PLANT-FUNGAL MUTUALISM AS A STRATEGY FOR THE BIOREMEDIATION OF HYDROCARBON POLLUTED SOILS

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
(Environmental Biotechnology)

of

RHODES UNIVERSITY

by

OLAJIDE MURITALA KESHINRO

FEBRUARY 2021

Abstract

Inasmuch as coal remains the linchpin for the generation of electricity and liquid petroleum products in South Africa, hydrocarbon waste and coal discard will continue to pose a threat to the environment. Therefore, the onus is on the associated industries to develop and implement efficient and sustainable strategies to mitigate the negative impacts of energy generating activities on the environment. Most conventional efforts in this regard, although successful for soil repair and the initiation of vegetation, have been deemed unsustainable. In an effort to find a sustainable remediation strategy a novel technology termed "FungCoal" was conceptualized and patented as a strategy for the rehabilitation of open cast coal mines, carbonaceous-rich spoils and coal wastes. This biotechnology, which exploits plant-fungal mutualism to achieve effective biodegradation of coal on discard dumps and the breakdown of the carbonaceous component in spoils, promotes revegetation to facilitate rehabilitation of mining-disturbed land. However, one limiting factor of the FungCoal bioprocess is that it requires oxidized weathered coal, a highly complex and variable resource for use as a co-substrate, for growth and proliferation of the coal degrading microorganisms. To fully exploit the potential of plant-fungal mutualism and its interaction for use in the remediation of coal contaminated soils, this study investigated the proposed relationship between plant roots, root exudate and the coal degrading fungus "*Aspergillus* sp." (previously *Neosartorya fischeri*) strain 84 in more detail, in an effort to gain further insight into the mechanisms underpinning plant-fungal mutualism as a strategy for re-vegetation of coal discard dumps and the rehabilitation of hydrocarbon-contaminated soil using the FungCoal approach. A pot-on-beaker (PoB) method was developed for the easy cultivation and collection of extracellular polymeric substance (EPS)-containing exudates from *Zea mays* L. (maize) and *Abelmuschus esculentus* (okra). Characterisation of the EPS material from these exudates was carried out using a combination of physicochemical and biochemical methods. The results from analysis of phenolics and indoles showed that exudates contain some form of indoles and phenolic compounds, although in little proportions, which may fulfil a signalling function, responsible for attracting soil microorganisms into the rhizosphere. Spectroscopic analysis of the exudates using FT-IR revealed vibrations corresponding to functional groups of alkanes, alkenes, alkynes, and carboxylic acids. These compounds likely provide an easily accessible source of carbon to soil microorganisms and are also a better alternative to the poly-aromatics which are an inherent component locked-up in the supposed recalcitrant coal material. The results from biochemical analyses also revealed the presence of carbohydrate, proteins, lipids, and low amounts of α-amino-nitrogen in the EPS

of maize and okra. These components of EPS are all essential for the stimulation of enzymatic activities in soil microorganisms and, which may in turn aid biodegradation. The action of the root EPS from maize was further tested on three coal-degrading fungal isolates identified as *Aspergillus* strain ECCN 84, *Aspergillus* strain ECCN 225 and *Penicillium* strain ECCN 243 for manganese peroxidase (MnP) and laccase (LAC) activities. The results revealed that the *Aspergillus* species, strains ECCN 84 and ECCN 225, showed with or without EPS, observable black halos surrounding each of the colonies after 7d incubation indicative of positive MnP activity, while no activity was observed for the *Penicillium* sp. strain ECCN 243. Analysis for LAC revealed little or no activity in any of the coal degrading fungi following addition of pulverized coal to the growth medium. Interestingly, the addition of EPS-containing exudate to the coal-containing medium resulted in increased LAC activity for all fungal isolates. This finding affirmed the positive contribution of EPS to extracellular LAC activity, purported as an important enzyme in the coal biodegradation process. Finally, the impact of plant-derived exudate on the colonisation and biodegradation of coal was investigated *in situ* using rhizoboxes, to simulate a coal environment, and was carried out for 16 weeks. Microscopic examination of coal samples after termination of the experiment showed fungal proliferation and attachment to coal particles. All of the rhizoboxes that contained plants had higher medium pH and EC, and the concentration of phenolics, indoles and humic acids was greater than that of control treatments. These observations indicated better rhizosphere colonisation, substrate biodegradation and humification. Therefore, root exudate appears to play a significant role in coordination of soil microorganisms within the rhizosphere and likely serves both as a scaffold for rhizospheric interactions by providing microorganisms with accessible carbon and as a likely 'trigger' for induction of coal-degrading enzymes such as fungal LAC for mobilisation of recalcitrant carbon. This study has shown that EPS exuded from roots of *Zea mays* together with coal degrading fungus *Aspergillus* strain ECCN 84 can alkalinise the coal substrate and facilitate introduction of oxygen, possibly as a result of increased laccase activity, and increase availability of nutrients (as indicated by higher EC) in a coal-polluted rhizosphere, to provide plants and their associated mycorrhizae and presumably other beneficial microorganisms a more mesic environment for sustained phytoremediation with enhanced rehabilitation potential. In conclusion, this study confirms the positive role of root exudate in mediating a mutualistic rehabilitation strategy involving plants and fungi such as the FungCoal bioprocess.

