v/v) of BTZ, Condensate-16 and Slop, compared to BHA control, calculated as the percentage of dish coverage by fungal mycelia.

| Strains | BTZ | Condensate-16 | Slop |
|---|-----|---------------|------|
| <i>Alternaria alternata</i> F1 | ++ | - | + |
| <i>Fusarium</i> sp.1 F2 | - | - | - |
| <i>Rhizopus</i> sp. F3 | - | - | - |
| <i>Cladosporium cladosporioides</i> F4 | + | - | - |
| <i>Aspergillus</i> sp.1 F5 | + | - | - |
| <i>Cladosporium sphaerospermum</i> F6 | ++ | + | + |
| <i>Alternaria</i> sp.2 F7 | + | + | + |
| <i>Chrysosporium</i> sp. F8 | - | - | - |
| <i>Scedosporium apiospermum</i> F10 | + | ++ | ++ |
| <i>Alternaria alternata</i> F13 | ++ | +++ | ++ |
| <i>Penicillium oxalicum</i> F15 | +++ | ++ | + |
| <i>Alternaria</i> sp.4 F21 | - | - | + |
| <i>Aspergillus</i> sp.5 F22 | - | - | - |
| <i>Aspergillus</i> sp.2 F23 | + | - | ++ |
| <i>Trichoderma</i> sp.1 F25 | + | - | + |
| <i>Trichoderma harzianum</i> F26 | +++ | ++ | + |
| <i>Penicillium</i> sp.1 F27 | + | - | + |
| <i>Curvularia aerea</i> F28 | ++ | +++ | + |

| | | | |
|--|-----|-----|-----|
| <i>Aspergillus</i> sp.6 F29 | + | - | + |
| <i>Aspergillus</i> sp.3 F30 | - | - | - |
| <i>Aspergillus</i> sp.4 F31 | + | - | - |
| <i>Cladosporium</i> sp.3. F32 | ++ | + | + |
| <i>Penicillium</i> sp.2 F34 | - | - | + |
| Mycelia sterilia F35 | + | - | - |
| <i>Coniochaeta</i> sp. F37 | ++ | + | ++ |
| <i>Fusarium solani</i> F39 | +++ | +++ | ++ |
| <i>Fusarium</i> sp.2 F40 | + | - | - |
| <i>Fusarium chlamydosporum</i> F41 | ++ | ++ | +++ |
| <i>Rhodotorula mucilaginosa</i> L1 | + | + | + |

(-) No increment in the coverage respect to the control; (+) from +10% to +25% of increment; (++) from +26% to +50% of increment; (+++) from +51% to +75% of increment.

3.3.2 Fungal production of ligninolytic enzymes

The tested strains have been *Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Trichoderma harzianum* F26, *Curvularia aeria* F28, *Coniochaeta* sp. F37, *Fusarium solani* F39 and *F. chlamydosporum* F41.

Generally, the gallic acid test was performed to screen the selected strains for ligninolytic enzyme secretion (phenol oxidases in particular), while the RBBR test aims to verify those with the ability

to degrade PAHs. All the tested strains on gallic acid, except for *Trichoderma harzianum* F26 and *Coniochaeta* sp. F37, produced the dark halo, more or less intensely, indicating the secretion of ligninolytic enzymes (Tab. 3.2). This suggests they used ligninolytic enzymes to exploit hydrocarbons in the previous screening. It also consistent with the literature; *Alternaria alternata* is a ligninolytic enzymes producer (Rajwar *et al.*, 2013; Kaur and Aggarwal, 2017), as well as *Penicillium* sp. (Jha and Patil 2011; Ayla *et al.*, 2018) and *Fusarium* (Saparrat *et al.*, 2000; Zhang *et al.*, 2020). In particular, the strains showing the highest enzymatic production were *Alternaria alternata* F13 and *Curvularia aerea* F28, which already after 3 days from the inoculum, showed an intense dark halo around the growing colony (Figs. 3.3; 3.4). *Curvularia* is a species that has not yet been extensively studied, especially for mycoremediation purposes. For this reason, not much information about its enzymatic activity is currently available.

In the RBBR test, the strains that showed the most intense activity, forming a transparent halo around the colony, were *Scedosporium apiospermum* F10, *Penicillium oxalicum* F15 (Fig. 3.5), and *Trichoderma harzianum* F26. Also, in this case, *Penicillium* and *Trichoderma* are reported as PAHs degraders (Leitão *et al.*, 2009; Zafra *et al.*, 2015; Park *et al.*, 2019). The other strains, in contrast, showed only slight or no activity.

The oxidation of guaiacol is indicative of Per and Lac activity in fungi. In this case, most of the strains reported low or no activity. Only *Alternaria alternata* F13 (Figs. 3.3) have shown high activity, in line with what is reported by many authors (Irfan *et al.*, 2018; Thakkar and Bhatt, 2020).

The results obtained indicate that the selected strains are almost all capable of producing ligninolytic enzymes but that these probably do not belong to the class of Lac and Per since these activities were the weakest recorded in this test. Moreover, in the case of non-white-rot fungi, P450s play an important role in xenobiotic detoxification, and these colorimetric tests do not record these enzymes.

Table 3.2. Results of the colorimetric test after 20 days of incubation.

| Strain | RBBR | Gallic acid | Guaiacol |
|--------|------|-------------|----------|
| F10 | + | ++ | 0 |
| F13 | 0 | +++ | ++ |
| F15 | ++ | ++ | + |
| F26 | ++ | 0 | + |
| F28 | ++ | +++ | + |
| F37 | 0 | 0 | + |
| F39 | 0 | ++ | 0 |
| F41 | + | ++ | + |

0 no activity; + activity; ++ high activity, +++ very high activity.

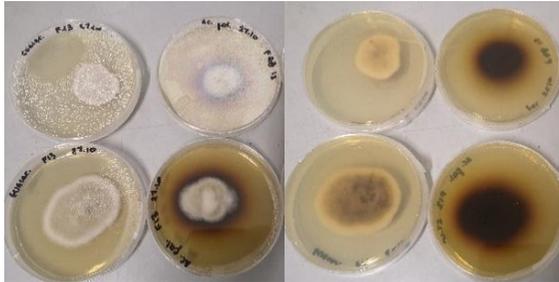


Figure 3.3. Results of the colorimetric test on F13 after three days of incubation, front and back. From the left: guaiacol test and gallic acid test.

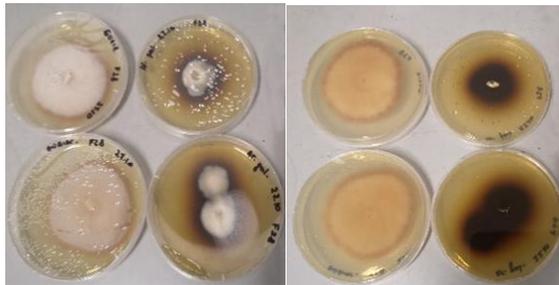


Figure 3.4. Results of the colorimetric test on F28 after three days of incubation, front and back. From the left: guaiacol test and gallic acid test.



Figure 3.5. Results of the RBBR test on F15 after seven days of incubation. It is possible to note the clearer halo around the colony.

Conclusions

All the isolated strains have been studied for their ability to use hydrocarbon complex mixtures as the sole carbon source. From the screening test, *Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Trichoderma harzianum* F26, *Curvularia aerea* F28, *Coniochaeta* sp. F37, *Fusarium solani* F39 and *F. chlamyosporum* F41 demonstrated this capacity and, therefore, will be the subject of the following analysis leading to the creation of the consortium. These results are consistent with the scientific literature that report most of these species as hydrocarbons degraders.

Subsequently, I have performed colorimetric enzymatic tests to detect the production of ligninolytic enzymes. *Alternaria alternata* F13 and *Curvularia aerea* F28 were the best producers, while no activity was recorded for *Trichoderma harzianum* F26 and *Coniochaeta* sp. F37. All the strains (except for *Alternaria alternata* F13, *Coniochaeta* sp. F37, and *Fusarium solani* F39) positively respond to RBBR, suggesting their ability to degrade PAHs.

In the future, it may be very useful to carry out more in-depth analyses to characterise better the enzymatic production of these strains and increase it.

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Chapter 4: Evaluation of the hydrocarbon-degrading capacity of the selected strains

Abstract

The selected fungal strains were studied to understand their degradative capacities on hydrocarbon complex substrates (used engine oil, Condensate-16 and Slop). This was demonstrated by evaluating their hydrocarbons composition before and after the fungal action using gas chromatography. All the fungal strains modified the substrates' composition, acting on different hydrocarbon families. Another result is that the GCxGC technique is more suitable for hydrocarbon degradation analysis, and dichloromethane can extract more hydrocarbon from the substrates than hexane. At the end of this chapter, a paper that reports the results obtained from studying four strains belonging to two *Trichoderma* species, including *T. harzianum* F26, is presented.

4.1 Introduction

4.1.1 Gas-chromatographic approaches to verify hydrocarbon biodegradation

Petroleum hydrocarbon biodegradation in contaminated substrates is usually monitored over time by collecting a sample and analysed it by gas-chromatographic (GC) separation (Dunkle *et al.*,

2019). GC is a convenient, rapid, precise, and accurate technique to detect classes and specific hydrocarbon compounds, helping determine a hydrocarbon mixture composition. There are different types of GC approaches, each with its strengths and disadvantages. One of the most used is the GC coupled to a mass spectrometer (MS) (GC-MS). MS is a sensitive mass detector that measures the mass-to-charge ratio of ions. It is universal for all compound classes but can also be selective through selected-ion monitoring (SIM). In GC-MS, the components with different numbers of carbon atoms are clearly separated. However, even using GC-MS, the high complexity of the hydrocarbon mixtures mostly prevents a reliable identification. Besides, GC-MS obtains qualitative information, and quantitative analysis can be difficult (Schoenmakers *et al.*, 2000). Other limitations include the analysis of labile and low molecular weight compounds, in addition to difficulties with differentiating between the many isomers that exist in liquid hydrocarbon streams (Dunkle *et al.*, 2019).

Multi-dimensional gas chromatography (GCxGC) is often preferred (Mao *et al.*, 2009). GCxGC is a powerful analytic technique that provides separations of complex mixtures that are not possible by conventional GC and improves peak capacity, resolution, and detectability (Dunkle *et al.*, 2019). The principle of GCxGC is based on the hyphenation of two capillary GC columns of different selectivity connected through a modulation device. This interface

enables sampling, focusing by trapping successive fractions of the effluent coming from the first column in narrow bands onto the second column and re-injection in a continuous way of sharp fractions of the first column's effluent into the second column. In that case, the entire sample is subjected to the two separation procedures and reaches the detector. Thus, the whole sample is separated on two different columns and no information gained during the first separation is lost during the second one (Vendeuvre *et al.*, 2007). On average, a GCxGC analysis has five times the sensitivity and gains three times the number of compound identifications of typical GC-MS runs. For these reasons, GCxGC has been applied to provide group type separation for several hydrocarbon complex mixtures, including heavy naphthas, diesel, marine diesel and oil, and gasoline (Schoenmakers *et al.*, 2000; Adam *et al.*, 2008a; Adam *et al.*, 2008b). GCxGC is a more precise and comprehensive methodology than GC-MS; however, the complicated nature of GCxGC instrumentation, data interpretation and management costs do not make it the most used technique (Dunkle *et al.*, 2019).

Commonly, the most used GC approach to detect the petroleum hydrocarbons biodegradation is the GC-MS (probably because of the GCxGC criticalities mentioned above) (Ameen *et al.*, 2016; Marchand *et al.*, 2017; Fayeulle *et al.*, 2019; Mikolasch *et al.*, 2019), but in this work, we decided to use both the methodologies to find the most suitable for our scopes. The GC-MS analysis was carried out at the

Centro Grandi Strumenti of the University of Pavia, while the GCxGC was performed in ENI S.p.A. laboratories.

4.2 Materials and Methods

The following paragraphs reported the protocols used to perform the hydrocarbons characterization in contaminated substrates pre and post the fungal action. As the contaminated substrate, Consedate 16, Slop and used engine oil were used. We decided to include also the engine oil to extend the analysis on other substances equally challenging to treat. Table 4.1 summarized the GC approach and the hydrocarbon contaminated substrate used to verify the degrading abilities of each strain selected from the screening test. The study was carried out on strains selected from the previous screening (Chapter 3). Data about *Trichoderma harzianum* F26 are presented at the end of the chapter in a published paper on Applied Sciences about the degrading abilities of four strains belonging to two *Trichoderma* species.

These tests were preliminary and aimed to find the most effective methodology and solvent to extract hydrocarbons from these complex substances to be used for future analysis of the consortium's capabilities. For this reason, the results between the strains are not comparable but are indicative only of the activity of the specific fungus tested.

Table 4.1. Used hydrocarbon substrates and type of GC analyses performed for each fungal strain tested.

| Fungal strains | Hydrocarbon substrates | GC methodology used |
|---|-------------------------------|----------------------------|
| <i>Scedosporium apiospermum</i> F10 | Used engine oil | GC-MS |
| <i>Alternaria alternata</i> F13 | Condensate 16 and Slop | GCxGC |
| <i>Curvularia aerea</i> F28 | Used engine oil | GC-MS |
| <i>Coniochaeta</i> sp. F37 | Condensate 16 and Slop | GCxGC |
| <i>Fusarium solani</i> F39 | Used engine oil | GC-MS |
| <i>Fusarium chlamydosporum</i> F41 | Used engine oil | GC-MS |

4.2.1 Comparison of hydrocarbons content in used engine oil before and after fungal action

This test analyses the engine oil-degrading abilities of the strains *Scedosporium apiospermum* F10, *Fusarium solani* F39 and *F. chlamydosporum* F41 by GC-MS. Fungi were inoculated using the technique of fungal mycelium suspension. The fungal mycelium suspension is collected from actively growing strains on PDA Petri plate; all the mycelium is scraped off the plates and introduced in a screw-cap tube containing 0,15% agar gel and sterile broken microscope slides. The mixture is then vortexed for 3 min at 3000 rpm to break hyphae and homogeneously disperse the spores. One mL of

fungi mycelium suspension was inoculated into a 100 mL sterile glass bottle with a crimp seal filled with 20 mL of BH and 1% (v/v) of used engine oil. The prepared bottles were incubated for 14 days on a rotary shaker at 26° C and 80 rpm. Control bottles were prepared without the fungal inoculum.

The amounts of hydrocarbon were determined by extracting the residual oil at the inoculum moment (T0) and after two weeks. The extraction was made by adding dichloromethane to the bottles in a ratio of 1:1 (v/v) and shaken vigorously for 1 min. Then the organic layer was allowed to separate for 5 min and 1 mL of the supernatant was collected. The extracted oil was evaluated by GC/MS carried out on a Thermo Scientific DSQII single-quadrupole GC/MS system. The injection of the samples was performed at 250°C in split mode. The oven temperature program was 70°C for 1 min, 70–120°C at 5°C/min, 120–260°C at 8°C/min, and held at 260°C for 5 min. A Restek Rxi-5Sil MS 30 m × 0.25 mm × 0.25 μm film thickness capillary column was used with helium as the carrier gas at a constant flow rate of 1.0 mL/min. The transfer line temperature was 270°C, and the ion source temperature was 250 °C. Electron ionization mode was used with 70 eV, and the ions were registered in full scan mode in a mass range of m/z 35–800 amu. The chromatogram acquisition, detection of mass spectral peaks, and waveform processing were performed using Xcalibur MS Software Version 2.1 (Thermo Scientific Inc., Waltham, MA, USA). The assignment of chemical structures to

chromatographic peaks was done based on comparison with the databases for the GC/MS National Institute of Standard and Technology (NIST) Mass Spectral Library (NIST 08) and Wiley Registry of Mass Spectral Data (8th Edition). Each component's percentage content was directly computed from the peak areas in the GC/MS chromatogram and classified following Hostettler *et al.*, 2013 classification.

4.2.2 Comparison of hydrocarbons content in Condensate-16 and Slop before and after fungal action

This test analyses the degradation of Condensate-16 and Slop by *Alternaria alternata* F13 and *Coniochaeta* sp. F37 through GCxGC. Fungi were inoculated using the technique of fungal mycelium suspension (par. 4.2.1), and 1 mL of suspensions were inoculated into a 100 mL sterile glass bottle with a crimp seal filled with 20 mL of BH medium and 1% (v/v) of Condensate-16 and Slop. The prepared bottles were incubated for 14 days on a rotary shaker at 26° C and 80 rpm. Control bottles were prepared without the fungal inoculum. The amounts of hydrocarbon were determined by extracting the residual hydrocarbons at the inoculum moment (T0), after one week, and after weeks. The hydrocarbon residues were extracted with hexane or dichloromethane in a ratio of 1:1 and shaken vigorously for 1 min. The organic layer was then allowed to separate for 5 min, 500µl

was collected and placed in 1 mL vials ready for the chromatography analysis.

The GCxGC analysis was carried out on an Agilent 7890B GC System. The samples' injection was performed at 380°C in split mode, ratio 5:1 and the flame ionization detector (FID) set up at 380°C. The oven temperature program was 50°C for 5 min, temperature increment up to 400°C at 4°C/min, and hold at 400°C for 5 min. Two columns J&W CP9092VF – 5ht Ultimet (30m x 250µm x 0,1 µm film thickness), carrier gas (H₂) flow = 0,3 mL/min and a Zebron 7HG – G025 – 11ZB – 35HT Inferno (5m x 250 µm x 0,25 µm film thickness), carrier gas (He) flow = 21 mL/min were used.

The GCxGC valve had a modulation time of 2,7 sec and a sample time of 2,55 sec. The data processing was performed using GC Image R2.5 GCxGC (GC Image, LLC, and the University of Nebraska).