Dedication

This research work is dedicated to Almighty Allah for guiding and guarding me throughout this PhD program despite the raging storm of the COVID-19 pandemic.

Acknowledgements

My profound gratitude goes to my supervisor Prof. A. K. Cowan for his tutelage, care, support, and constructive criticism throughout this research. I am also grateful to the Institute for Environmental Biotechnology (EBRU), and Rhodes University (RU scholarship) for the Doctoral bursary and scholarship, respectively.

My appreciation goes to the members of staff at the Institute for Environmental Biotechnology, Rhodes University (EBRU); Gila Eustace, Xolisa Maganca, Richard Laubscher, Dr. Yinka Titilawo, Andile Magaba, Olwethu Baba, and Norman Singapi for their respective support throughout my study. I also appreciate the love and support from my colleagues; Jimoh Taobat, Linda Sibelo, Anele Dube, Kuhle Nkangala and Masudi Wiya. My sincere appreciation also goes to my cousin, Olawunmi Kolawole who assisted me with some of my schematics.

My heartfelt thanks go to my parents Mr Sheriffdeen Olabode Keshinro and Mrs Abosede Kikelomo Keshinro for their prayers, support, and sacrifice, may Almighty Allah reward you abundantly. I appreciate the encouragements from my siblings; Mrs Monsurat Suleiman, Adebisi Keshinro, Damilola Keshinro, Akorede Keshinro and Taiwo Keshinro, and my cousins; Olugbenga Har-yusuph and Oluwaseun Har-yusuph. I also appreciate the unflinching supports from my dear aunties and uncles (Mrs Kudirat Har-yusuph and her hubby, Arc. D.A.K. Har-yusuph, Mr and Mrs Kolawole, Mr and Mrs Saheed Kareem and Mr and Mrs Alaba Okunbanjo) who threw their weights behind me throughout my studies, may Allah continue to support you all.

Finally, I would like to thank my beauteous wife who doubles up as my colleague, Taobat Adekilekun Keshinro (*Nee* Jimoh) for her love, kindness, support and understanding throughout my studies, may our love continue to wax stronger (Amin). I can never forget to appreciate my in-laws; Dr Mahboob Jimoh, Mrs Ganiyat Jimoh, and their entire family for their pivotal support throughout this study, may Almighty Allah continue to bless and reward you all.

Table of Contents

List of Figures

List of Tables

List of Abbreviation

ABTS:	2.2’ [azino-bis-(3-ethylbonzthiazoline-6-sulphonic acid) diammonium salt]
ANOVA:	Analysis of Variance
ATR:	Attenuated Total Reflectance
BLAST:	Basic Local Alignment Search Tool
BSA:	Bovine Serum Albumin
CPD:	Critical-point drying
CRD:	Completely randomized design
DAS:	Days After Sowing
DMRT:	Duncan Multiple Range Test
DNA:	Deoxyribonucleic acid
EBRU:	Institute for Environmental Biotechnology-Rhodes University
E.C:	Electrical Conductivity
ECCN:	EBRU Culture Collection Number
EDS:	Energy Dispersive x-ray Spectroscopy
EPS:	Extracellular polymeric substance
FD:	Freeze-drying
FT-IR:	Fourier Transform Infrared
GBSM:	Glutamate basal salt medium
GPS:	Global Positioning System
HMDS:	Hexamethyldisilazane
ITS:	Internal transcribed spacer
KBr:	Potassium Bromide
LAC:	Laccase

LiP: Lignin peroxidase

MnP: Manganese peroxidase

MOS84: Maize + Okra + Strain 84 treatment

MS84: Maize + Strain 84 treatment

NCBI: National Centre for Biotechnology Information

P-coal: Pulverized coal

PCR: Polymerase chain reaction

PDA: Potato dextrose agar

PDB: Potato dextrose broth

PoB: Pot-on-Beaker

PVC: Polyvinyl Chloride

SE: Standard Error

SEM: Scanning Electron Microscopy

SPV: Sulfo-phospho-vanillin

TEM: Transmission Electron Microscopy

Chapter One: General Introduction

1.1 Background

In recent years, many mineral-rich countries have experienced strong economic growth, and this largely comes from contributions from their respective mining sectors. In South Africa, mining has always been the mainstay of the economy with almost 90 % of its energy coming from coal-fired power stations. The Integrated Reports Plan (IRP) - 2019 predicted that South Africa will continue to depend on coal until at least 2050. A recent survey carried out by Global Energy Monitor and The Centre for Research on Energy and Clean Air (2020) showed that China, which is currently the largest producer of coal in the world, just proposed an additional 40.8 GW of new coal plants which is about the same size as the entire coal fleet of South Africa (41.4 GW), despite coal pollutants being directly and scientifically linked to climate change. Indeed, the contribution of mining to the economy may be significant but the social and environmental impact of post-mining activities continue to pose a huge challenge. In an effort to mitigate the environmental hazard posed by mining in South Africa, the Land Rehabilitation Society of Southern Africa (LaRSSA) and CoalTech (2018) published a guideline for the rehabilitation of coal contaminated sites. However, a major oversight has always been the apparent omission or de-emphasis of biotic factors that underpin successful and sustainable re-vegetation. It is pertinent to note that mining not only visibly disrupts the aesthetics of a landscape, but also disrupts all the soil components including soil horizons and structure, soil microbe populations, and nutrient cycles that are crucial to sustaining a healthy ecosystem. All ecosystems function optimally as a process that is dependent on nutrient recycling, which is the balance between growth and decomposition of microbial, plant and animal matter. Soil houses a scaffold of organic and inorganic elements that sustains this process, so soil structure, composition and density directly affect the stability of any supported plant community post-rehabilitation. Reclamation strategies must therefore address soil sub-structure, microbe populations, and nutrient cycling to transform the land from a disturbed condition to some ‘new’ repurposed self-sustaining ecosystem. In lieu of this, the quest for sustainable bioprocesses that aid the degradation and depolymerization of pollutants such as coal has become an iterative process, and one sought after technology available to the mining sector in South Africa is Rhodes University-developed and patented process called FungCoal.

About a decade ago, a joint initiative between Anglo Thermal Coal and the Institute for Environmental Biotechnology (EBRU) resulted in a patented technology called, “FungCoal”

(Rose *et al*., 2010). FungCoal was developed as a bioprocess for the treatment of coal waste or discard to facilitate rapid soilification and rehabilitation of waste dumps. The science behind the FungCoal concept is based on the mutualism between plants belonging to the grass family (Poaceae) and arbuscular mycorrhizal fungi, which in the presence of weathered coal and coal degrading saprophytic fungi effectively biodegrade the polyaromatic-containing carbonaceous spoils into soil-like material, the products of which are capable of supporting vegetation sustainably (Igbinigie *et al*., 2008; Igbinigie *et al*., 2010; Rose *et al*., 2010). This technology was developed primarily to support sustainable rehabilitation through re-vegetation of South African coal discard (waste) dumps and mining-disturbed environments including, open cast spoil. Currently, coal mining companies in South Africa translocate large quantities of topsoil across vast distances to meet the needs of its extensive land rehabilitation program and at enormous financial and environmental costs. Preliminary large-scale commercial trials using FungCoal showed that it is possible to commence rehabilitation of coal discard dumps without the addition of topsoil. However, this bioprocess was not ideal and relatively slow (Cowan *et al*., 2016). For efficiency of the FungCoal technology, a co-substrate in the form of a humic rich material such as weathered coal was confirmed as ideal. However, as a co-substrate or activator, this material is in short supply and therefore, unfortunately, must also be translocated over considerable distances. More recently, humics, sourced from commercial suppliers and at great cost, are being considered as replacement for either topsoil or weathered coal (Anglo Coal; personal communication). Nevertheless, while FungCoal technology can be adopted immediately to off-set costs incurred through topsoil and/or humics acquisition, a better bioprocess application strategy is required to eliminate transportation of materials altogether. Current data strongly suggest that fortification of the biocatalyst with coal-degrading bacteria, fungi, small molecules and/or oxidative enzymes can 'ready' the carbonaceous substrate such that no additional material (i.e., either weathered coal or topsoil) is required. However, one aspect that has not been well-researched in the development of FungCoal is the contribution of plant root exudate to the bioprocess.