4.3 Results and Discussion

4.3.2 *Used engine oil consumption by Scedosporium apiospermum F10, Fusarium solani F39 and F.chlamyosporum F41*

The GC-MS analysis of the used engine oil before the fungal treatment provided its percentage composition (Fig. 4.1). The peaks characterising the chromatogram (Fig. 4.1S, Supplementary material) were short-chain alkanes (C₁-C₂₀), long-chain alkanes (C₂₀-C₅₀), monoaromatic hydrocarbons (e.g. benzene, xylene), diaromatics

hydrocarbons (naphthalenes and substituents), triaromatics hydrocarbons (e.g. fluorene, phenanthrene and anthracene), pyrene and opane derivatives. This classification is based on Hostettler *et al.*, 2013. The most abundant families were BTEX and alkanes, representing more than 70% of the engine oil composition.

It was observed that the fungi modified the hydrocarbon composition of the used engine oil. After a 14-days incubation *Scedosporium apiospermum* F10 reduced the alkane family by almost 10% (Fig. 4.2), while *Fusarium solani* F39 showed a similar reduction in the benzene family (Fig. 4.3) and a slight reduction of alkanes. *Fusarium chlamydosporum* F41, as *Fusarium solani* F39, acted on alkanes and benzenes but with a weaker activity (Fig. 4.4). As already discussed in previous chapters, it has already been demonstrated that *Scedosporium* can degrade hydrocarbon complex mixtures (Yuan *et al.*, 2018), but Morales *et al.* (2017) found in its genome the metabolic pathways to elaborate, among others, alkanes, alkenes and cycloalkanes. Many *Fusarium* species are reported as aromatic hydrocarbons degraders (Hong *et al.*, 2010; Wu *et al.*, 2010; Hesham *et al.*, 2017), and the *Fusarium* used in this work demonstrated a degrading action especially on BTEX fraction.

In some cases, some hydrocarbons families seem to increase respect to the control, probably resulting from fungal secondary metabolite production. Recently, indeed, different studies have been published concerning the ability of several species of ascomycetes to

synthesise a broad spectrum of volatile organic compounds but also C₆–C₁₉ extracellular hydrocarbons, naphthalene and other polycyclic aromatic hydrocarbons (Ahamed *et al.*, 2011; Naik, 2018; Roy and Banerjee, 2019).

These results, even preliminary, are promising from the point of view of the assemblage of a consortium. Microorganisms performing different actions on the same substrate certainly increases the efficiency of the degradation process.

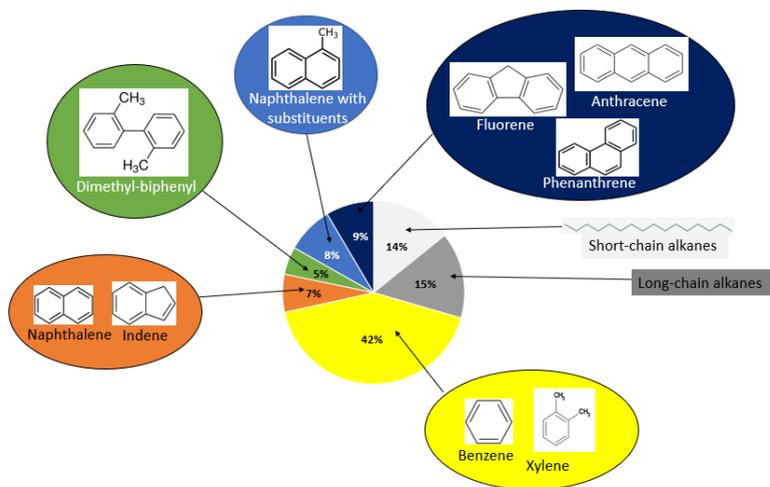


Figure 4.1. Percentage composition of the used engine oil

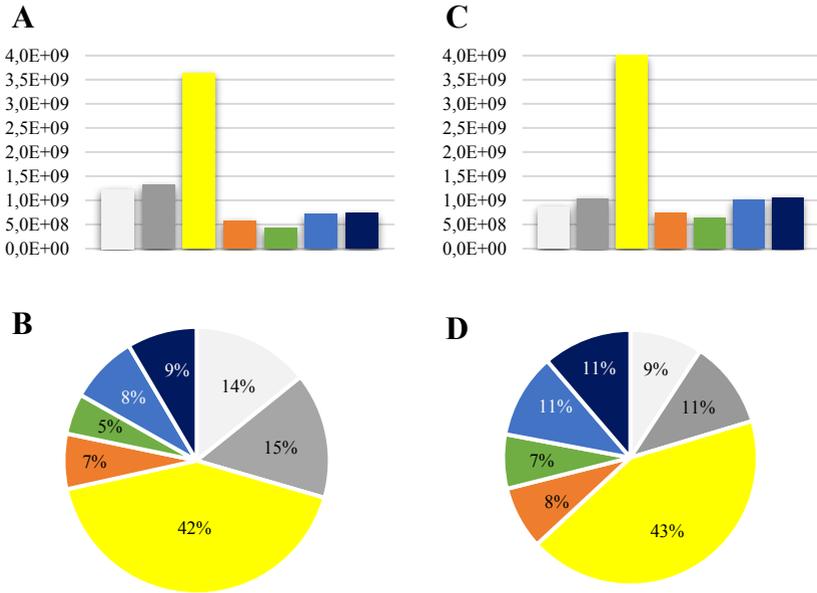


Figure 4.2. A; B: bar graph of the absolute areas and percentage composition of the hydrocarbon families present in control, after 14 days. C; D: bar graph of the absolute areas and percentage composition of the hydrocarbon families present in the supernatant of F10, after 14 days. The compounds were grouped in classes of benzene (yellow), naphthalene and indene (orange), dimethyl-biphenyl (green), naphthalene and substituents (light-blue), the group of anthracene, fluorene and phenanthrene (dark-blue), short-chain alkanes (white) and long-chain alkanes (grey).

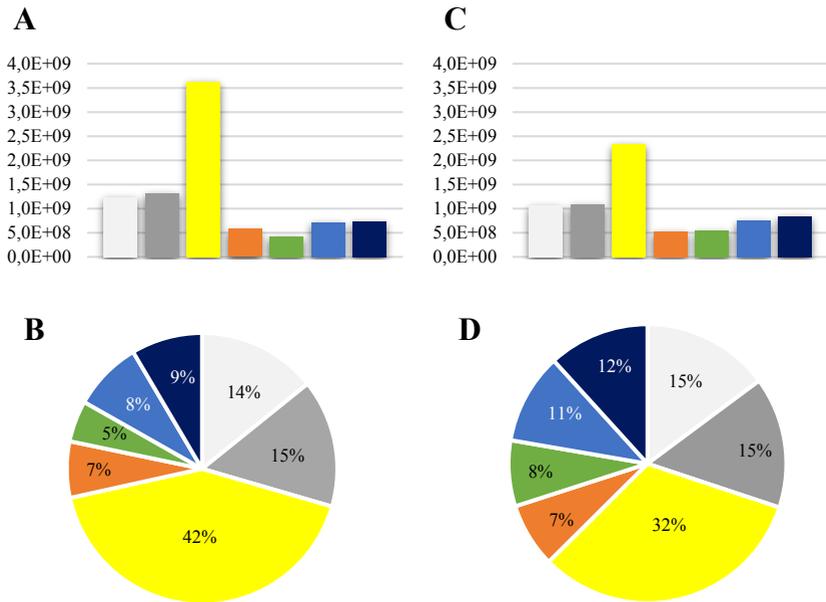


Figure 4.3. A; B: bar graph of the absolute areas and percentage composition of the hydrocarbon families present in control, after 14 days. C; D: bar graph of the absolute areas and percentage composition of the hydrocarbon families present in the supernatant of F39, after 14 days. The compounds were grouped in classes of benzene (yellow), naphthalene and indene (orange), dimethyl-biphenyl (green), naphthalene and substituents (light-blue), the group of anthracene, fluorene and phenanthrene (dark-blue), short-chain alkanes (white) and long-chain alkanes (grey).

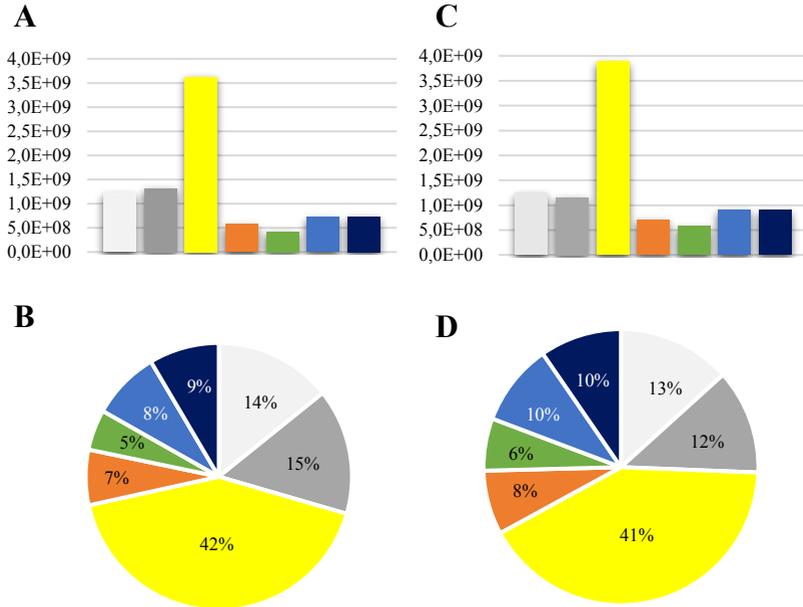


Figure 4.4. A; B: bar graph of the absolute areas and percentage composition of the hydrocarbon families present in control, after 14 days. C; D: bar graph of the absolute areas and percentage composition of the hydrocarbon families present in the supernatant of F41, after 14 days. The compounds were grouped in classes of benzene (yellow), naphthalene and indene (orange), dimethyl-biphenyl (green), naphthalene and substituents (light-blue), the group of anthracene, fluorene and phenanthrene (dark-blue), short-chain alkanes (white) and long-chain alkanes (grey).

4.3.4 Degrading abilities of *Alternaria alternata* F13 and *Coniochaeta* sp. F37

Figure 4.5 shows the most common oil hydrocarbons' spectra detected by the diesel oil analysis conducted in an unpublished study by Dr Carpani. The closest lines on the left refer to the shorter aliphatic hydrocarbons, in the centre the longest ones, and in the upper part are the aromatic hydrocarbons. The figure allows us to understand the position of the hydrocarbon families on the following chromatograms. Figures 4.6-4.9 show the spectrum of hydrocarbons present in Condensate-16 and Slop at the T0 and after one week of fungal action. The spectra of 14 days of action are not presented because they do not differ from 7 days.

Alternaria alternata acted mainly on Condensate-16 long-chain aliphatic hydrocarbons (Fig. 4.6), while in Slop, short-chain aliphatic hydrocarbons and PAHs were more reduced (Fig. 4.7). A similar activity was also demonstrated by Loretta *et al.* (2017). They reported that *Alternaria* sp. can degrade long-chain aliphatics in crude oil efficiently. *Coniochaeta* sp. F37 results on Condensate-16 did not show remarkable differences between T0 and one week of action (Fig. 4.8) but it seemed to considerably reduce the lighter aliphatic fraction of Slop (Fig. 4.9). In addition, the pre-treatment and post-treatment chromatograms of *Coniochaeta* sp. F37 shows that new spectra appear in the post-treatment chromatogram. This may be due to the possible production by the strain of secondary metabolites detected by the

instrument or to the presence in the treated supernatant of shorter hydrocarbon chains formed after fungal action.

The results of *Alternaria alternata* F13 and *Coniochaeta* sp. F37 are not comparable because the hydrocarbons in the two contaminated substrates were extracted with dichloromethane for *Alternaria alternata* F13 and with hexane for *Coniochaeta* sp. F37. However, this test aimed not to demonstrate what strain was the most performant but was made to find the most suitable protocol for future analyses on the consortium. Thus, the analyses showed that the extraction with hexane is less suitable with these substrates because it extracts fewer hydrocarbons than those extracted with dichloromethane.

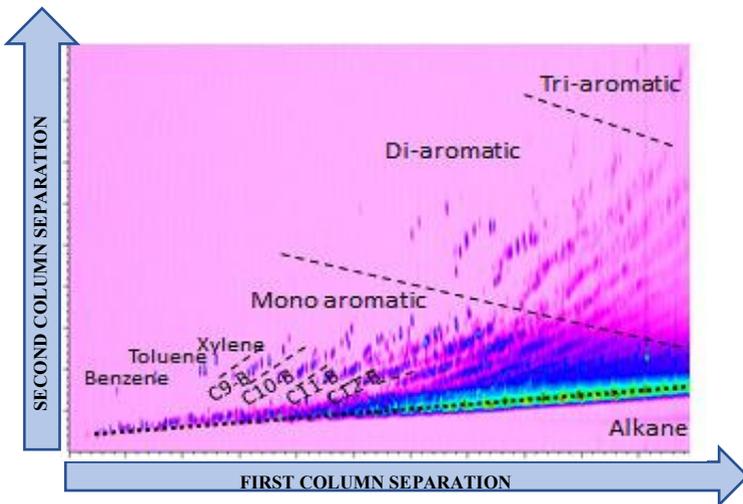


Figure 4.5. General separation of hydrocarbons obtained by GCxGC analysis of diesel oil (ENI S.p.A.)

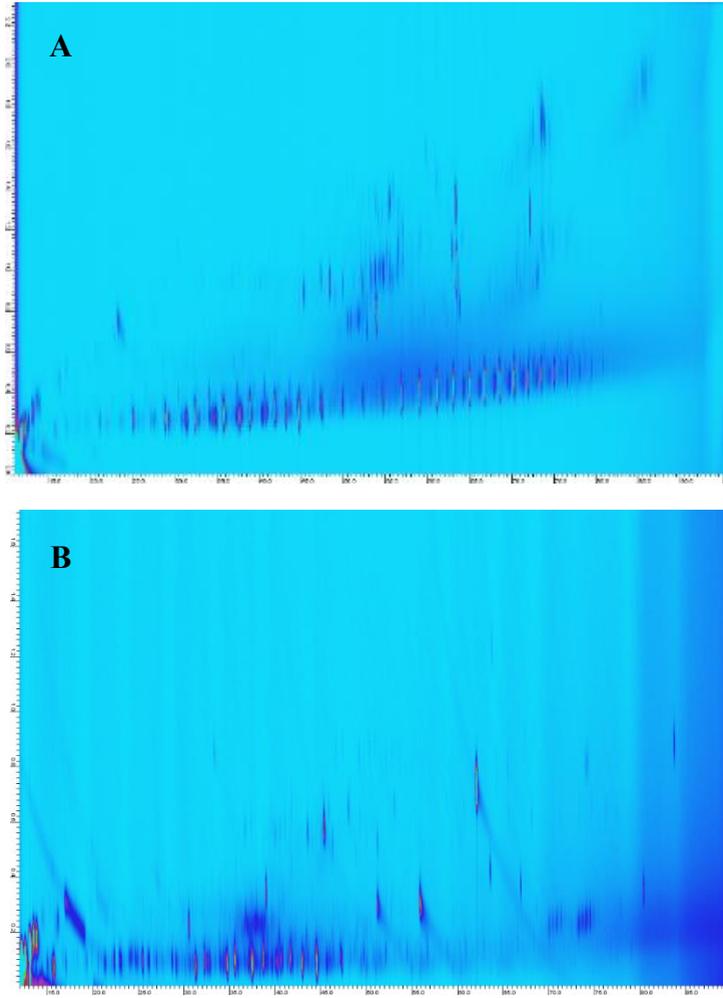


Figure 4.6. A: Condensate-16 spectrum at inoculum moment;
B: Condensate-16 spectrum after one week of treatment with F13.
The hydrocarbons were extracted with dichloromethane.

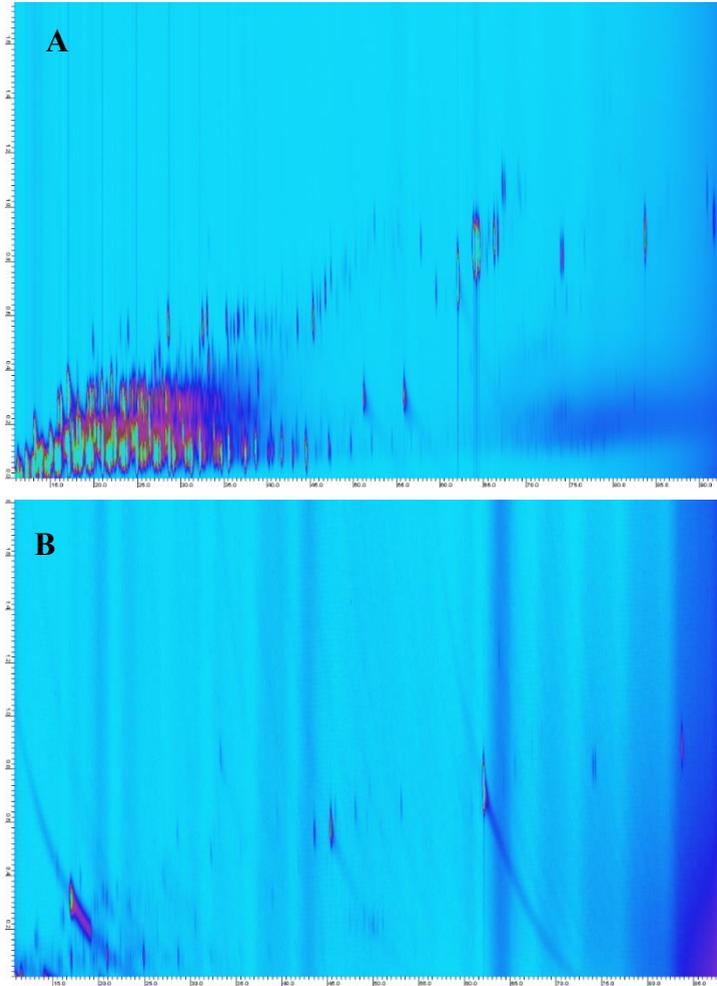


Figure 4.7. A: Slop spectrum at inoculum moment;
B: Slop spectrum after one week of treatment with F13.
The hydrocarbons were extracted with dichloromethane.