Plant root exudate has been reported to produce, in addition to secondary metabolites and polymeric carbon, ligninolytic enzymes which are responsible for degrading lignin-like materials like the aromatic compounds present in the coal structure (Yoshida, 1883; Wong, 2009; Plácido and Capareda, 2015). Root exudation is a rhizodeposition process, which serves as a major source of soil organic carbon released into an external environment by intact plant roots (Nguyen, 2003; Thomas, 2014). The composition of root exudate depends on plant

species and prevailing abiotic and biotic conditions, and this influences the community composition of root-associated organisms (Wieland *et al*., 2001; Marschner *et al*., 2004; Micallef *et al*., 2009; Mitchell *et al*., 2010; Lange *et al*., 2015). It is generally assumed that root exudate plays important roles in the rhizosphere dialogue between plants and microbes (Neal *et al*., 2012; Sun *et al*., 2012). In the Poaceae family (which is the plant family specified for the patented FungCoal bioprocess), enzymes such as laccase and peroxidases are typically exuded (Wang *et al*., 2015; Janusz *et al*., 2020). However, and at the same time, exudate enhances the removal of complex aromatics (such as those in coal and lignite) from the soil which may be a consequence of extracellular oxidative enzymes present. Indeed, peroxidases from root exudate of sorghum (*Sorghum bicolor* L. Moench) and alfalfa (*Medicago sativa* L.) oxidize native aromatics and polyaromatics confirming that these enzymes are involved in rhizosphere xenobiotic degradation (Dubrovskaya *et al*., 2017). These enzymes together with other oxidizing enzymes produced by the suite of microbial organisms typically present is soils, could be a good biocatalyst for carbonaceous wastes such as coal. Therefore, the underlying molecular physiology of root exudate in the mutualistic grass-microorganism association, and their role in the biodegradation of coal needs further investigation. Knowledge thus garnered will help in the optimization of FungCoal as a bioprocess and enable other protocols for soil pollutants other than coal, and in particular hydrocarbons, to be managed in an environmentally friendly manner.

1.2. Environmental pollution

Pollution of the environment persists as a consequence of life and will do so for centuries to come. Although some of the pollutants occur naturally due to unforeseen circumstances such as earthquakes, landslides, hurricanes, volcanic eruptions and so on, which in most cases we have little control over, most pollutants that are damaging the environment to date have been anthropogenic in origin. The soil environment which is the layer of the Earth that supports terrestrial life and human activity has been adversely affected by the many different forms of domestic, industrial, and agricultural wastes (Wang *et al*., 2020). These contaminants have led to the influx of harmful chemicals such as nitrogen oxides, polycyclic aromatic hydrocarbons (PAHs), heavy metals, pesticides, plasticizers, polychlorinated biphenyls (PCBs), dioxins, furans, endocrine disrupting organics, and antibiotics into the soil, rendering it unviable for farming activities and posing a huge health risk to humans (Wang *et al*., 2020). Also, mining, the economic mainstay of many developing and developed countries, generates copious amounts of waste during excavation and processing of products such as crude oil, natural gas,

coal, platinum, gold and so on. This waste is a major contributor to atmospheric pollution, surface and groundwater deterioration by acid leachate runoff, erosion, and sedimentation of particulates into adjacent rivers and dams, spontaneous combustion of carbonaceous residues, and landslides. If left unmanaged, the effects of this waste material can and does have a severe impact on human, environmental health, and quality of life. Furthermore, when mining and processing operations cease, the vacated land is typically unsuitable for agriculture, habitation and risks being lost through erosion. Therefore, it is important to have in place appropriate strategies to mitigate the effects of industrial 'wealth creation' such that disturbed and/or polluted environments can be restored from some original condition to a new and beneficial condition. The process it is suggested must return the land to a stable condition, capable of supporting permanent use as directed by a mine plan (Coppin 2013; Limpitlaw and Briel, 2015). While this new, rehabilitated state must also allow for alternative land use opportunities, not contribute to environmental deterioration, and be consistent with surrounding aesthetic values, it can be significantly different from its historical state.

In the present work, emphasis will be on coal and waste coal as the pollutants. This is in part due to coal mining being integral to the development and advancement of South Africa's economy and its potential contribution to economic transformation within the country. In 2013, Prof. Rosemary Falcon stated, "It (coal) is the mainstay of South Africa's industrialization, quality of life, employment and income". Thus, a need to reduce the impact of coal mining, coal utilisation, and waste using effective strategies may help facilitate the uninterrupted transition from fossil fuel to a renewable energy-based economy.

1.3. Coal

Coal is essentially a sedimentary rock, organoclastic, and generated largely but not exclusively from helophytic plant debris and plant derivatives (Schweinfurth, 2009; O'Keefe *et al.*, 2013). Coal formation begins as peat in a mire or swampy environment which enables it to aggregate into thick beds (Wheelers and Proctor, 2000; Hazrin-Chong *et al.*, 2020). Peat is naturally formed through a process of anaerobic decay, whereby the presence of water truncates the supply of oxygen and allows thermal and microbial decomposition of plant material to take place, instead of the completion of the carbon cycle. Peat gradually transitions to coal through physical and chemical processes which occur due to compaction and heat with prolonged burial at depths over periods of up to several hundred million years. Coal contains a wide range of inorganic compounds which were likely introduced into the mire from wind or water-borne

sediments, volcanic ash, or its original vegetation; for example, after the decomposition of plants, all the resultant inorganic compounds present in the plant remain in the resulting peat (Finkelman and Brown, 1991). Some of those elements combine to form discrete minerals, such as pyrite. Other sources of inorganic compound used by the plants may be the mud that coats the bottom of the mire, sediments introduced by drainage runoff, dissolved elements in the mire water, and wind-borne sand, dust, or ash (Schweinfurth, 2009). On the other hand, organic compounds in coal are composed of the macro elements; carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulphur (S), and trace amounts of a variety of other elements.

1.3.1. Classes of coal

An integral factor in determining the quality of coal is through its rank, which is a slow and natural process called diagenesis or coalification. (Bustin, 1998; Li *et al*., 2020). Over time, buried plant matter gradually metamorphose into an ever denser, drier, more carbon-rich, and harder material. The degree of diagenesis that coal undergoes determines its coal rank. However, coal is generally classified into four categories or ranks based on time-temperature history and widely differing properties (Figure 1.1). These are, from lowest to highest, lignite, sub-bituminous, bituminous, and anthracite (Sekhohola *et al*., 2013; Mochida *et al*., 2014). The higher the coal rank the more deeply it was buried, and therefore, the higher the temperature it was subjected to during and after burial. Hence, why older coals such as the anthracites and bituminous coals are classified as higher rank is related to depth and age of burial. In fact, the higher the rank of the coal, the more the fixed carbon and heating value, and the lesser the moisture contents, hydrogen, and oxygen content they possess.

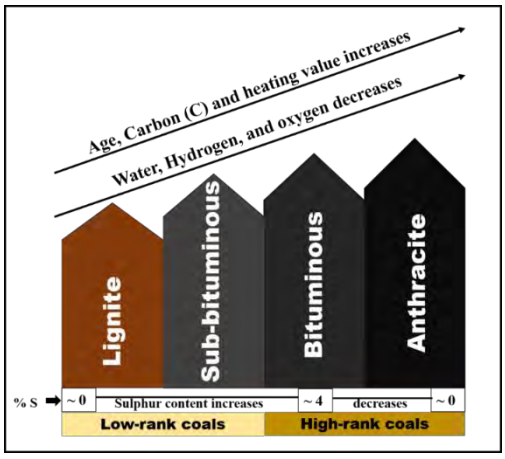


Figure 1.1. Classification of coals by ranks (Sondreal and Wiltsee, 1984; American Society for Testing and Materials, 1999; Mochida *et al*., 2014).

The two most recognized and oldest forms of high-rank coals are bituminous and anthracite coals, respectively. These have been subjected to the highest grade of metamorphism and are both black, hard, and brittle but anthracite is harder and with a shiny appearance. These coals have low moisture content and volatility but contain high carbon. Bituminous coal has the highest sulphur content (~4) more than the low-rank coals and the anthracite coal (Figure 1.1). The sulphur could be in an organic form such as thiol, sulphide, disulphide, and thiophene rings or in an inorganic form such as pyrite (Sondreal and Wiltsee, 1984; Mochida *et al.*, 2014). It could also be emitted during the combustion of coal as SO_2 which could be hazardous to the atmosphere. However, bituminous coal remains the fastest growing product in the coal market with a heating value of 28 MJ/Kg and is used primarily for the generation of electricity and coke for the steel industry (Hodek, 1994). Anthracite has the highest fixed-carbon content of approximately 86–98 % and a low combustion process which is largely due to its low volatile matter. The heating value of most anthracite coal which is the highest across the ranks is about 34.890 KJ/Kg, and they combust with hot, clean flame, containing low content of sulphur (Hodek, 1994). The two most common low-rank coals are lignite and sub-bituminous coal. These are the youngest coal types, which simply implies that they have been subjected to the least metamorphic changes during the coal forming process and possess the lowest energy content. Low-rank coals are crumbly in texture upon air drying and have higher moisture and volatile contents than the high-rank coals (Sondreal and Wiltsee, 1984). In most cases, these low-rank coals are extensive, consumed in poorer economies or stockpiled as waste and are as a consequence, the major source of pollutants generated by the industry.