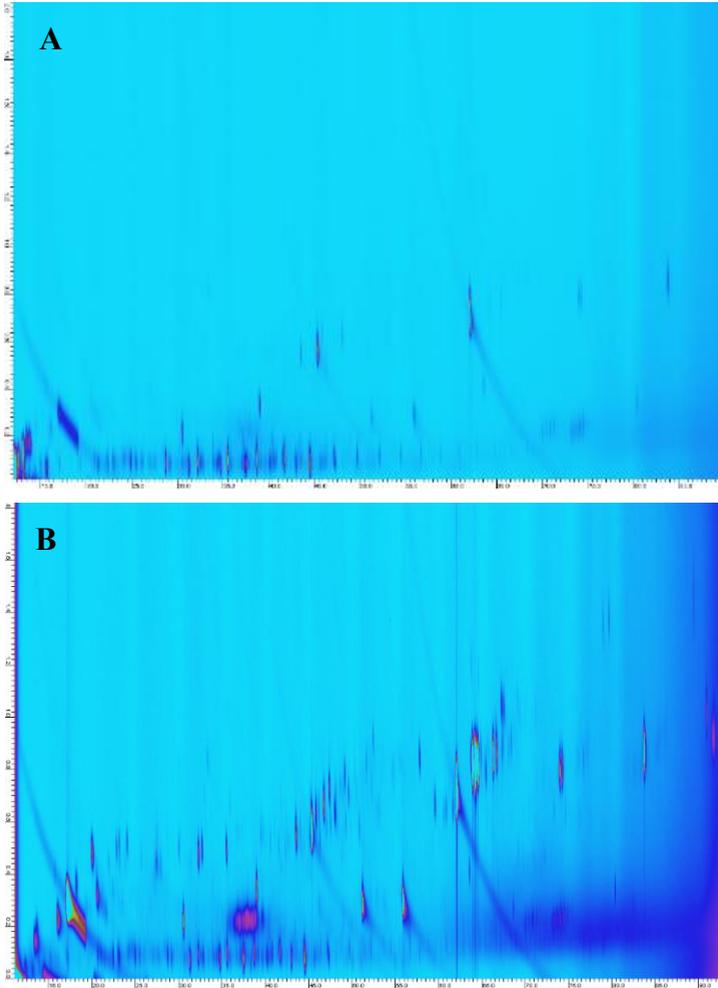


Figure 4.8. A: Condensate-16 spectrum at inoculum moment;
B: Condensate-16 spectrum after one week of treatment with F37.
The hydrocarbons were extracted with hexane.

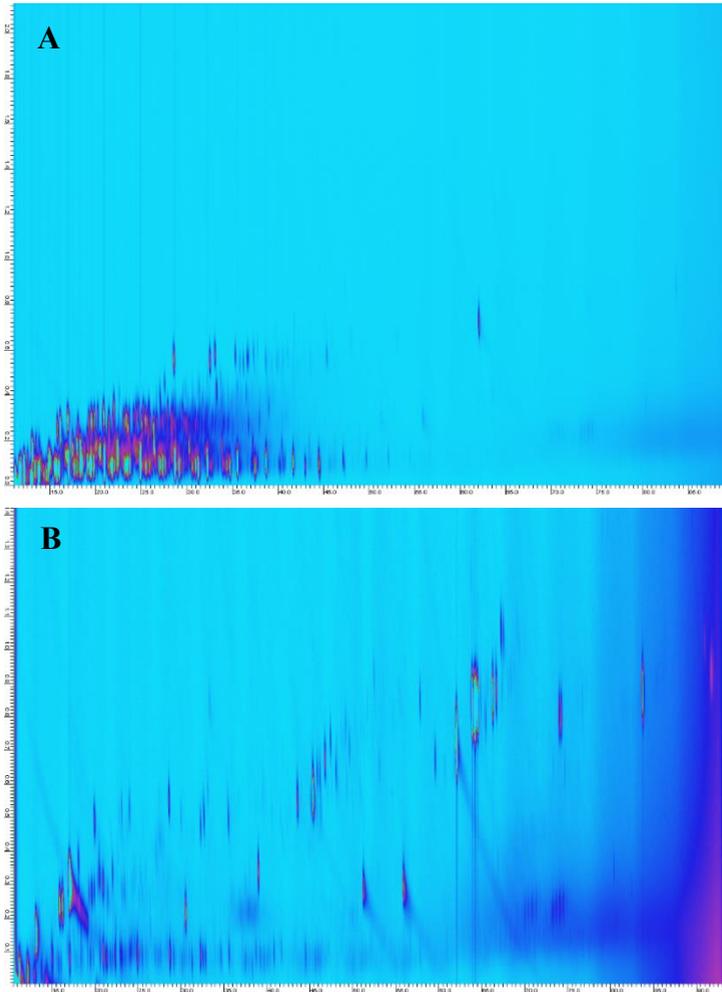


Figure 4.9. A: Slop spectrum at inoculum moment;
B: Slop spectrum after one week of treatment with F37.
The hydrocarbons were extracted with hexane.

Conclusions

The strains *Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Trichoderma harzianum* F26, *Curvularia aerea* F28, *Coniochaeta* sp. F37, *Fusarium solani* F39 and *F. chlamydosporum* F41. were studied to understand their degradative capacities on used engine oil, Condensate-16 and Slop. The hydrocarbon consumption was demonstrated by gas chromatography analysing the substrates hydrocarbons composition before and after the fungal attack. Although the different analysis results are not comparable overall, it is evident that all the fungal strains modified the substrates' composition, acting on different hydrocarbon families. Moreover, these analyses aimed not to demonstrate what strain was the most performant but what GC techniques and solvent were the most suitable for future analyses on the consortium. The results showed that GCxGC is the best methodology for these contaminated substrates and that dichloromethane can extract more hydrocarbon than hexane.

Supplementary material

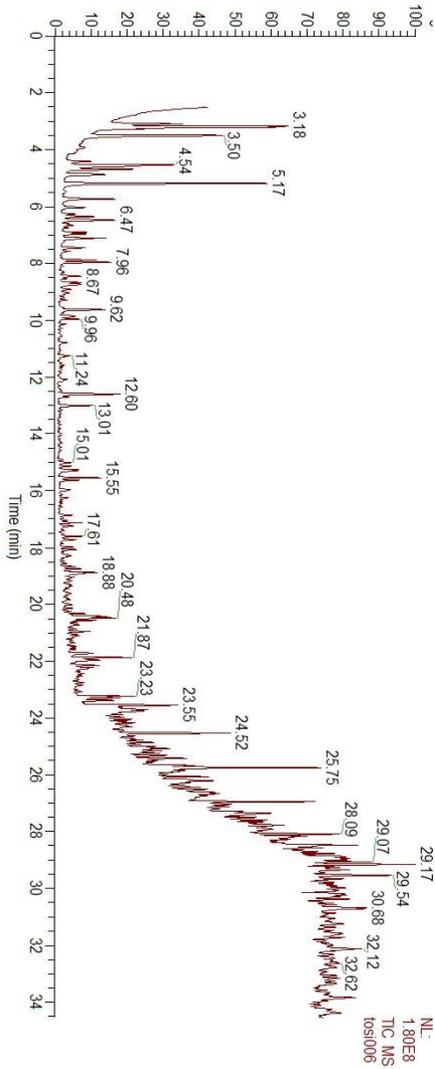


Figure 4.1S. Chromatogram for GC-MS analysis of used engine oil.

Acknowledgements

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Paper III. *Trichoderma*: evaluation of its degrading abilities for the bioremediation of hydrocarbon complex mixtures

***Trichoderma*: Evaluation of Its Degrading Abilities for the Bioremediation of Hydrocarbon Complex Mixtures**

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Abstract

Hydrocarbons can have very harmful effects on organisms and the environment, and conventional techniques for their removal are expensive and require the use of chemicals and long-term actions. *Trichoderma* is an ascomycete genus known to be active on different recalcitrant substrates, since it can produce a set of nonspecific extracellular enzymes generally involved in the degradation of lignin. However, the literature concerning the use of *Trichoderma* to degrade hydrocarbons is still limited. In this work we aimed to investigate the ability of *Trichoderma* to exploit used engine oil as its sole carbon source for prospective bioremediation of contaminated substrates. Four different strains belonging to *Trichoderma asperellum* and *Trichoderma harzianum* species were tested. The fungi were inoculated in direct contact with used engine oil, and after 45 days the samples were analyzed by gas chromatography/mass spectrometry (GC/MS). The results showed that all strains (except *Trichoderma asperellum* F1020) significantly changed the oil composition, decreasing the aromatic fraction in favor of the aliphatic one. *T. harzianum* F26, especially, showed a significant reduction of the BTEX (benzene, toluene, ethylbenzene, and the three xylene isomers) and alkylbenzenes fraction and an increase in short-chain aliphatics C₁–C₂₀. Enzymatic tests for laccase and peroxidase were also carried out, demonstrating that every strain seems to express a different mode of action.

Keywords: fungi; contaminated substrates; hydrocarbons bioremediation; enzymes

1. Introduction

Fuel oils are commonly used worldwide. Despite significant improvements in handling, transportation, and containment, they still enter water and soil environments. The most severe damage to natural ecosystems has been reported after accidental releases [1]. Engine oil is a complex mixture of hydrocarbons, engine additives, and metals like aluminum, chromium, copper, iron, lead, manganese, nickel, and silicon. Engine oil contains hundreds of aliphatic, linear, or branched and aromatic hydrocarbons [2], most of which are toxic to living organisms [3,4]. Hydrocarbons can accumulate in animal organs directly or indirectly through the food chain [5]. In humans, prolonged exposure to or contact with high concentrations of hydrocarbons can cause liver or kidney disease, bone marrow damage, reproductive disorders, and increased risk of cancer [6,7]. Oil products can also have important repercussions on the environment and plants. In fact, they can create a thick layer on plant organs, reducing their metabolic activities and physical capacities [8].

Conventional treatments to remove hydrocarbons from the environment are efficient but very expensive and often require the use of chemicals in operational steps that alter the structure and composition of soil and subsoil, as well as changing the autochthonous

microbial population [9,10]. An unconventional and environmentally friendly method of treating hydrocarbon-contaminated soils is bioremediation, which is the exploitation of natural microbial metabolism and co-metabolism to degrade toxic contaminants [4]. For these reasons, bioremediation is a “green” strategy, simple to design, and cost-effective.

Filamentous fungi are potential agents of degradation because they possess branched hyphae with which they can reach the deepest portions of contaminated substrates, digesting it through the secretion of extracellular enzymes [11]. Most fungi produce enzymes that include manganese peroxidase (MnP), laccases (Lac), cytochrome P450 monooxygenases, and epoxide hydrolases [12]. These extracellular enzymes, usually involved in the oxidation of lignin, are not substrate-specific and can therefore oxidize a wide range of xenobiotics, including pesticides, plastics, and hydrocarbons. Moreover, fungi can grow under different and extreme environmental conditions [13,14].

Among the different species of filamentous fungi, *Trichoderma* represents a genus with high potential. *Trichoderma* species belong to the Hypocreales order of the phylum Ascomycota, usually present in soils and plant roots [15]. This genus is one of the most widely distributed fungal groups in terrestrial and aquatic ecosystems [16]. It is resistant to most agrochemicals [17], heavy metals, pesticides, and polycyclic aromatic hydrocarbons (PAHs) [18]. The genus *Trichoderma* is often used as a biocontrol agent of plant pathogens

since its species are mycoparasites and antibiotic producers. They can regulate the growth and incidence of microorganisms that cause diseases in several horticultural plants [19,20]. During the last few decades, many research teams started to focus their attention on the possibility of exploiting the properties of *Trichoderma* in the bioremediation of petroleum hydrocarbons. Chaîneau *et al.* [21] showed that some *Trichoderma* species contributed to the degradation of a saturated hydrocarbons fraction in a petroleum sample. *T. harzianum*, *T. pseudokoningii*, and *T. viride* can utilize pyrene as a carbon source [22,23]. Hadibarata and Tachibana [24] demonstrated that the strain *Trichoderma* S019 can degrade 73% of n-eicosane when glucose is applied as a carbon source. Other researchers reported the ability of *T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride*, *T. virens*, and *T. asperellum* in the degradation of low-molecular-weight PAHs such as naphthalene and phenanthrene, or more complex PAHs such as anthracene, benzo[a]anthracene, benzo[a]fluoranthene, benzo[a]pyrene, and chrysene [12,25,26]. Argumedo-Delira *et al.* [27] reported the tolerance of 11 *Trichoderma* isolates to crude oil, naphthalene, phenanthrene, and benzo[a]pyrene using in vitro systems. Probable mechanisms for PAH degradation have been hypothesized for *Trichoderma*, including the production of laccases [28], peroxidases (Per) [29], and dioxygenases [30].

In light of these results, in this work we aimed to investigate the ability and the properties of different strains of *Trichoderma*

(belonging to *T. asperellum* and *T. harzianum*) to be used as bioremediation agents, exploiting used engine oil as the sole carbon source. The strains were isolated from different environments and substrates. The degree of transformation of the motor oil used was evaluated by gas chromatography coupled to mass spectrometry (GC/MS), a technique commonly used for this purpose [31–33] and applied in this study to identify the classes of hydrocarbons mainly attacked by the different *Trichoderma* species.

2. Materials and Methods

2.1. Used Engine Oil

Used engine oil was chosen for the growth and degradation tests; it was a complex mixture of hydrocarbons, engine additives, and metals, provided by an Italian private company. Its composition was defined by gas chromatography coupled to mass spectrometry (GC/MS).

2.2. Biological Material

Trichoderma Strains and Their Phylogenetic Relationships.

Four strains of *Trichoderma* (F12, F26, F1020, and F58) belonging to the Mycology Laboratory of the University of Pavia were utilized in this study. The fungal strains were isolated from four different environments: F12 and F26 from hydrocarbon-contaminated soils in Ecuador and Pakistan, respectively; F1020 from the rhizosphere of a plant growing in a hydroponic setting (toxin free) in Italy; and F58

from soil polluted with pesticides. The Mycology Laboratory previously used F12 and F1020 for tolerance tests with different types of hydrocarbon substances. They were chosen for this work to study their potential in the treatment of used engine oil. The strains were grown on potato dextrose agar (PDA) Petri dishes and incubated at 27 °C for one week. After this time, all the fungal strains were identified by morpho-dimensional examination under a light microscope, based on the nature of the mycelium and the different reproductive structures. Molecular characterization was performed to confirm the morphological identification. The fungal genomic DNA was extracted using the NucleoSpin Plant II by Macherey-Nagel (Bethlehem, PA, USA), with one hour of incubation at 65 °C after the addition of RNase, and then subjected to PCR amplification of the internal transcribed spacer (ITS) region of the ITS1-5.8S-ITS2 rDNA gene. The PCR reaction was performed on a Thermocycler Bio-Rad T100 in 20 µl reaction mixture containing 1× DREAM Taq Green PCR MasterMix reaction buffer (Thermo Scientifics, Pittsburg, PA, USA), 2 µl (5 µM) of each primer, 2 µl of DNA sample, and 4 µL of Nuclease Free water. The PCR program was as follows: denaturation by heating for 5 min at 95 °C, then 35 cycles of 30 s at 95 °C, 45 s at 50 °C, and 1 min at 72 °C, and a final elongation step for 10 min at 72 °C. The primers used were ITS1 (5'-TCCGTAGG TGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') [34]. PCR products were purified with ExoSAP-IT (Applied Biosystems, Foster City, CA,

USA) according to the manufacturer's protocol. The amplified and purified DNA was sent to BMR Genomics (Padova, Italy), and the sequences were compared with target sequences using BLAST online (<https://blast.ncbi.nlm.nih.gov/>) and MEGA X 10.1.7.

The DNA sequences generated in this work, together with representative ITS sequences of other *Trichoderma* strains, were aligned using MEGA X [35]. The ITS sequences of 28 strains belonging to seven species were downloaded from Genbank. To show the phylogenetic positions of our strains, strains belonging to the *Trichoderma* clade *Pachybasium* A and the *Pachybasium* B clade *Lixii/catopton* were included in this analysis. The maximum likelihood tree was obtained with 100 bootstrap replications, using the Jukes–Cantor model. The rates among sites were gamma-distributed with invariant sites (G+I).

2.3. Engine Oil Tolerance Test on the Fungal Strains

The ability of the *Trichoderma* strains to tolerate and grow on used engine oil was tested on Bushnell Haas mineral agar medium (BHA) Petri dishes complemented with 1% (v/v) of used engine oil. All the fungi were inoculated in the Petri dishes, and each one was replicated three times; BHA dishes with the inoculum but without the used engine oil were considered as control. After one week at 27 °C, the dishes were visually examined to compare the growth on the controls and the growth on the BHA and used engine oil. For evaluation of the tolerance, the dish coverage percentage by fungal mycelia growing on

used engine oil compared to the coverage in dishes without used engine oil was assessed. In fact, *Trichoderma* is a genus that shows nonregular and non-diametric growth. In the early stages of growth, its development is submerged in the medium; it only secondarily emerges and on the surface develops scattered conidiophores aggregated into fascicles or pustules [36]. For these reasons, it is difficult to determine the growth of the strains based on their radius of growth; alternatively, to evaluate the coverage percentage of the dish can be helpful.