1.3.2. Coal mining in South Africa

According to the Integrated Resource Plan (2019), South Africa is the 6th largest coal-producing/exporting nation and holds 3.5 % of the world's coal resources. However, coal mining activity has huge socio-economic, physical, and biophysical impacts on soil, water, air, other ecological habitats, and regional landscapes. Coal mining activities are usually carried out through any of the following methods, the underground method; open cast method; or the specialized method. In the underground method, mining activity is either carried out with minimal surface disturbance (board and pillar mining), or with significant surface disturbance (longwall mining). In the open cast method, the pit overburden material is either relocated to permanent overburden dumps (open-pit mining) or replaced in an adjacent open, mined-out section (surface strip-mining). About 49 % of South African coal mining is produced using open-cast methods. There are also specialized methods of hydraulic mining and gasification.

All these afore-mentioned mining activities generate a copious amount of mining wastes which eventually results to mine dumps that directly or indirectly affect the health, safety, environment, and society. Mine dumps are often referred to as a large mound of mining waste comprising of a multifaceted mixture of metals and dust particles (Agboola *et al*., 2020). Sometimes they may contain squashed, sandy by-product waste material, called tailings, which is majorly formed from metal mining operations. Most times during mining activities, sulphide minerals which are naturally buried deep in the earth are exposed and as a result of that are oxidized to form sulphuric acid, metal ions and sulphate, which, if untreated, are leached into groundwater and surface water to commence a destructive process known as acid mine drainage (Akcil, and Koldas, 2006; Wei *et al*, 2011; Kruse *et al*., 2012); and hence, affect agricultural land and aquatic life.

1.4. Current challenges for the rehabilitation of South African coal discard dumps

The inability to provide a sustainable rehabilitation strategy/solution to the disposal of waste coal-generated from coal mining activity has been a challenge for South African coal mining companies. The megatons of coal waste generated from mining are dumped at designated disposal sites where they adversely contribute to atmospheric pollution, surface and groundwater deterioration, erosion, and sedimentation of particulates into adjacent rivers and dams, spontaneous combustion, and landslides. In 2002, South African coal mining and beneficiation processes generated about 66 million tons of discard (Prevost, 2003). Coal discard contains large amounts of carbonaceous material which has an adverse effect on the environment. According to South African legislation on mine closure, coal mining companies are obliged to return mined land to a viable post-mining state that caters to alternative land use and mitigates any deleterious environmental impact (Limpitlaw and Briel, 2015). The conventional approach to rehabilitation land disturbed by mining activity, opencast spoil and discard coal dumps in South Africa involves the use of topsoil to create a microenvironment to facilitate plant growth (Salt *et al*., 1998). This approach is not sustainable but also hardly degrade the underlying carbonaceous waste (Cowan *et al*., 2016). Precise, topsoil itself is a scarce and expensive commodity, thus, translocating it for rehabilitation purposes is not financially and environmentally sustainable. Therefore, developing a sustainable approach for the remediation of coal discard remains imperative.

1.5. Bioremediation

According to the National Research Council, Washington D.C. (1993), bioremediation is the exploitation of naturally occurring biodegradative process for the clean-up of contaminated sites which could be carried out either *in-situ* or *ex-situ*. Naturally, this process involves living organisms with the ability to degrade toxic contaminants. Bioremediation is often specified in terms of the catalysts employed e.g., bacteria = bactoremediation, fungi = mycoremediation plant = phytoremediation (See figure 1.2). However, in most cases, all these processes work in tandem with one another. Although most studies have claimed that microorganisms play the greatest role in bioremediation due to their ability to secrete extracellular oxidative enzymes capable of degrading organic pollutants (Alkorta *et al*., 2004; Pilon-smits, 2005; Pande *et al*., 2020), they are most effective when in close proximity to plant roots (i.e., rhizosphere) where they depend on the root exudate for energy. Bioremediation can be by any of the following methods: *in situ* which is the in-place treatment of polluted or contaminated sites, *ex-situ* which is the treatment of contaminated materials off-site; and intrinsic bioremediation or natural attenuation, which is the indigenous level of pollutant degradation that occurs without any treatment or stimulation (Maier *et al*., 2000).

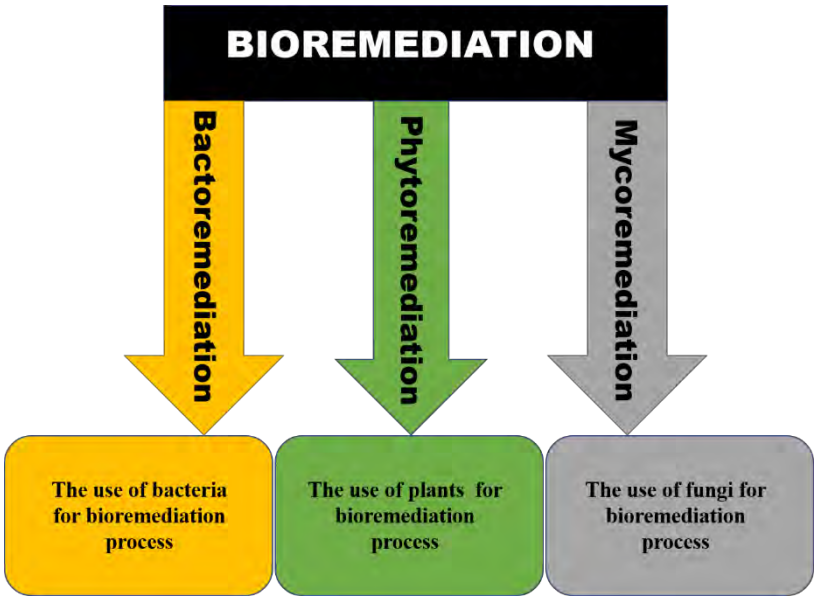


Figure 1.2. The major sub-divisions of bioremediation. (Alkorta *et al*., 2004; Pilon-smit, 2005).

1.5.1. Bacto-remediation

The use of bacteria for bioremediation of contaminated soils is now a contemporary area of research, and this is largely due to microbial activity, reaction mechanisms and the dissolution

of the process of degradation (Wang *et al*., 2010). More recently, the ability of bacteria to produce ligninolytic enzymes have been linked to the biodegradation of carbonaceous wastes such as coal/coal discard (Pawlik *et al*., 2016; Olawale *et al*., 2020). The enzyme, laccase, which has been identified as the chief enzyme responsible for the biodegradation of waste coal (Igbinigie *et al*., 2008; Sekhohola *et al*., 2013), has been detected in bacteria such as *Sinorhizobium meliloti* (Pawlik *et al*., 2016), *Streptomyces lavendulae* REN-7 (Suzuki *et al*., 2003), *Azospirillum lipoferum* (Diamantidis *et al*., 2000) and *Bacillus subtilis* (Martins *et al*., 2002), while manganese peroxidase (MnP) activity has been reported in fungi such as *Bacillus pumilus* (de Oliveira *et al*., 2009), *Azospirillum brasilense* (Kupriashina *et al*., 2012), *Streptomyces psammoticus* (Niladevi and Prema, 2005). However, for the effectiveness of these enzymes on the biodegradation of the substrates (coal or other carbonaceous wastes), the bacterium/bacteria have to be in contact with the substrates, and studies have shown that the attachment of bacteria to its substrate initiates the process of biodegradation (Videla and Herrera, 2005; Hazrin-Chong *et al*., 2014; Hazrin-Chong *et al*., 2020). In fact, a recent study by Olawale *et al*. (2020) reported that bacteria, *Exiguobacterium* strain ECCN 21b; *Serratia* strain ECCN 24b; *Proteus* strain ECCN 23b, *Citrobacter* strain ECCN 19b; *Bacillus* strain ECCN 18b; *Escherichia* strain ECCN 25b which were isolated from slurries of coal tailings and diesel-contaminated soil and screened for coal biodegradation competence, both colonized and degraded bituminous discard coal and weathered coal substrates. In this study, the biodegradation of the substrates was believed to be the consequence of colonisation and an increase in preferential metabolic activities on the substrates.

1.5.2. Myco-remediation

The importance of fungi in the biodegradation and biosolubilization of coal and its derivatives has been emphasized in many studies (Belcarz *et al*., 2005; Rezacova and Gryndler, 2006; Igbinigie et al. 2008; Sigoillot *et al*., 2012; Ghani *et al*., 2015; Akhtara and Mannan, 2020). These fungi are regarded as ligninolytic fungi and appear to be very efficient lignin degraders. Many possess an ability to produce extracellular oxidative enzymes, chelators and alkaline substances used in degrading the aromatic compounds present in a coal structure (Ghani *et al*., 2015; Akhtara and Mannan, 2020). The study by Igbinigie *et al.* (2008) on the biodegradation of coal by *Neosartorya fischeri* was tested in a perfusion fixed-bed bioreactor, to simulate a coal dump environment, revealed oxidation of the coal surface and nitration of condensed aromatic structures of the coal macromolecules. More recently, Smolenova *et al*. (2020) studied the ability of *Trichoderma* strains (isolated from the underground of lignite mine Záhorie,