2.4. Analysis of Used Engine Oil Degradation

The *Trichoderma* strains were tested for their ability to degrade used engine oil by analyzing its composition before and after the fungal action. A 5 mm disk of each *Trichoderma* strain was collected from the outer part of the fungal colony grown for seven days on PDA Petri dishes, and each was aseptically inoculated into a 20 mL sterile glass bottle with a crimp seal filled with 5 mL of Bushnell Haas mineral medium (BH) and 1% (v/v) of used engine oil. The degradation study bottles were incubated at room temperature and 80 revolutions per minute (rpm) for 45 days. The amounts of hydrocarbon were determined by extracting the residual oil at the inoculum moment (T₀) and after 45 days (T₄₅), with four replicates for fungal strain each time. T₀ was considered as control. At the moment of analysis, diethyl ether was added to the degradation bottles in a ratio of 1:1. The samples were shaken vigorously for 1 min, and the organic layer was

allowed to separate for 5 min. At this point, 1 mL of the supernatant was collected from the degradation bottles, placed in a 1.5 mL Eppendorf tube, and centrifuged for 3 min at 3000 rpm. The supernatant was then transferred to a 1 mL GC/MS glass vial. The extracted oil was evaluated by GC/MS carried out on a Thermo Scientific DSQII single-quadrupole GC/MS system. The injection in the GC/MS system was performed at 250 °C in split mode. The oven temperature program was 70 °C for 1 min, 70–120 °C at 5 °C/min, 120–260 °C at 8 °C/min, and hold at 260 °C for 5 min. A Restek Rxi-5Sil MS 30 m × 0.25 mm × 0.25 μm film thickness capillary column was used with helium as the carrier gas at a constant flow rate of 1.0 mL/min. The transfer line temperature was 270 °C and the ion source temperature was 250 °C. Electron ionization mode was used with 70 eV, and the ions were registered in full scan mode in a mass range of m/z 35–800 amu. The chromatogram acquisition, detection of mass spectral peaks, and waveform processing were performed using Xcalibur MS Software Version 2.1 (Thermo Scientific Inc., Waltham, MA, USA). The assignment of chemical structures to chromatographic peaks was done based on comparison with the databases for the GC/MS National Institute of Standard and Technology (NIST) Mass Spectral Library (NIST 08) and Wiley Registry of Mass Spectral Data (8th Edition). The percentage content of each component was directly computed from the peak areas in the GC/MS chromatogram.

2.5. Colorimetric Screening for the Detection of Ligninolytic Enzymatic Activities

The presence of ligninolytic enzymes useful for the degradation of used engine oil was tested by three different approaches using Petri dishes supplemented with different substrates (Remazol Brilliant Blue R (RBBR), gallic acid, and guaiacol). Fungal cultures in malt extract agar (MEA) without the addition of the different substrates were used as controls and incubated under the same conditions. Three replicates of each culture were analyzed in each experiment. Each test was evaluated visually, and the difference between the tested dishes and the control ones was estimated qualitatively, giving a mark from 0 (no colorimetric difference with the control) to +++ (colorimetric difference considerably higher than the control).

Petri dishes were used with MEA and 5 g/L of gallic acid as a culture medium to determine the ligninolytic enzyme production [37,38]. The production of a brown shaded color produced in the agar has previously been strongly correlated with the ability of fungi to oxidize gallic acid by ligninolytic enzymes [39]. The strains were inoculated in the medium and incubated for seven days at 26 °C. Three replicates of each culture were analyzed. The Petri dishes were visually examined daily to monitor the production of a brown halo due to the oxidation of gallic acid [40].

Also, other Petri dishes with MEA and 0.2% (v/v) of guaiacol were used as a screening method to determine the Lac and Per

production in the fungi. Guaiacol is a phenolic natural product, and the oxidation of guaiacol to its reddish-brown-colored form in agar is indicative of Lac and Per activity in fungi [41]. A plug of 7 mm diameter of each fungus was inoculated in the above medium, and cultures were incubated for seven days at 26 °C. Petri dishes were visually examined daily to monitor the production of a reddish-brown color due to the oxidation of guaiacol.

An RBBR dye-decolorization test was used to determine whether *Trichoderma* strains were capable of degrading PAHs and can also suggest the presence of Lac [37]. This is possible because RBBR is an anthracene derivative structurally similar to certain PAHs, and it is strongly decolorized by lignin-degrading fungi [42,43]. MEA Petri dishes supplemented with 50 mg/L of RBBR were used as culture medium. Each strain was inoculated onto the medium and incubated for seven days at 26 °C. Petri dishes were visually examined daily [44].

2.6. Statistical Analysis

GC/MS data were processed statistically using Past3 software [45], and one-way multivariate analysis of variance (MANOVA) or one-way nonparametric MANOVA (PERMANOVA) tests were applied to the data, according to the normality and homoscedasticity of the data, tested via Mardia's multivariate skewness and kurtosis, Doornik–Hansen test, and Box's M test (with significance at $P < 0.05$). The differences between each class at Time 0 and Time 45 were assessed

using the two-tailed (Wilcoxon) Mann–Whitney U test. GC/MS data were also evaluated by principal component analysis (PCA).

3. Results

3.1. Identification of *Trichoderma* Strains and Analysis of Their Phylogenetic Relationships

The morphological analysis of the selected strains based on observations under a light microscope of the conidia and conidiophore morphology led to the identification of the four strains as F1020 *T. asperellum* and F12, F26, and F58 *T. harzianum* (Table 1). Moreover, the sequencing of the ITS regions, compared with target sequences with BLAST, confirmed this morphological identification. The tree obtained in the phylogenetic analysis (Figure 1) contains 32 taxa. Our strains of *T. harzianum* (F26, F58, and F12) formed a clade together with the other strains of *T. harzianum* obtained from Genebank. In particular, *T. harzianum* F26 and *T. harzianum* F58 seem to be closely related from a phylogenetic point of view. On the other hand, *T. asperellum* 1020 is grouped with other *T. asperellum* strains and other strains from the *Trichoderma Pachybasium* A complex (*T. hamatum* and *T. pubescens*).

Table 1. Strains of *Trichoderma* identified by morphological and molecular identification.

| Code | Species |
|-------------|----------------------|
| F1020 | <i>T. asperellum</i> |
| F12 | <i>T. harzianum</i> |
| F26 | <i>T. harzianum</i> |
| F58 | <i>T. harzianum</i> |

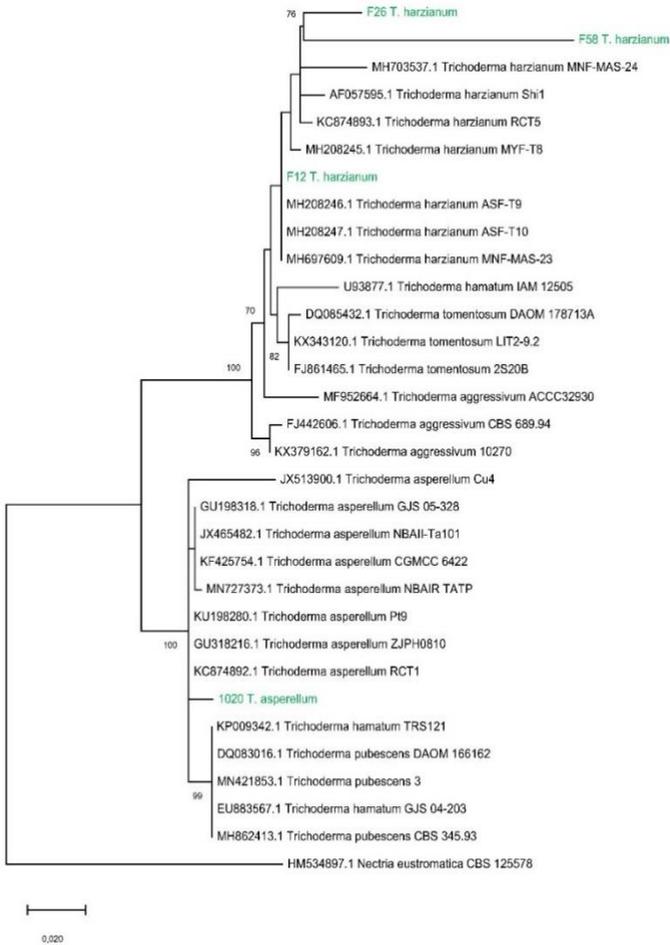


Figure 1. Phylogenetic tree based on maximum likelihood analysis of the internal transcribed spacer (ITS) sequences. *Nectria eustomatica* was used as the outgroup. Maximum likelihood bootstrap values greater than 70% are given at the nodes. The four strains used in this work are highlighted in green.

3.2. Used Engine Oil Tolerance Test on *Trichoderma* Strains

After one week of incubation at 27 °C, the dishes were visually examined. All the fungi inoculated on BHA and used engine oil showed more abundant growth than did those on BHA alone (Table 2).

Table 2. Results of the tolerance test on *Trichoderma* strains. The table reports the fungal growth on BHA + 1% (v/v) used engine oil compared to the BHA control and calculated as the percentage of dish coverage by fungal mycelia.

| Increment in the Coverage Percentage of the Dish Compared to the Control | |
|---|-----|
| <i>T. asperellum</i> F1020 | + |
| <i>T. harzianum</i> F12 | ++ |
| <i>T. harzianum</i> F58 | ++ |
| <i>T. harzianum</i> F26 | +++ |

(+) from +10% to +25%; (++) from +26% to +50%, (+++) from +51% to +75%.

3.3. Used Engine Oil Composition

The hydrocarbon composition of the used engine oil was determined by GC/MS analysis (Figure 2). The compounds were grouped into the following classes according to Hostettler *et al.* [46]: C₁–C₂₀ aliphatics, C₂₀–C₅₀ aliphatics, BTEX (benzene, toluene, ethylbenzene, and the three xylene isomers), Alkyl Benzenes, Alkyl Indenes, Alkyl Tetralines, Alkyl Biphenyls, Polycyclic Aromatics, and Other Compounds (Table S1). The most abundant families in used motor oil

are BTEX, C₂₀-C₅₀ aliphatics, and PAHs, which together make up 67% of the present compounds.

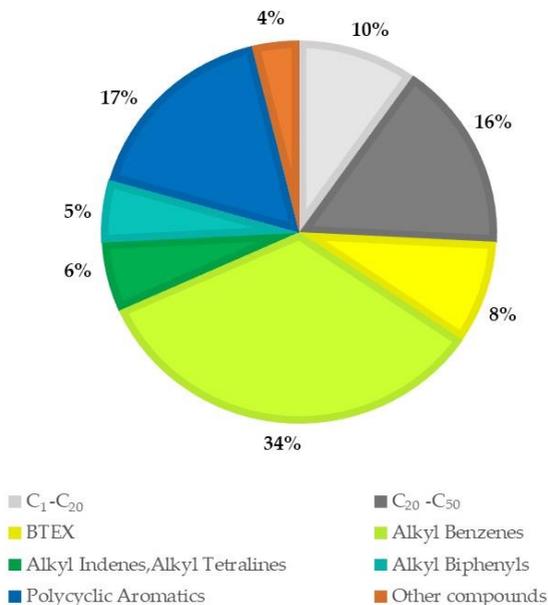


Figure 2. Composition of the used engine oil determined by GC/MS analysis.

3.4. Analysis of Used Engine Oil Fungal Degradation

The percentage composition of the engine oil used by fungi was compared between T0 and T45 for each fungal strain by GC/MS analysis. The peaks detected on the chromatogram, indicative of the various compounds present in the used engine oil, were initially

grouped into the macroclasses “Aliphatic”, “Aromatics”, and “Other Hydrocarbons” in order to identify the most degraded families of compounds. The three strains of *T. harzianum* (F12, F26, and F58) all significantly modified the composition of the used engine oil macroclasses (MANOVA or PERMANOVA, $P < 0.05$) (Figure 3). *T. asperellum* F1020 was the only strain that did not significantly modify the oil composition (Figure 3). The compounds were then grouped into the following microclasses: C₁–C₂₀ aliphatics, C₂₀–C₅₀ aliphatics, BTEX, Alkyl Benzenes, Alkyl Indenes, Alkyl Tetralines, Alkyl Biphenyls, Polycyclic Aromatics, and Other Compounds. The only fungal strain that produced significant changes in these groups after 45 days of action was *T. harzianum* F26 (PERMANOVA, $P < 0.05$, Figure 4), which reduced the presence of BTEX from $4.33\% \pm 0.38\%$ to $2.98\% \pm 0.50\%$ and that of Alkyl Benzenes from $14.45\% \pm 0.87\%$ to $10.99\% \pm 1.57\%$ significantly, while C₁–C₂₀ compounds increased significantly from $3.44\% \pm 0.17\%$ to $4.49\% \pm 0.14\%$ (two-tailed Mann–Whitney U test, $P < 0.05$) (Figure 5). The chromatograms are shown in Figures S1 and S2.

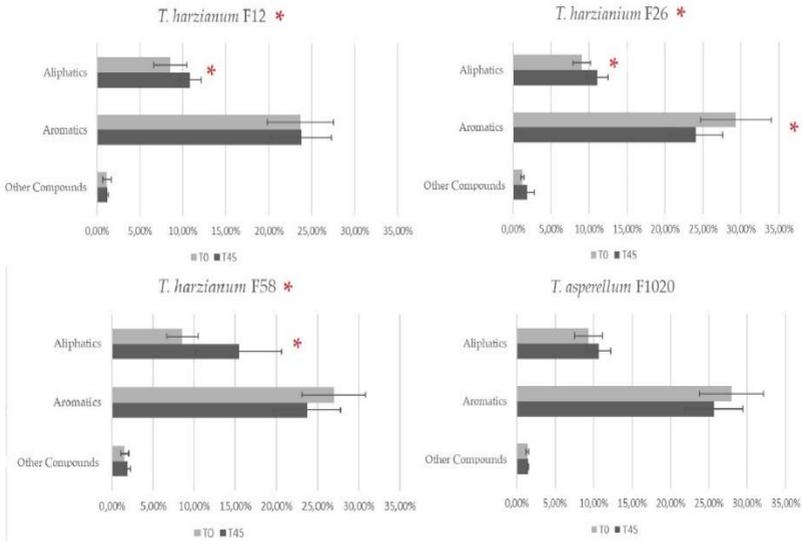


Figure 3. Degradation of used engine oil by *T. harzianum* F12, F26, and F58 and *T. asperellum* F1020. The compounds were grouped in the macroclasses Aliphatics, Aromatics, and Other Compounds. A red asterisk next to the strain name indicates statistically significant data tested by multivariate analysis of variance (MANOVA) or nonparametric MANOVA (PERMANOVA). A red asterisk next to the bar indicates statistically significant data via two-tailed (Wilcoxon) Mann–Whitney U test between T0 and T45 for the macroclass.

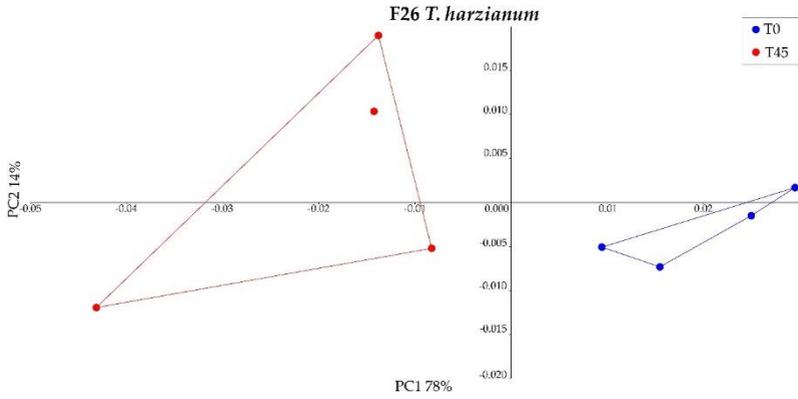


Figure 4. Principal component analysis (PCA) of used engine oil microclass degradation by *T. harzianum* F26 at Time 0 (T0) and after 45 days (T45).

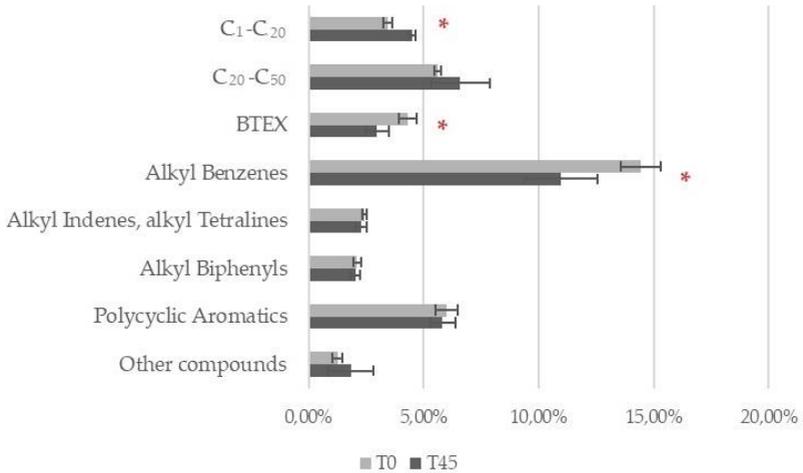


Figure 5. Degradation of used engine oil by *T. harzianum* F26 at Time 0 (T0) and after 45 days (T45). The compounds were grouped into microclasses. A red asterisk indicates statistically significant data (two-tailed (Wilcoxon) Mann–Whitney *U* test).

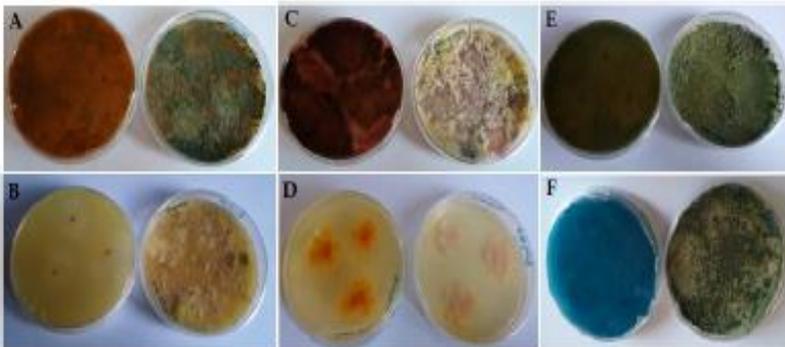


Figure 6. Colorimetric screening. (A) gallic acid test for *T. asperellum* F1020 (very high activity); (B) gallic acid test for *T. harzianum* F26 (no activity); (C) guaiacol test for *T. asperellum* F1020 (very high activity) (D) guaiacol test for *T. harzianum*

F58 (no activity); (E) RBBR test for *T. harzianum* F26 (very high activity); (F) RBBR test for *T. asperellum* F1020 (no activity); It is possible to appreciate the dark halo around the colonies for gallic acid and guaiacol test, and the decolorization of the medium for RBBR test.

Table 3. Results of the colorimetric test on *Trichoderma* strains.

| | Guaiacol | Gallic Acid | RBBR |
|----------------------------|-----------------|--------------------|-------------|
| <i>T. asperellum</i> F1020 | +++ | +++ | 0 |
| <i>T. harzianum</i> F12 | +++ | + | + |
| <i>T. harzianum</i> F58 | + | 0 | + |
| <i>T. harzianum</i> F26 | ++ | 0 | +++ |

0 no activity; + activity; ++ high activity, +++ very high activity.

4. Discussion

Over the years, many researches focused on the selection of fungal strains capable of degrading hydrocarbons, and the potential of *Trichoderma* in this field [26; 27; 47; 48]. The aim of our work, however, was to compare the bioremediation action of *Trichoderma* strains coming from different areas of the world and isolated from contaminated and non-contaminated substrates. Moreover, while most of the works already published showed the ability of *Trichoderma* to degrade crude oil or PAH mixtures [49-51], our investigation focused on the biodegradation of used engine oil. This pollutant is certainly very easy to find in the environment, as it is used daily all over the

world, so finding appropriate bioremediation remedies for used engine oil could have a great beneficial impact.

The tolerance test showed that all strains could grow on used engine oil, meaning that the pollutant does not totally inhibit their growth and that they could use it as a nutrient source. However, there were differences in the growth of the strains. *T. harzianum* F26 was the best performing, while *T. asperellum* F1020 was the least one. Moreover, in the GC/MS analysis of hydrocarbon degradation after 45 days, *T. asperellum* F1020, was the only one that did not produce statistically significant changes in the used engine oil macroclasses, while *T. harzianum* F26, F12 and F58 changed them significantly. The isolation substrate origin can explain this result: *T. asperellum* F1020 was isolated from toxic-free hydroponic plant cultivation; on the contrary, the three strains of *T. harzianum* were isolated from contaminated substrates with pesticides or hydrocarbons and could be naturally adapted to the use of recalcitrant substances for their growth.

The literature reports several cases of *T. asperellum* able to degrade hydrocarbons efficiently. For example, Zafra *et al.* [26] report the case of a *T. asperellum* removing large amounts of PAHs in soils contaminated with 1,000 mg Kg⁻¹ of a mixture of phenanthrene, pyrene and benzo[a]pyrene degrading the 74%, the 63% and the 81% respectively, in 14 days. Similarly, Husaini *et al.* [52] show how *T. asperellum* TUB F-1067 (SA4), *T. asperellum* Tr48 (SA5), *T. asperellum* TUB F-756 (SA6) strains can grow on a minimal substrate

containing 1% (v/v) of used engine oil. However, these strains had been isolated from heavily contaminated crude-oil soil or used motor oil. Probably, highly tolerant organisms, having the capacity to use hydrocarbons as the only source of carbon, are more likely to adapt to a more polluted environment [53].

We also analyzed the composition of the used engine oil by grouping the compounds into microclasses. In this case, the only strain that showed statistically significant results was *T. harzianum* F26, which showed better degrading action on the BTEX and alkyl benzene family, increasing that of C₁-C₂₀ alkanes. An explanation may be that *T. harzianum* F26 is more selective and prefers the degradation of BTEX and alkylbenzenes, while the other strains have a more widespread and less marked degradation in all microclasses making it more challenging to appreciate the results from a statistical point of view. Many authors indicate *T. harzianum* as one of the *Trichoderma* species able to degrade unsaturated hydrocarbons, BTEX, and resins as well as PAHs [54; 55]. This species, in fact, can degrade up to about the 10% of anthracene at the concentration of 400 mg kg⁻¹ and the 24,7% of pyrene at the concentration of 10 mg kg⁻¹ [56]. The results obtained by the action of *T. harzianum* F26 partially agree with the literature as it acts more on BTEX and Alkyl Benzenes. Other works, however, have also demonstrated its ability to grow even on a mixture of complex hydrocarbons such as crude oil up to a concentration of 3% [57].

Finally, the colorimetric tests were aimed at identifying ligninolytic enzymatic activities often associated with hydrocarbon degradation. Regarding the gallic acid test, only *T. asperellum* F1020 and *T. harzianum* F12 showed an indication of ligninolytic enzymes production, and the guaiacol test showed that *T. asperellum* F1020 and *T. harzianum* F12 were able to produce Lac and Per. However, the guaiacol test also revealed that *T. harzianum* F26 and *T. harzianum* F58 showed a slight activity. This result suggests that the guaiacol test may be more sensitive to the production of Lac and Per than that the gallic acid, even detecting milder activity. RBBR test was used to determine the strains' ability to degrade PAHs and indicate the presence of Lac [40]. *T. harzianum* F58 and *T. harzianum* F12 showed positive results, but *T. harzianum* F26 was the most active. F1020 showed no activity, consistently with the results obtained by GC/MS. The production of Lac and Per enzymes by these *Trichoderma* species has already been demonstrated by other authors [42; 56]. Zafra *et al.* [26] reported that fungal catechol 1,2 dioxygenase, laccase, and peroxidase are involved in the degradation of PAHs by *T. asperellum*. Considering the results of colorimetric screening, *T. asperellum* F1020 was an excellent producer of Lac and Per. However, this result was not demonstrated in the GC/MS test, suggesting the action of other enzymes in the degradation of used engine oil. Many authors have shown metabolic pathways activated by fungi to degrade hydrocarbons and what emerges is that microfungi, in particular,

mainly use cytochrome P450 (CYP) monooxygenases (EC:1.14.13.12) to degrade recalcitrant substances [11; 57-61]. Moreover, as already mentioned, adaptation to the pollutant is fundamental for its efficient degradation. On the contrary, the strains that generated significant results to GC/MS did not lead to equally evident signals in colorimetric tests. The reasons may be that *T. harzianum* F12, *T. harzianum* F26 and *T. harzianum* F58, could take more advantage of the CYP complex mentioned above or could increase the production of Lac and Per in the presence of used engine oil. In fact, the colorimetric tests were performed on MEA without the pollutant presence, as required by the protocol, but this may not have stimulated the production of the enzymes of interest. It is known that the concertation of lignin products and organic pollutants can induce the regulation of Lac and Per, especially in white-rot fungi [62; 63], but a similar effect has been noted by Jonathan *et al.* [58] in *T. harzianum*. They found that the concentrations of Lac and Per produced by a strain of *T. harzianum* increased when the fungus grew in a polluted medium of crude oil than in the oil-free medium.

Further research should focus on exploring the enzymatic mechanisms of hydrocarbon degradation in-depth, especially for the strain *T. harzianum* F26, which was the most successful.

The experiments carried out in this work have shown that the *Trichoderma* strains tested tolerate the presence of used engine oil and that they can use it for their growth. The degradation efficiency,

however, varies greatly depending on the single strains and species, as demonstrated in GC/MS tests. The causes may be different environmental growing conditions and substrate types, but especially adaptation to the presence of toxic agents such as pesticides, PAHs and chlorinated agents [63-66]. *T. harzianum* F26 was the strain that best responded to all the tests, and it was hypothesized that this strain might have developed adaptive mechanisms to be able to degrade this type of hydrocarbon pollutants.

Further studies are certainly needed to obtain more information on the chemical processes involved in bioremediation by *Trichoderma*. For this reason, an approach that integrates genetic, molecular, biochemical and ecological studies can be a useful tool in understanding the behavior of this fungus and its metabolic processes. Besides, genetic engineering could improve the degradative pathways and the substrate specificity in *Trichoderma* also in the prospective to develop modified strains capable of degrading specific families of pollutants.

In conclusion, we can summarize the results of this work in four main messages:

1. the efficiency of degradative activities seems to vary greatly depending on the different species and single strains.
2. The use of strains isolated from contaminated environments provides an advantage in bioremediation activities. This may depend on the adaptation of fungi to stress conditions that

limit their growth and on an improved enzymatic response to the degradation of recalcitrant substances.

3. *T. harzianum* F26 can be subject to further studies to deepen and enhance their potential as bioremediation agent, including field studies.

Author Contributions:

Conceptualization: D.C., T.M.E.E., N.L., T.S., M.B.; methodology: D.C., T.M.E.E., N.L., M.B., C.F.; software: N.L., T.M.E.E., D.C.; data curation: T.M.E.E., N.L., D.C; writing-original draft preparation: C.D, N.L.; writing-review and editing: N.L, D.C, T.S., C.G.; visualization: T.M.E.E., D.C., N.L.; supervision: T.S.; project administration: T.S.; funding acquisition: T.S. All authors have read and agreed to the published version of the manuscript.

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Chapter 5: Assembling of a fungi-bacteria consortium and inquiring its efficacy

Abstract

Once the degradative capacities of the selected strains and their enzymatic activities were investigated, I evaluated the possibility to use them in a consortium. An antibiosis test was performed to ensure the strains compatibility, and those compatible were combined in a consortium with the selected bacterial strains. Consortium degrading abilities were then tested to demonstrate its efficacy in Condensate-16 degradation by GCxGC. Finally, strains preservation methods were tested to find the most suitable one for preserving and transporting the consortium over long distances.

5.1 Introduction

5.1.1 Microbial consortia for bioremediation processes

A consortium is defined as a group of different microbial populations that interact and function together as a community (Bao *et al.*, 2012). Functional differentiation and metabolite exchange during ecological interactions, particularly in co-operative relationships, allow consortium species to efficiently access nutrients

and achieve strong resistance and resilience to environmental perturbations, ensuring protection and development for all consortium members (Kato *et al.*, 2008). This lifestyle is so favourable that in nature, microorganisms typically occur in complex communities containing several populations that can interact metabolically with each other. Despite the advantages of this life form, there remain some challenges for natural microbial consortia, such as the low population stability and controllability, which interfere with their practical applications (Ding *et al.* 2016). These are the key points that researchers need to work on to obtain multispecies cultures useful in bioremediation processes.

The advantages of using mixed instead of pure cultures in bioremediation have been widely demonstrated (Das and Chandran, 2011; Varjani *et al.*, 2013). The challenge for microbes to degrade all components of an oil mixture is immense. Although many microorganisms can metabolise various petroleum hydrocarbons, no single microorganism possesses the enzymatic capacity to degrade all, or even most, of the hydrocarbon components of petroleum pollutants. The different metabolic activities of the species that make up a consortium make these systems an excellent candidate for the degradation of oil pollutants. For this reason, in a bioremediation process of a complex mixture of hydrocarbons, a microbial consortium consisting of several taxonomic microorganisms with different metabolic properties is preferable. Mixed populations harbouring

different sources of active enzymes are responsible for further increasing the oil biodegradation rate and speed (Priya *et al.*, 2015). The effects of synergistic interactions between consortium members are crucial. The mechanisms by which oil degraders benefit from synergistic interactions can be complex. One species can remove some toxic metabolites, and another species can degrade compounds that the first can only partially (Alexander, 1999). This is why it is necessary to understand the roles of individual members in influencing the effectiveness of a microbial association.

Various reports are available on the metabolic versatility of mixed cultures that have demonstrated the superiority of consortia to pure cultures to utilise in hydrocarbons biodegradation (Das and Chandran, 2011; Varjani *et al.*, 2013). Recent studies have also shown that a well combined microbial consortium will share complementary catabolic pathways aimed explicitly at degrading hydrocarbons (McGenity *et al.*, 2012). For example, the bacterial and fungal consortium has shown improved degradation rates of diesel oil and many polyaromatic hydrocarbons (PAHs) under laboratory conditions (Varjani and Upasani, 2013). The results obtained by Li and Li (2011) revealed that the introduction of a consortium of fungus-bacteria to petroleum hydrocarbons bioremediation was more efficient than the sum of the individual removal obtained in pure culture of fungus and bacteria. A consortium composed of *Acremonium* sp. and *Bacillus subtilis* efficiently degraded naphthalene, fluorine, phenanthrene,

anthracene, and fluoranthene individually (50 mmol/L) over ten days (Ma *et al.*, 2018). A consortium of three alkane-degrading bacterial strains (*Arthrobacter oxydans* ITRH49, *Pseudomonas* sp. ITRI73, *Pseudomonas* sp. MixRI75) degrades the 80% of diesel in a soil contaminated with 1,5 g kg⁻¹ of diesel (Ummara *et al.*, 2020). Another study evaluated a mixed microbial consortium (*Mycobacterium fortuitum*, *B. cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, *Microbacteriaceae* bacterium and *Fusarium oxysporum*) isolated from a PAHs contaminated land farm site to degrade and mineralise different concentrations of anthracene, phenanthrene and pyrene in soil: the consortium degraded on average, 99%, 99%, and 96% of the PAHs different concentrations in 70 days, respectively (Jacques *et al.*, 2008).

The degradative capacity of any microbial consortium is not necessarily the result of merely adding together the capacities of the individual strains forming the association. Many groups researching consortial biodegradation observed this. Komukai–Nakamura and co-workers (1996) reported the sequential degradation of Arabian light crude oil by two different genera. *Acinetobacter* sp. T4 biodegraded alkanes and other hydrocarbons producing the accumulation of metabolites. Following that, *Pseudomonas putida* PB4 began to grow on the metabolites and finally degrade aromatic compounds in the crude oil.

The interactions that take place during a bioremediation process are complex. The process depends on many factors such as the type of contaminant, the microorganisms used, the interaction between the selected microorganisms, their ability to produce biosurfactants, and any other factors able in increasing their activity.

5.1.2 Strains preservation methods

In order to use the consortium where there is a need to carry out bioremediation, it is necessary to find a method that would make it easily conservable, transportable and that would not damage the vitality of the microorganisms present. Many methods are available to preserve microorganism, maintaining them in a state of vitality without modifying their genetic, physiological or anatomical characteristics, but the two widely used are cryopreservation and freeze-drying.

Cryopreservation is an excellent method, providing appropriate care during freezing and thawing because the strains will not undergo any phenotypic or genetic modification. Although microorganisms, especially fungi, are often resistant to ice-induced damage, cooling must be controlled to achieve optimal survival. The choice of the right cryoprotectant is also crucial and varies depending on the organism type (Ryan and Smith, 2007).

Freeze-drying is the removal of ice or other frozen solvents from a material through sublimation and the removal of bound water

molecules through the process of desorption (Mellor, 1978; Wolkers and Oldenhof, 2015). Freeze-drying is a reliable and successful conservation technique, especially for sporulating fungi (Ryan and Smith, 2007). Compared to other methods, the advantages of freeze-drying include good stability, long shelf life, convenient storage of vials in a laboratory environment under environmental conditions, and easy distribution (Tan, 1997). In order to achieve optimal freeze-drying, attention must be paid to several factors such as the cooling rate, which is fundamental for the process to take place uniformly and with not excessively long times, the final temperature and the rate of heat input during drying. Excessive drying will be lethal to cells or induce mutation by damaging the DNA (Rey *et al.*, 1977). However, having too high a residual moisture will result in rapid deterioration during storage (Smith and Kolkowski, 1992).

5.2 Materials and Methods

5.2.1 Antibiosis test in dual culture to test fungal interactions

Antibiosis tests is a method used to study the fungi interactions on each other. This test was performed based on the protocol proposed by Skidmore and Dickinson (1976).

The strains interactions were examined in culture by inoculating all possible paired combinations (Tab. 5.1) (or single, as the control) on PDA. The fungal strains were aseptically inoculated by taking a

small portion of the colony grown for seven days on PDA. In the test plate, two strains were inoculated at 1,5 cm from the edge at the opposite sides along the diameter, and in the control plate, one strain was inoculated alone in the same position (Fig. 5.1). The plates were then incubated at 26°C for seven days. After this time, the interactions were visually assessed using the reported key (Skidmore and Dickinson, 1976) (Fig. 5.2). The fungal interactions can be:

- i) Mutually intermingling growth where both fungi grow into one another without any visible signs of interaction.
- (ii) Overgrowth by antagonism where one strain grows on the other colony that has stopped growing or is heavily inhibited.
- (iii) Slight inhibition where the fungi approached each other until almost in contact and a narrow demarcation line between the two colonies is visible.
- (iv) Mutual inhibition at a distance of > 2 mm.

The colony growth radius was also calculated with the following formula:

$$\% \text{ growth} = \frac{R_c - R_t}{R_c} \times 100$$

R_c: radius of the strain inoculated alone.

R_t: radius of the strain inoculated with another one.

Chapter 5

Table 5.1. Fungal strain pairs used in the antibiosis test. With this system, all the strains' interactions are tested.

| Fungal strain pairs | | | |
|---------------------|-----------|-----------|-----------|
| F10 + F13 | F13 + F15 | F15 + F28 | F26 + F41 |
| F10 + F15 | F13 + F26 | F15 + F37 | F28 + F37 |
| F10 + F26 | F13 + F28 | F15 + F39 | F28 + F39 |
| F10 + F28 | F13 + F37 | F15 + F41 | F28 + F41 |
| F10 + F37 | F13 + F39 | F26 + F28 | F37 + F39 |
| F10 + F39 | F13 + F41 | F26 + F37 | F37 + F41 |
| F10 + F41 | F15 + F26 | F26 + F39 | F39 + F41 |

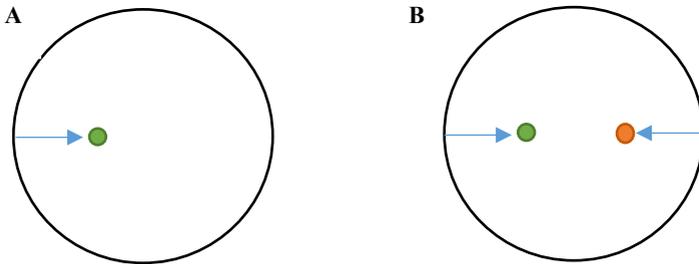


Figure 5.1. Schema followed to perform the antibiosis test.

A. control plate in which it is inoculated the strain alone. B. test plate with the strains in pairs.

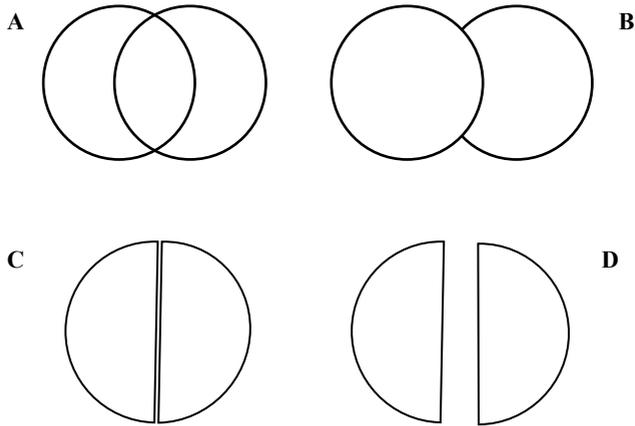


Figure 5.2. Proposed fungal interactions. A. Mutually intermingling growth; B. Overgrowth by antagonist; C. Mutual slight inhibition; D. Mutual inhibition at a distance.

5.2.2 *Assembling of fungi-bacteria consortium*

The best performing fungal and bacterial strains from this work were combined to assemble a consortium with high degradative capacities. The bacterial consortium was set up by Dr Carpani, who selected the most suitable bacterial strains for the purpose after carrying out hydrocarbon-degrading and compatibility tests. The fungal strains were chosen based on the results of the antibiosis test.

5.2.3 Testing the consortium abilities to degrade hydrocarbon complex mixtures

Once the fungi-bacteria consortium was set up, it was tested in its abilities to degrade complex hydrocarbon substrates. To this end, GCxGC analysis, which was the most suitable method to verify hydrocarbons degradation, was carried out to assess the Condensate-16 hydrocarbon composition *pre-* and *post-* treatment with the consortium. Condensate-16 was used because it is the consortium's final target, the substrate on which it should work.

The consortium suspension (200 μ l) was inoculated into a 100 mL sterile glass bottle with a crimp seal filled with 60 mL of BH medium and 1% (v/v) of Condensate-16. The consortium inoculum was prepared by growing the selected fungi and the selected bacteria in BH + 1% of Condensate-16 for 7 days on a rotary shaker at 26° C and 80 rpm and inoculated. Condensate-16 was sterilised under UV light for 30 minutes. A control was prepared in the same condition, without the consortium inoculum, and with the addition of 2 mL of 10% sodium azide (NaN_3) to prevent microorganisms' growth. The test bottles were incubated at 26°C at 80 rpm for 30 days.

The analysis was performed at the inoculum moment (T0) and every seven days for 28 days, extracting the hydrocarbon residues with dichloromethane in a ratio of 1:1 and shaken vigorously on vortex for 1 min at 3000 rpm. The organic layer was then allowed to separate for 5 min, 500 μ l was collected and placed in 1mL vials ready for the GC

analysis. The GCxGC analysis was carried out on an Agilent 7890B GC System. The samples' injection was performed at 380°C in split mode, ratio 5:1 and the flame ionization detector (FID) set up at 380°C. The oven temperature program was 35°C for 5 min, temperature increment up to 400°C at 4°C/min, and hold at 400°C for 5 min. Two columns J&W CP9092VF – 5ht Ultimetel (30m x 250µm x 0,1 µm film thickness), carrier gas (H₂) flow = 0,3 mL/min and a Zebron 7HG – G025 – 11ZB – 35HT Inferno (5m x 250 µm x 0,25 µm film thickness), carrier gas (He) flow = 21 mL/min were used. The GCxGC valve had a modulation time of 2,7 sec and a sample time of 2,55 sec. The data processing was performed using GC Image R2.5 GCxGC (GC Image, LLC, and the University of Nebraska.).

5.2.4 Preservation of the consortium

The microbial consortium was assembled to be used for *in-situ* bioremediation. Therefore, it was necessary to find a method of storing and transporting the consortium strains and the consortium itself that would not damage the viability of the microorganisms, and that was both simple and economical. Two methods were tested for this purpose: cryopreservation and freeze-drying.

The protocol followed for bacterial and fungal strains cryopreservation, modified from Homolka (2013), planned to inoculate the strain in a PDA Petri plate and after seven days, or when the strain has grown on almost the entire plate, it can be stored. The

mycelium, maintaining the sterility, is scraped off from the plate and placed in a 10 mL tube containing a 15% solution of glycerol. The solution is homogenized through vortexing for 30/60 seconds at 3000 rpm. For the fungal strains, this step is made in a tube containing a 15% solution of glycerol and broken cover slides previously autoclaved to obtain a propagule suspension. Then, 1 mL of the suspension is placed in 1,5 mL cryotubes previously autoclaved. Individual fungal and bacterial strains were both cryopreserved separately and combined in a fungal-only and a bacterial-only consortium. Four copies are stored at -80°C

About freeze-drying, two different protocols were used for fungi and bacteria, respectively. The freeze-drying protocol for fungi was modified from Bunse and Steigleder (1991). Again, the strains were inoculated in Petri plates with rich medium, and when the strain grew on almost the entire plate, it was collected and inserted into sterile freeze-drying vials. The strains were then frozen for 24h at -21°C and then placed on the Büchi Lyovapor L-200 rack to begin the freeze-drying process. This step lasted 24h at a pressure of 1 mbar and a temperature of -50°C . The bacterial strains were inoculated into a rich liquid medium until an optical density at 600nm (OD_{600}) = 4 was obtained. Then, an equal volume of freeze-drying solution (Na-glutamate 2% + sucrose 14%) was added and the mix divided into freeze-drying vials to be lyophilized. Even in this case, individual

fungus and bacterial strains were lyophilized separately and combined in a fungus-only and a bacterial-only consortium.

5.2.6 Consortium revitalisation tests

Revitalisation tests were then carried out for all these preservation methods to ensure that the strains were still viable and undamaged.

For cryopreservation, the viability test first involved thawing the strain gradually in ice to avoid thermal shock. After thawing, the contents of cryovials were divided into two approximately equal portions each and placed in sterility on PDA Petri plates. The plates were then incubated at 26°C, and it was observed whether or not the colony would resume growth. Particular attention was also given to the possible presence of contamination.

Freeze-dried fungus strains were rehydrated with approximately 5 mL of sterile water and left to stand for at least two hours. They were then transferred into Petri plates with rich medium and incubated at 26 °C at complete revitalization. Instead, the bacterial strains were rehydrated in a volume of rich liquid medium adequate to the amount of lyophilized and incubated at 30°C. All the viability tests were conducted after two weeks and after a month.

5.3 Results and Discussion

5.3.1 Results of the antibiosis test

Figure 5.3 graphically shows the partition of the Petri plate space between the two inoculated strains. The growth reduction percentage has been calculated; the results are reported in Supplementary materials at the end of this chapter (Tab. 5.1S).

Almost all strains establish positive relationships (growth reduction <75%) without significantly inhibition observed. The only two strains that gave no positive results were *T. harzianum* F26 and *Coniochaeta* sp. F37. The growth of *Coniochaeta* sp. F37 was inhibited (growth reduction >75%) with almost all strains; thus, it was not suitable for life in a consortium. *T. harzianum* F26, on the other hand, is the strain that most inhibited the growth of the others, causing growth reduction >75% for 5 out of 7 strains. This result is not unexpected since F26 is *T. harzianum*; *Trichoderma* is a genus known to be a hyperparasitic species of other fungi, *T. harzianum* particularly (Juliatti *et al.*, 2019; Węgrzyn *et al.*, 2019; Inayati *et al.*, 2020).

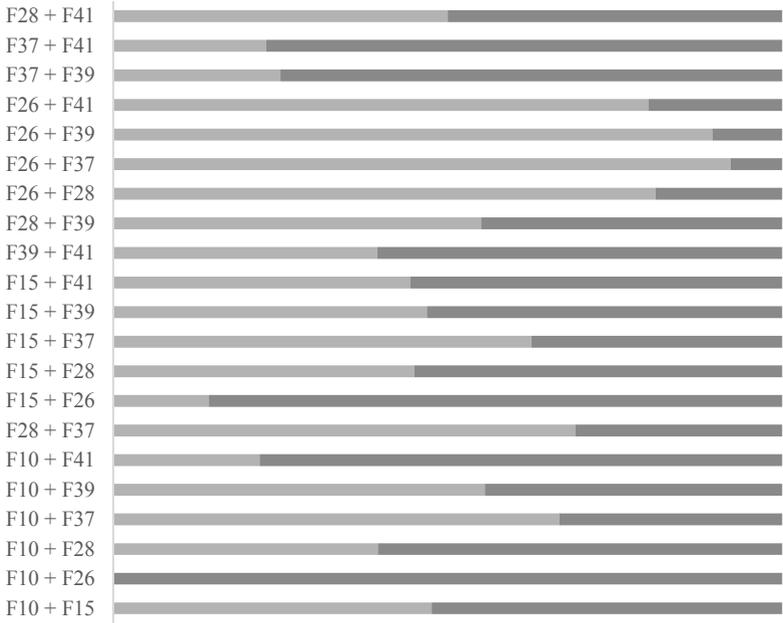


Figure 5.3. Results of the antibiosis test. The figure showed the partition of the Petri plate between the two inoculated strains.

5.3.2 Fungal and bacterial strains selected to assemble the microbial consortium

In the light of the obtained results, it was possible to assemble a fungal consortium with the six strains that established positive interactions: *Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Curvularia aerea* F28, *Fusarium solani* F39 and *F. chlamydosporum* F41 (Tab. 5.2). The microbial

consortium was made-up with fungal consortium and the bacterial one set up by Dr. Carpani (Tab. 5.3).

As already said, *Scedosporium apiospermum* is a group 2 agent and can cause severe infections in immunocompromised patients (rarely also in immunocompetent people) (Guarro *et al.*, 2006). For this reason, further analyses will be necessary to rule out the possibility that the *Scedosporium apiospermum* F10 strain is potentially pathogenic. If analyses indicate that it is dangerous, it will be immediately excluded from the consortium.

The fungal consortium is maintained in flasks with malt extract broth (MEB) and the bacterial consortium in flasks with MEB pH 6.5.

Table 5.2. Species composition of the fungal consortium

| Fungal consortium | |
|--------------------------|---------------------------------|
| Strain | Species |
| F10 | <i>Scedosporium apiospermum</i> |
| F13 | <i>Alternaria alternata</i> |
| F15 | <i>Penicillium oxalicum</i> |
| F28 | <i>Curvularia aeria</i> |
| F39 | <i>Fusarium solani</i> |
| F41 | <i>Fusarium chlamydosporum</i> |

Table 5.3. Species composition of the fungal consortium

| Fungal consortium | |
|--------------------------|--|
| Strain | Species |
| Myc 7 | <i>Pseudomonas balearica</i> strain Z8 |
| Myc 27 | <i>Pseudomonas stutzeri</i> USW-CAP-1 |
| Myc 38 | <i>Stenotrophomonas</i> sp. strain MRC 2-1 |
| 1.5 | <i>Pseudomonas</i> sp. strain H16 |
| 1.19 | <i>Pseudomonas stutzeri</i> strain FM22 |
| 1.29 | <i>Pseudomonas stutzeri</i> strain NCTC10451 |
| 2.1 | <i>Pseudomonas balearica</i> strain 56 |
| 3.1 | <i>Gordonia</i> sp. strain N-6-2 |
| 3.6 | <i>Pseudomonas</i> sp. strain 38M1 FL01 |
| 3.9 | <i>Gordonia</i> sp. strain hbs1 |
| 3.48 | <i>Pseudomonas plecoglossicida</i> strain MR70 |
| 4.8 | <i>Pseudomonas</i> sp. strain 09 |
| 4.26 | <i>Pseudomonas</i> sp. strain R2A2 |
| 4.27 | <i>Pseudomonas</i> sp. strain THAF7b |
| 5.1 | <i>Pseudomonas</i> sp. strain TW2-101 |
| 5.6 | <i>Pseudomonas</i> sp. strain ISA 15 |
| 5.10 | <i>Pseudomonas stutzeri</i> strain MN1 |
| 6.1 | <i>Pseudomonas</i> sp. strain RMR33 |
| 6.18 | <i>Pseudomonas</i> sp. strain ZGLJ8 |
| 6.35 | <i>Pseudomonas stutzeri</i> strain EGY-SCi2 |
| 7.2 | <i>Pseudomonas stutzeri</i> strain CUMB KMR-05 |
| 8.2 | <i>Leucobacter chromiireducens</i> Kh.C. M7 |
| 8.6 | <i>Gordonia</i> sp. strain N-6-2 |
| 8.10 | <i>Gordonia amicalis</i> |
| 8.11 | <i>Pseudomonas</i> sp. PrPco79 |
| 8.12 | <i>Pseudomonas taiwanensis</i> WTB6 |
| 8.19 | <i>Pseudomonas balearica</i> strain OT17 |
| 8.21 | <i>Pseudomonas</i> sp. strain SA03 |

5.3.3 Consortium degrading abilities on hydrocarbon complex mixtures

The GCxGC analysis of the hydrocarbons present in the Condensate-16 allowed to verify the fungi-bacteria consortium activity. The control's chromatograms remained almost unchanged throughout the analysis period, while the analysis of treatments reported fascinating changes. Figure 5.4 shows the chromatogram of Condensate-16 at the inoculum moment. After one week of incubation, almost all the detected hydrocarbons in Condensate-16 had been degraded (Fig 5.5). In the following weeks of the test, the results did not show any further noticeable changes in the composition of Condensate-16; for this reason, only the chromatograms of the last week of analysis are presented here (Fig. 5.6). The consortium seems to be more active and efficient in the degradation of this substrate. After a week of incubation, the synergistic activity of fungal and bacterial strains led the consortium to act widespread on the Condensate-16, making the degradation process faster and more efficient.

Many studies have already shown that the simultaneous action of several microorganisms with different metabolic properties is preferable (Bacosa *et al.*, 2010; Zafra *et al.*, 2016; Kumari *et al.*, 2018). Different species have different enzymatic activities that a microbial consortium could use to metabolise a wide range of substances. Despite this, not many works using a mixed fungi-bacteria

consortium are available. Most of them focus on using only bacterial consortia or only fungal consortia (Ghanem *et al.*, 2016; Garrido-Sanz *et al.*, 2019; Phulpoto *et al.*, 2021). However, some works are available, and Li and Li (2011) demonstrated that using a fungi-bacteria consortium to petroleum hydrocarbons bioremediation was more efficient than the sum of the individual removal obtained in pure culture of fungus and bacterium. Also, Zanaroli *et al.* (2010) saw that enriched consortia of bacteria and a fungus could degrade a broad range of the hydrocarbons composing diesel fuels. Finally, Zafra *et al.* (2015) reported a mixed microbial consortium's efficiency to degrade high amounts of PAHs in soils; this high activity is presumably due to increased co-metabolic degradation.

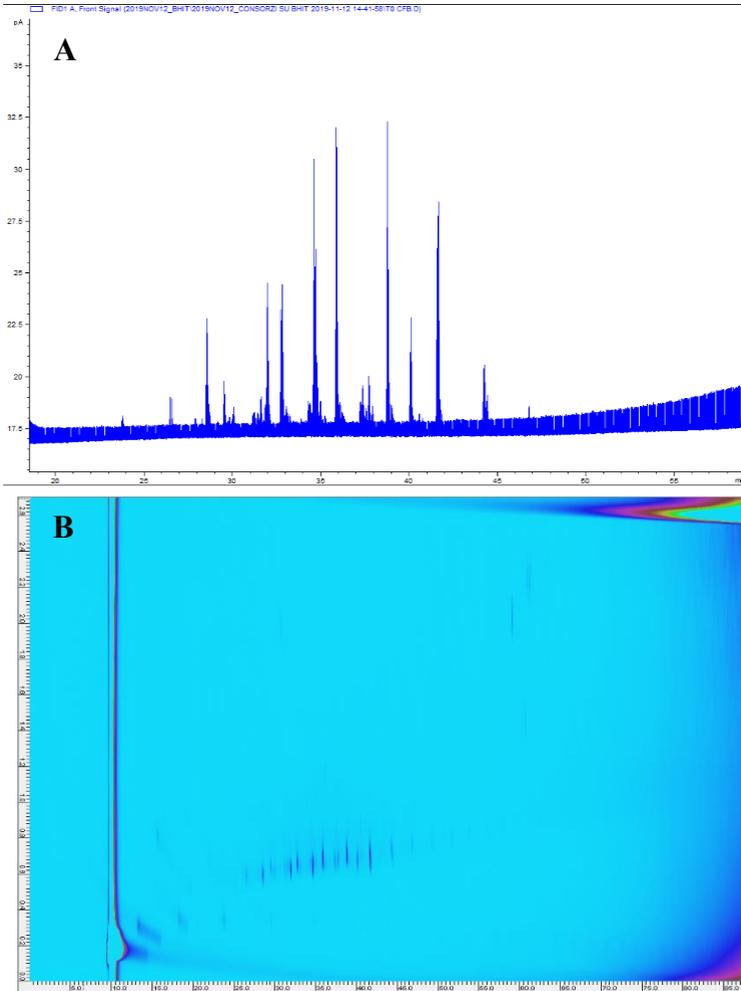


Figure 5.4. Condensate-16 hydrocarbons present in the treatment with the consortium at T0. A: monodimensional chromatogram; B: bidimensional chromatogram.

Chapter 5

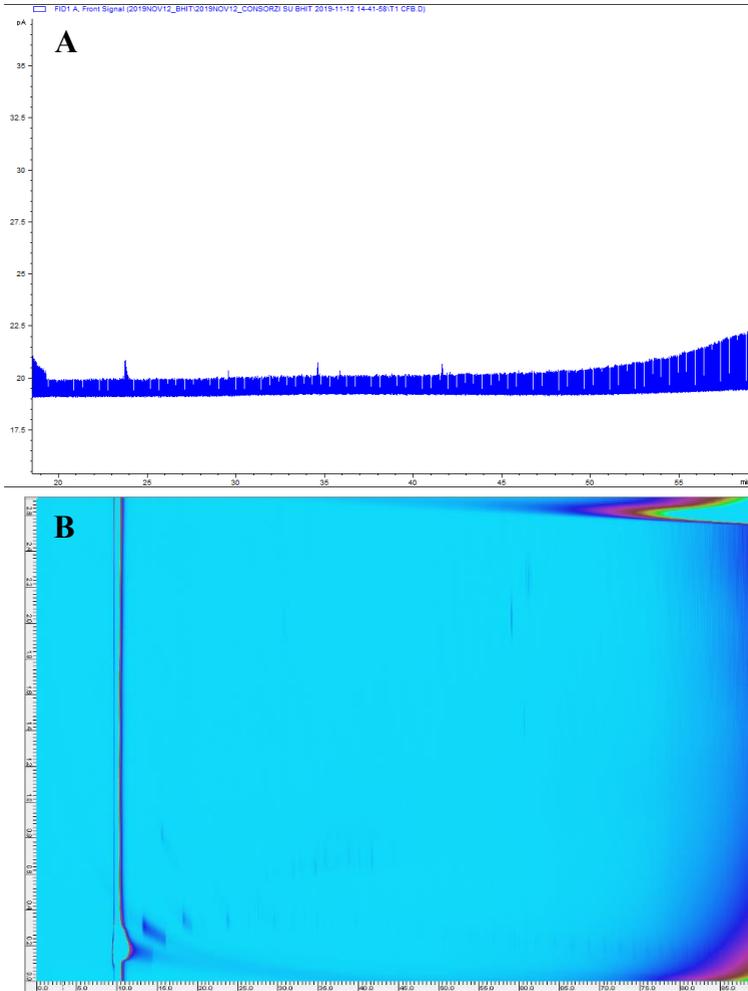


Figure 5.5. Condensate-16 hydrocarbons present in the treatment with the consortium after seven days. A: monodimensional chromatogram; B: bidimensional chromatogram.

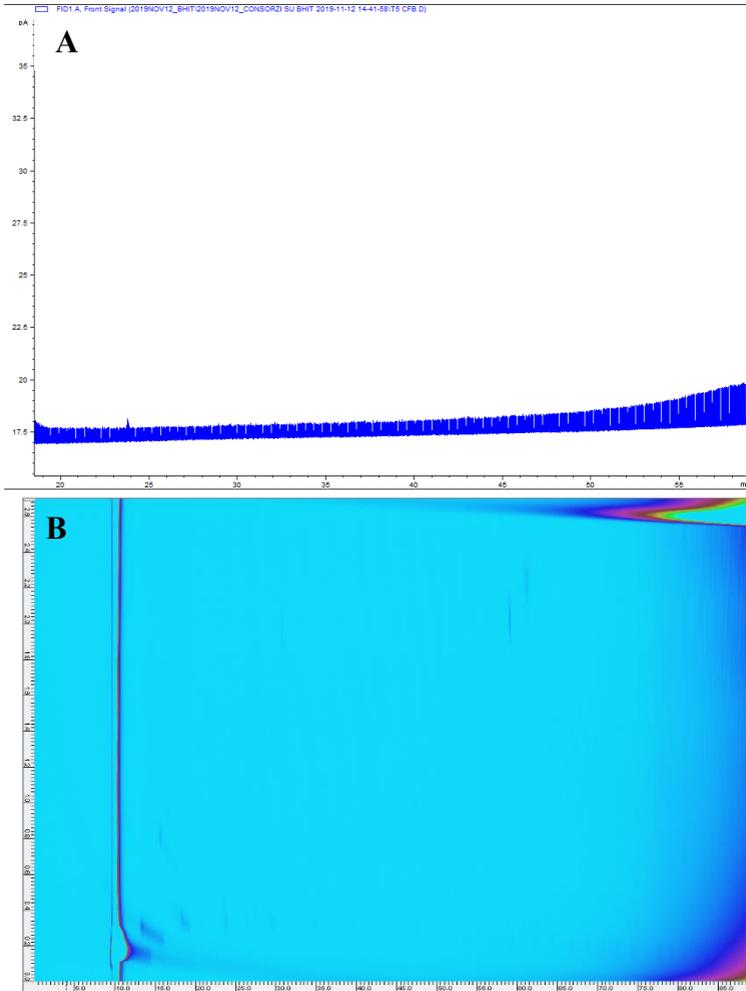


Figure 5.6. Condensate-16 hydrocarbons present in the treatment with the consortium after 30 days. A: monodimensional chromatogram; B: bidimensional chromatogram.

5.3.4 Revitalisation of the consortium's strains

Cryopreservation and freeze-drying are both excellent methods of preserving strains economically, quickly and easily. The viability tests carried out on both the cryopreserved and the freeze-dried consortium strains gave positive results. The strains all started to grow again after 2/3 days from inoculation in the plate. The test was positive also revitalizing the strains after more than a month.

However, I decided to use the two methods for different purposes. Cryopreservation was chosen to store copies of the strains in the laboratory for an extended period. This technique can be applied to sporulating and non-sporulating cultures and, with proper care during freezing and thawing, the strains will not be damaged (Ryan and Smith, 2007). Cryopreservation also allows the strain to be preserved in combination with other microorganisms.

On the other hand, freeze-drying proved to be the best method of transporting the strains and the consortium even over long distances. Freeze-dried strains do not take up much space and do not require any special precautions during shipment, which would have been required to transport frozen samples.

Conclusions

An antibiosis test was performed on the selected fungal strains to ensure their compatibility with a consortium's life. *Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Curvularia aerea* F28, *Fusarium solani* F39 and *Fusarium chlamydosporum* F41 were combined in a consortium with the bacterial strains selected by Dr Carpani. Consortium degrading abilities were then tested to demonstrate its efficacy in Condensate-16 degradation by GCxGC. The consortium has been efficient in Condensate-16 degradation. After a week of activity, the synergistic work of fungal and bacterial strains led the consortium to act widespread on the Condensate-16, degrading almost all the hydrocarbons present in the substrate. Finally, strains preservation methods were tested to find the most suitable one for preserving and transporting the consortium over long distances. The most effective method to preserve individual strains and the complete consortium was found to be cryopreservation. However, freeze-drying is the most suitable method for transporting consortium because freeze-dried samples do not need any special handling to be transported.

Supplementary material

Table 5.1S. Results of antibiosis test. Growth reduction is expressed in percentage and calculated as the difference between the radius of the strain grown together with another one and the strain grown individually.

| Strains | % growth reduction | |
|-----------|--------------------|-----|
| F10 + F13 | 20% | 6% |
| F10 + F15 | 0% | 27% |
| F10 + F26 | 100% | 8% |
| F10 + F28 | 15% | 35% |
| F10 + F37 | 0% | 78% |
| F10 + F39 | -25% | 43% |
| F10 + F41 | 65% | 44% |
| F13 + F15 | 0% | 2% |
| F13 + F26 | 78% | 5% |
| F13 + F28 | 0% | 70% |
| F13 + F37 | 50% | 81% |
| F13 + F39 | 10% | 13% |
| F13 + F41 | 23% | 2% |
| F15 + F26 | 67% | 8% |
| F15 + F28 | 40% | 45% |
| F15 + F37 | 17% | 67% |
| F15 + F39 | 50% | 51% |
| F15 + F41 | 33% | 44% |
| F26 + F28 | 54% | 83% |
| F26 + F37 | 8% | 89% |
| F26 + F39 | 8% | 80% |
| F26 + F41 | 8% | 67% |
| F28 + F37 | 28% | 71% |
| F28 + F39 | 45% | 49% |
| F28 + F41 | 50% | 56% |
| F37 + F39 | 78% | 40% |
| F37 + F41 | 82% | 40% |
| F39 + F41 | 57% | 49% |

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Chapter 6: Ecotoxicological test

Abstract

An ecotoxicological test was set up to demonstrate the bioremediation capacity of the assembled fungi-bacteria consortium. A phytotoxicity test was performed using *Lepidium sativum* L., the common watercress, as the bioindicator of the removal of the hydrocarbons from contaminated soil. Watercress seeds were planted in soil artificially contaminated with used engine oil after being treated with the consortium for a week, two weeks and three weeks. The seed germination and root length of watercress plants were measured. The test showed that the consortium could remediate the polluted soil, returning it almost to an uncontaminated state by acting for at least three weeks.

6.1 Introduction

*6.1.1 Ecotoxicological tests and the bioindicator *Lepidium sativum**

Ecotoxicology evaluates the toxic effects of chemical and physical agents on living organisms, including how these agents spread and their interactions with the environment (Pavel *et al.*, 2013). Ecotoxicological tests allow assessing environmental contamination caused by different pollution sources. They have the ability to detect

deleterious effects produced by a toxic agent, or mixture, on living organisms. Thus, these tests allow evaluating how hazardous these substances are (Chasin and Pedrozo, 2013; Clasen and Moura Lisbôa, 2019). The response of organisms to different concentrations of pollutant is monitored for one or more fixed periods of exposure. At least one group of organisms (control group) is not subjected to the pollutant but is treated exactly in the same way as the exposed organisms. The parameter observed and measured (endpoint) in the target organisms may be mobility, survival, size or growth, number of eggs or offspring, or any physiological variable that can be reliably quantified. The aim is to establish what kind of relationship exists between an endpoint and test substance concentration or sample concentration (source: ispra.cnr.it).

Lepidium sativum is a plant commonly used as bioindicator for phytotoxicity tests because it is a high sensitivity species, with a fast germination time and a very low cost (Pavel *et al.*, 2013; Covino *et al.*, 2015; Manãs and De las Heras, 2018). Petroleum hydrocarbons are known to affect the germination and growth of plants adversely and through the formation of a film of hydrocarbons around the seeds producing a physical barrier to water and oxygen uptake (Adam and Duncan, 2002). Normally in ecotoxicological tests with *L. sativum* two endpoints are tested: seeds germination and roots elongation.

6.2 Materials and Methods

6.2.1 Preparation of the ecotoxicological test

The test is aimed at demonstrating the bioremediation capacity of the fungi-bacteria consortium. The test was carried out in plastic Compact Disc (CD) cases carefully washed with dish soap, 70 % alcohol and then left under UV rays for 30 minutes. It has been chosen to use CD cases instead of standard plastic pots because they allow easy observation of the plant's growth without necessarily eradicating or damaging it. Spheres of expanded clay were placed on the bottom to allow water drainage. (Fig. 6.1). A total of eight CD cases, each containing expanded clay, 100 g of polluted soil, and the consortium inoculum, were prepared. The polluted soil (1 Kg) was artificially polluted with 5% (w/v) of used engine oil. The unpolluted soil was previously sterilized for two cycles in the autoclave at 121° C for 1 hour. Five mL of fungi-bacteria consortium were inoculated using 1 mL Pasteur pipette, distributing the inoculum in all the soil volume. The consortium was pre-adapted to the hydrocarbon presence by growing the consortium's strains in BH + 1% of used engine oil for 7 days on a rotary shaker at 26° C and 80 rpm. The action time of the consortium in the contaminated soil was one week, two weeks and three weeks. Three different controls were prepared: (i) non-contaminated soil; (ii) non-contaminated soil and the consortium

inoculum; (iii) polluted soil. Each test was conducted in duplicate. Figure 6.2 shows a schematic representation of the test set-up.

Ten *Lepidium sativum* seeds were immediately planted in the controls. In the treated soil, they were planted after one week, two weeks and three weeks of consortium action. *Lepidium sativum* is an annual plant widely used in toxicity tests because of its sensitivity, rapidly growing, cheap and easy to analyze (Pasini *et al.*, 2001). Ten seeds were sown in each replication, for a total of 20 seeds per treatment. The seeds were examined and selected, which means that the discoloured or damaged seeds were removed. For all the experiment time, the system was kept moist by frequent watering.



Figure 6.1. CD case prepared for the test; spheres of expanded clay and paper were placed on the bottom to allow water drainage and humid the system.

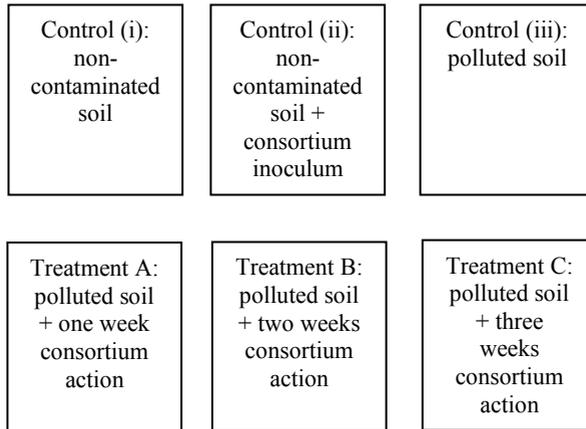


Figure 6.2. Schematic representation of the test set-up. All the trials were in duplicates.

6.2.2 *Lepidium sativum* germination test

The sensitivity of *L. sativum* germination to hydrocarbons was assessed based on the number of the germinated seeds and the root length measurement. The number of germinated seeds was monitored two times a week for two weeks, and the lengths of each seedling's radicle (L) grown in the controls and the treatments were carefully measured two weeks. The measurement of radicle elongation is taken from the node (the thickest transition region between the radicle and the hypocotyl) to the root's tip.

The germination index (GI) was calculated as follows (Hoekstra *et al.*, 2002):

$$GI \% = 100 \times (Gs/Gc) \times (Ls/Lc)$$

Gs: average number of germinated seeds in the sample

Gc: average number of germinated seeds in the control of clean soil

Ls: average radicle lengths of seedlings in the sample

Lc: average radicle lengths of seedlings in the control of clean soil

6.2.3 Statistical analysis

The experimental design used six treatments (including three different controls) with two replicates (each CD case was considered a replicate). Data were processed statistically using ANOVA analysis and Past3 software. To verify that the normal distribution of the data, standardized skewness and kurtosis values were checked.

6.3 Results and Conclusions

6.3.1 Results of ecotoxicological test

The unpolluted soil was used as the reference control (Fig. 6.3). Significant differences ($P < 0,05$) were found for all the collected data.

No clear and official statements exist, but according to Aguerre and Gavazzo 2012, there is the criterion that $GI > 80\%$ values indicate no phytotoxic substances or they are found at very low concentrations; values between 50 and 80% could indicate a moderate presence of

pollutants and GI <50% indicate that the presence of phytotoxic substances is strong.

The GI of the control with unpolluted soil and the consortium inoculum is 91,7%, close to the GI 100% of the unpolluted soil without consortium, indicating favourable growing watercress conditions. The GI of the seeds planted in the contaminated soil, on the other hand, is 37,5%, a very low value that indicates strong phytotoxicity. Finally, the seeds sown in the polluted soils treated with the consortium for the three periods of time exhibited a variable behaviour, displayed by the different GI. The one-week treatment had a GI of 50,7, just above the high toxicity threshold of 50%, while the three-weeks treatment had a GI of 80,1%. This allows us to state that the consortium acts on the toxicity of the oil by reducing it and making the soil almost as fertile as uncontaminated soil. All the germination indices are reported in table 6.1 (the two-weeks treatment was not calculated due to lack of data).

Figure 6.4 shows the number of germinated seeds in all trials over 15 days. The germination time of seeds planted in unpolluted soil is consistent with that indicated by the seed producer; most of the seeds germinated in less than a week. Besides, seeds sown in the unpolluted soil inoculated with the consortium, compared to those planted in unpolluted soil alone, developed a longer rootlet and a more vigorous shoot, suggesting a consortium's supplementary action on watercress germination. Several microorganisms, in fact, are known

to colonize plant roots, improving their health and growth, enhancing their ability to tolerate biotic and abiotic stress and increasing nutrient availability and absorption (Morrissey *et al.*, 2004; Jain *et al.*, 2012; Mondal *et al.*, 2020). These include *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Trichoderma*, *Penicillium*, *Fusarium* etc. (Noori and Saud 2012; Lenin and Jayanthi 2012; Panwar *et al.*, 2014; Radhakrishnan *et al.*, 2014; Bitas *et al.*, 2015), many of which are genera that belong to the consortium assembled in this thesis. Moreover, also the beneficial effects of microbial consortia are well known, as reported, among the others, by Kalaiyararsi and Victoria (2016), Schoebitz *et al.* (2016) and Mondal and collaborators (2020).

The seeds sown in the polluted soils treated with the consortium for different times exhibited a variable behaviour. The seeds in the polluted soil treated with the consortium for one week started to germinate after ten days, while those in the soil treated for three weeks showed the first vital signs only two days after sowing (Fig. 6.3). At the end of the experiment, the seed sown in the soil treated for two weeks had still not germinated. This phenomenon may indicate that the pollutant concentration in the soil treated for two weeks is a more toxic dose for watercress than that in the soil treated for only one week, which should be higher due to the shorter consortium action period. However, this phenomenon will have to be demonstrated by further analysis, as no data are currently available in the scientific literature for comparison. The seeds sown in control with only polluted soil,

which successfully germinated, were the first compared to all the others in polluted soils, showing the first radicle already after two days. However, in the following days, the plant became much longer, but the root remained short and thin, and at the end of the first week, the seedlings were dead. This suggests that the soil pollutant may have initially enhanced the plant growth, but its high toxicity limited plant viability. This phenomenon is reminiscent of hormesis, a biphasic dose-response relationship in which low doses of a pollutant induce stimulatory effects on an organism, while higher doses may induce inhibitory responses that at even higher doses often become toxic (Agathokleous and Calabrese, 2020). It has been observed that the presence of hydrocarbon contaminants, such as benzo[a]pyrene, PHAs, crude oil and vehicle fuels, often induces this behaviour in other plant species (Zhang *et al.*, 2011; Li *et al.*, 2019; Agathokleous *et al.*, 2020). However, this phenomenon does not correspond exactly to the seeds' reaction in the ecotoxicological test, as the seedlings do not survive once they have germinated. Therefore, further investigations will be needed to fully understand this behaviour's cause, although hormesis is undoubtedly a good reflection point.

In conclusion, under controlled conditions, the consortium can bioremediate polluted soil, but it needs an action time of at least three weeks because the degradative effects on the pollutant became evident and the soil suitable for plant growth again.



Figure 6.3. Germinated seeds in control with unpolluted soil.

Chapter 6

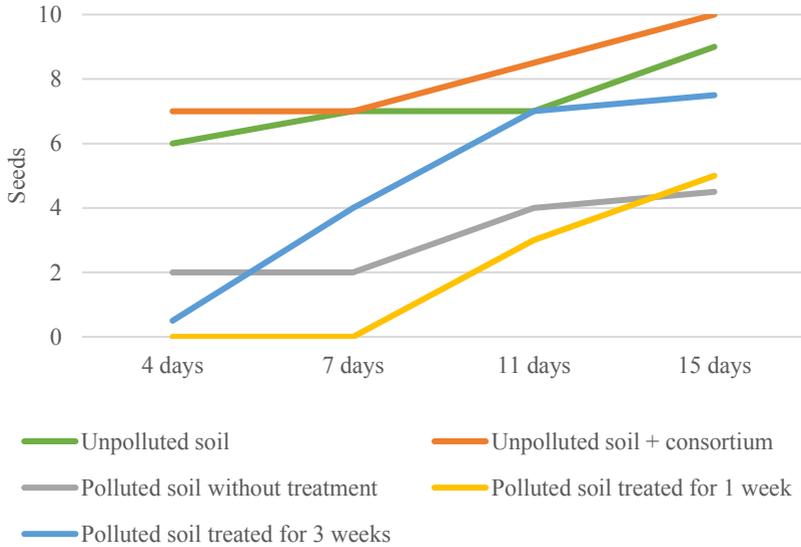


Figure 6.4. Number of seeds germinated in the different tests monitored twice a week for two weeks.

Table 6.1. Germination index (GI) obtained for *Lepidium sativum* seeds.

| | GI (%) |
|-----------------------------------|--------|
| Unpolluted soil | 100 |
| Unpolluted soil + consortium | 91,7 |
| Polluted soil without treatment | 37,6 |
| Polluted soil treated for 1 week | 50,7 |
| Polluted soil treated for 3 weeks | 80,1 |

Conclusions

The bioremediation capacity of the assembled fungi-bacteria was demonstrated with an ecotoxicological test, in which *Lepidium sativum* L. was used as the bioindicator. The seeds were planted in soil artificially polluted with used engine oil after being treated with the consortium for a week, two weeks, and three weeks. The results showed that the consortium can remediate the polluted soil to almost an uncontaminated state by acting for at least three weeks and that it can have beneficial effects on the watercress growth, improving the plant health.

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Chapter 7: General conclusions and future perspectives

7.1 General conclusions

In the oil industry, large tanks are often used to store raw materials, intermediate and finished products. The sludge and heavy oil residues accumulated in the tanks have to be cleaned periodically, usually by manual means. This is dangerous, time-consuming and labour-intensive. Besides, substances resulting from cleaning must be disposed of specifically as they are rich in hazardous substances. An alternative method is the bioremediation process, which involves selected microorganisms to degrade toxic compounds in the polluted substrate.

This project aimed to set up a consortium of fungi-bacteria, isolating and selecting microorganisms directly from contaminated substrates to treat waste products and polluted soils from the oil industry's production processes. In the project, 29 fungal and 50 bacterial strains were isolated and identified from contaminated substrates supplied by ENI. Then I set up a screening test to select promising fungal species, and the best ones were *Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Trichoderma harzianum* F26, *Curvularia aeria* F28, *Coniochaeta* sp. F37, *Fusarium solani* F39 and *F. chlamydosporum*

F41. The scientific literature study then allowed me to focus on some ligninolytic enzymes involved in the degradation processes of recalcitrant substances. I performed colorimetric tests that showed *Alternaria alternata* F13 and *Curvularia aerea* F28 as the best ligninolytic enzymes producers, while no activity was recorded for *Trichoderma harzianum* F26 and *Coniochaeta* sp. F37. All the strains (except for *Alternaria alternata* F13, *Coniochaeta* sp. F37, and *Fusarium solani* F39) responded positively to the RBBR test, suggesting their ability to degrade PAHs. Subsequently, I evaluated their action on the hydrocarbon components of the complex mixtures tested. The hydrocarbon composition was analysed before and after fungal action using gas chromatography. All the fungal strains modified the composition of the contaminated substrates, acting on different hydrocarbon families. This result is significant because assembling a consortium with microorganisms able to act on different hydrocarbon families provides a broad spectrum of action.

The main goal of this project was to set up a microbial consortium with high degrading abilities. The consortium was assembled with 28 bacterial strains (belonging to *Stenotrophomonas*, *Pseudomonas*, *Gordonia* and *Leucobacter* genera) and 6 of the 8 fungal strains (*Scedosporium apiospermum* F10, *Alternaria alternata* F13, *Penicillium oxalicum* F15, *Curvularia aerea* F28, *Fusarium solani* F39 and *F. chlamydosporum* F41), which showed antibiosis positive interactions each other. The consortium demonstrated high

degradative abilities against hydrocarbon pollutant sources, degrading all the hydrocarbons present within a week. Moreover, its activity was confirmed by the ecotoxicological test on *Lepidium sativum*. The main result showed by the germination index demonstrated that the bioremediation processes was almost complete.

Microbial consortia for bioremediation processes is a well-known technique, but few research works have included the use of mixed fungal-bacterial assemblage. This work demonstrates that the consortium we have obtained can certainly be an excellent bioremediation agent thanks to the synchronous enzymatic action of fungi and bacteria against the different pollutants. Moreover, it has been shown that autochthonous microorganisms isolated from a contaminated area are the best tools to bioremediate the area itself

7.2 Future perspectives

As has already been pointed out several times, microbial consortia in bioremediation is a promising methodology. However, some points not yet understood will need to be further investigated. For example, many details on the metabolism of fungi and the enzymes involved in hydrocarbon degradation processes, especially for non-ligninolytic species, are still unknown. Besides, many questions remain about the substances resulting from the degradation action, and understanding their nature is essential to avoid substances that are more toxic than the original ones.

More specifically about this work, field trials will be carried out to confirm the consortium abilities in the coming months. Nonetheless, before proceeding with this step, it will be necessary to deepen the investigations. For example, further analyses should be conducted on *Scedosporium apiospermum*. It has been recognized as a potential etiologic agent of severe infections in immunocompromised and occasionally immunocompetent patients. Before using it in a consortium that will be distributed to the industry, it will be necessary to understand whether our particular strain can be dangerous or not.

Moreover, due to the hydrophobic of petroleum hydrocarbons, some biostimulation strategies, including surfactants or nutrients added, should be employed to improve the hydrocarbons availability and microbial biomass. For these reasons, other analyses should also be performed to verify if the isolated strains can produce biosurfactants. Biosurfactants are secondary metabolites involved in increasing the solubility and availability of various water-immiscible substrates, like hydrocarbons. Their production can enhance the consortium biodegradation rates accelerating the remediation process.

Finally, the DNA sequences of all the consortium strains will certainly be deposited, and metagenomic studies will be carried out to understand how the consortium population changes and how to maintain the perfect balance among the species over time

All these analyses were planned for the last year of my PhD, but unfortunately, the agenda has been changed due to the impossibility of regularly accessing the laboratories because of the Covid-19 pandemic. However, the results obtained are encouraging and open the door to many possibilities. Also, they add to the already flourishing published literature on the subject, implementing it with new data on the role of fungi-bacteria consortia in the bioremediation of substrates contaminated by complex hydrocarbon mixtures. This work also highlights how collaboration between different researchers with different training and experience, specialised in various scientific fields, is essential to find greener and low environmental impacts methodologies.

Supplementary material: Composition of culture media and antibiotic mix

Table 1S. Media and antibiotics used.

| Agar gel 0,15% | | Antibiotics mix | |
|---|--------|---|---------|
| Agar | 1,5 g | Penicillin | 50 ppm |
| dH ₂ O | 1 L | Streptomycin | 40 ppm |
| Mix well and autoclave at 121 °C for 20 min | | Tetracycline | 30 ppm |
| | | Neomycin | 100 ppm |
| | | Chloramphenicol (in ethanol) | 100 ppm |
| Büshnell-Hass broth (BH) | | Büshnell-Hass agar (BHA) | |
| MgSO ₄ | 0,2 g | MgSO ₄ | 0,2 g |
| KH ₂ PO ₄ | 1 g | KH ₂ PO ₄ | 1 g |
| NH ₄ NO ₂ | 1 g | NH ₄ NO ₂ | 1 g |
| CaCl ₂ | 0,02 g | CaCl ₂ | 0,02 g |
| K ₂ HPO ₄ | 1 g | K ₂ HPO ₄ | 1 g |
| FeCl ₂ | 0,05 g | FeCl ₂ | 0,05 g |
| dH ₂ O | 1 L | Agar | 15 g |
| Mix well and autoclave at 121 °C for 20 min | | dH ₂ O | 1 L |
| | | Mix well and autoclave at 121 °C for 20 min | |

Supplementary material

| | | | |
|---|---------|---|--------|
| Malt extract broth (MEB) | | Malt extract agar (MEA) | |
| Malt extract | 20,0 g | Malt extract | 20,0 g |
| Dextrose | 20,0 g | Dextrose | 20,0 g |
| Peptone | 6,0 g | Peptone | 6,0 g |
| dH ₂ O | 1 L | Agar | 15 g |
| Mix well and autoclave at 121 °C for 20 min | | dH ₂ O | 1 L |
| | | Mix well and autoclave at 121 °C for 20 min | |
| M9 mineral medium (M9) | | Sabouraud dextrose agar (SAB) | |
| Na ₂ HPO ₄ ·7H ₂ O | 64 g | Dextrose | 40 g |
| KH ₂ PO ₄ | 15 g | Peptone | 10 g |
| NaCl | 2,5 g | Agar | 15 g |
| NH ₄ Cl | 5,0 g | dH ₂ O | 1 L |
| MgSO ₄ 1M | 2 mL | Mix well and autoclave at 121 °C for 20 min | |
| Glucose | 20% | | |
| CaCl ₂ 1M | 100µl | | |
| dH ₂ O | 1 L | | |
| Mix well and autoclave at 121 °C for 20 min | | | |
| Rose Bengal agar (RB) | | Potato dextrose agar (PDA) | |
| Peptide digestion (animal tissue) | 5 g | Dextrose | 20 g |
| Dextrose | 10 g | Potato extract | 4 g |
| KH ₂ PO ₄ | 1 g | Agar | 15 g |
| MgSO ₄ | 0,5 g | dH ₂ O | 1 L |
| Rose Bengal | 0,025 g | Mix well and autoclave at 121 °C for 20 min | |
| Agar | 15 g | | |
| dH ₂ O | 1 L | | |
| Mix well and autoclave at 121 °C for 20 min | | | |

Curriculum vitae

Chiara Daccò

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| Date and Place of birth | 05 August 1991 Casorate Primo, Pavia, Italy |
| Nationality | Italian |

Work experiences

2017 – present

Tutor, support, and assistance to the teaching of Botany (BIO/03) and Systematic Botany (BIO/04). Department of Biology and Biotechnology and Department of Earth and Environmental Sciences, University of Pavia, Italy.

Education

2017 - present

PhD student in Earth and Environmental Sciences, XXXIII cycle. University of Pavia, Pavia, Italy.

Thesis title: Selection of new fungal strains and development of a microbial consortium for the bioremediation of complex hydrocarbon mixtures. Collaboration with ENI S.p.A.

2016

Master's degree in Applied and Experimental Biology and Biodiversity.

University of Pavia, Pavia, Italy.

Thesis title: Selection of useful fungi for the treatment of lignocellulosic biomass

2014

Bachelor's Degree in Biological Science

University of Pavia, Pavia, Italy

Thesis title: Leukocyte formula and immune system activation in the wall lizard *Podarcis muralis*.

Extracurricular activities

- Course "Rappresentazione e analisi dati"
Working environment in R, descriptive statistics, inferential statistics.
- Course "Linguaggi, problemi e metodi della comunicazione della ricerca scientifica"
Writing in english for scientist, presentation making, public speaking
- Spanish Course
- Diving course (PADI Diver Certified, level: Open Water)

Experiences abroad

2017

Erasmus Traineeship Postgraduate

University of Copenhagen, Copenhagen, Danmark.

Took part in the project named “Fungicide resistance in potato early blight (*Alternaria solani*)”.

Adviser

Adviser Bachelor’s Degree in Biological Sciences and Science and Technology for Nature. University of Pavia, Pavia, Italy

- Fungal species associated with the rhizosphere: assessment of their hydrocarbon-degrading potential. Martina Fassina, 2017.
- Selection of fungal strains for the removal of hydrocarbon residues from solid surfaces. Anna Melati, 2018.
- Selection of fungal strains useful in the degradation of plastics. Ilaria Zanotto, 2018.
- Fungi from continental antarctica: assessing their potential for bioremediation. Greta Tacconi, 2019.
- The importance of fungi in the bioremediation of aquatic and transitional environments. Lorenzo Signorini, 2020.

Adviser Master’s Degree thesis in Advanced Biotechnology. University of Pavia, Pavia, Italy.

- Soil pollution in Italy: The European 'Life Biorest' project and the evaluation of *Trichoderma* potential in mycoremediation. Edoardo Henzen, 2018.

Publication

- Asemoloye, M.D., Tosi, S., Daccò, C., Wang, X., Xu, S., Marchisio, M.A., Gao, W., Jonathan, S.G., Pecoraro, L., 2020. Hydrocarbon Degradation and Enzyme Activities of *Aspergillus oryzae* and *Mucor irregularis* Isolated from Nigerian Crude Oil-Polluted Sites. *Microorganisms* (Q2; IF 2019 4.152) 1–19. <https://doi.org/10.3390/microorganisms8121912>
- Daccò, C., Nicola, L., Elisabetta, M., Temporiti, E., Mannucci, B., Corana, F., Carpani, G., Tosi, S., 2020. *Trichoderma*: Evaluation of Its Degrading Abilities for the Bioremediation of Hydrocarbon Complex Mixtures. *Applied Sciences* (Q2; IF 2019 2.474) 10, 1–15. <https://doi.org/10.3390/app10093152>
- Daccò, C., Girometta, C., Asemoloye, M.D., Carpani, G., Picco, A.M., Tosi, S., 2020. Key fungal degradation patterns, enzymes and their applications for the removal of aliphatic hydrocarbons in polluted soils: A review. *International Biodeterioration and Biodegradation* (Q1; IF 2019 4.074) 147, 104866. <https://doi.org/10.1016/j.ibiod.2019.104866>
- Daccò, C., Elisabetta, M., Temporiti, E., Nicola, L., Tosi, S., 2019. Fungi Useful for the Purification of the Lignin Fraction from Residues of Bioethanol Production. *Acta Microbiol. Bulg.* 3/35.

Awards

Diploma for “excellence scientific research” for the work “Fungi on hydrocarbon complex mixtures: evaluation of their degradation potential - M.E.E. Temporiti, C. Daccò, M.D. Asemoloye, B. Mannucci, F. Corana, S. Tosi.” presented at 6th International Conference, Ecological Engineering and Environmental Protection, 2019.

Congress Participation

9-11 Sept 2020

115° Congresso della Società Botanica Italiana, Online

Temporiti M.E.E, Nicola L., Daccò C., Tosi S. *Fusarium* sp. VS plastica: una storia di degradazione fungina.

6-7 Feb 2020

CYBO – Conference of Young Botanists, Genova, Italia

Daccò C., Temporiti M.E.E, Nicola L., Mannucci B., Corana F., Tosi S. The fungal genus *Trichoderma*: can be useful for the biodegradation of used engine oil?

16-21 Sept 2019

18th Congress of European Mycologist, Varsavia – Białowieża, Polonia

Daccò C., Temporiti M.E.E, Nicola L., Mannucci B., Corana F., Tosi S. Evaluation of different *Trichoderma* species in their ability of degrading engine oil.

4-7 Sept 2019

114° Congresso della Società Botanica Italiana Onlus, Padova, Italy
Daccò C., Manassero A., Pasinetti G., Tosi S. Mycoremediation of soil polluted by insecticides and fungicides.

5-7 June 2019

6th International Conference, Ecological Engineering and Environmental Protection, Burgas, Bulgaria

Daccò C., Temporiti M.E.E., Tosi S. Selection of fungal strains useful for the purification of the lignin fraction from residues of bioethanol production.

M.E.E. Temporiti, C. Daccò, M.D. Asemoloye, B. Mannucci, F. Corana, S. Tosi. Fungi on hydrocarbon complex mixtures: evaluation of their degradation potential.

6-8 Sept 2018

UMI – Convegno Nazionale di Micologia, Siena, Italy

Daccò C., Faè M. Study of the microfungi activity on ligninocellulosic substrates.

28 – 29 Aug 2017

2nd Symposium on Plant Biomass Conversion by Fungi, Utrecht, Netherlands

Daccò C., Faè M., Cella R., Picco A.M., Tosi S. Selection of fungal strains useful for the purification of the lignin fraction from residues of bioethanol production.

14 -17 May 2017

Euroblight 2017 Workshop, Aarhus, Denmark

Petersen N.S., Daccò C., Abuley I., Nielsen B., Kjøller R. Species diversity, pathogenicity and fungicide resistance of *Alternaria* in Denmark.

Technical competences

- In-depth knowledge of the fungal world gained during university training and consolidated with the PhD.
- Excellent knowledge of laboratory techniques (growth of in vitro cultures, preparation of culture media, isolation of microorganisms).
- Excellent knowledge of molecular and morphological identification techniques.
- Excellent ability to use the spectrophotometer for different types of investigations.
- Excellent knowledge of Microsoft Office and Open Office packages, internet browsers, image editors (Photoshop and GIMP).
- Good capacity in the use and knowledge of gas chromatography instruments.
- Good ability to study fungal enzymes and their action on complex substrates.
- Good knowledge of software for statistical analysis (R, Past3)

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