Slovakia) on the biodegradation of lignite coal, and the result showed that the fungi were able to solubilize the coal substrates effectively. It was suggested that the solubilization process might be due to the action of enzymes such as cellulase, laminarinase, laccase, and lignin-peroxidase-like enzyme, and alkaline pH. Some other examples of coal degrading fungi which have been reported include *Polyporus versicolor* (Cohen *et al*., 1987), *Phanerochaete chrysosporium* (Raeder and Broda, 1984), *Penicillium simplicissimum* (Achi, 1994), *Penicillium citrinum* (Polman *et al*., 1994), *Bjerkandera* sp. strain BOS55 (Mester and Field, 1997), *Bjerkandera adusa* 59 (Belcarz *et al*., 2005), *Alternaria Alternaria* (Rezacova and Gryndler, 2006), and *Penicillium* sp P6 (Yuan *et al*., 2006).

1.5.3. Phytoremediation

The word phytoremediation is derived from an amalgam of the Greek Phyto- meaning “plant”, while remediation was derived from the Latin word *remedium* - meaning “restoring balance”. Therefore, phytoremediation can easily be described as the use of plants and their associated microbes for environmental clean-up (Pilon-Smits, 2005; Akhtara and Mannan, 2020). Plants, through their roots, release a variety of photosynthetic-derived organic compounds into the rhizosphere that can serve as sources of carbon for heterotrophic fungi and bacteria. Unlike other remediation strategies, phytoremediation may occur naturally without human interference (natural attenuation) and may be engineered for specific purposes. However, this remediation process is relatively slow and may take several years to reduce soil pollutants/contaminants to a safe and acceptable condition (Khan, 2005; Shahzad *et al*., 2020). The microbial interaction within the plant holobiont enables the degradation and sequestration of both organic and inorganic pollutants. Some examples of organic pollutants which have been efficiently phyto-remediated include herbicides such as atrazine (Burken and Schnoor, 1997), trichloroethylene (TCE) (Newman *et al*., 1997) and petroleum hydrocarbons (Aprill and Sims, 1990; Schnoor *et al*., 1995). Phytoremediation can be used as an efficient biofiltration mechanism in constructed wetlands (Horne, 2000). Plants can also prevent erosion and leaching in the soil, stabilize pollutants in the soil, and sometimes extract pollutants such as metals and other toxic inorganics from the soil through roots, and accumulate these in tissues (Banuelos and Meek, 1990; Blaylock and Huang, 2000). The latter is often referred to as phytomining or phytoextraction. After uptake into plant tissue, certain pollutants can exit the plant in a volatile form (Pilon-Smits, 2005), while others can be easily harvested. Plants also facilitate the biodegradation of organic pollutants by microbes in the rhizosphere through exudation of extrapolymeric substances or degradative or hydrolytic enzymes (McCutcheon

and Schnoor, 2003). However, phytoremediation does have its limitations. One of these is that plants involved in phytoremediation need to be close to the rhizospheric pollutants to be effective; another is that the process may be slower than other established remediation methods like excavation, incineration, or pump-and-treat systems, limiting applicability. A very good example is a study carried out by Igbinigie *et al*. (2010) on the phyto-bioconversion of hard coal in the *Cynodon dactylon*/coal rhizosphere. In this study, the activity of non-mycorrhizal fungi in the *C. dactylon*/coal rhizosphere was investigated and the result revealed a bioconversion of coal had taken place. The authors suggested that this bioconversion must have been influenced by low molecular weight organics and nitrogen moieties. Another study by Mukasa-Mugerwa *et al*. (2011) on the role of a plant/fungal consortium in the degradation of bituminous hard coal suggested that the interaction between *C. dactylon, N. fischeri* and other coal degrading fungi could result in the biodegradation of bituminous coal. Despite all these observations and suggestions, the role of *C. dactylon* and its root exudate remains obscure. In 2014, a study conducted by Thomas *et al*. (2014) on the complexation of lead by the root exudate of Bermuda grass (*C. dactylon*) showed that the root exudate of *C. dactylon* possesses the ability to solubilize lead in aqueous media. In this study, carboxylic acids and amine functional groups were identified in the root exudate of *C. dactylon*, therefore suggesting these functional groups as the mechanism by which metals are complexed by the root exudates of *C. dactylon*.

1.5.3.1. Exudation in plants

Plants play a major role in the biogeochemical function of the environment and do so by exuding a range of organic compounds from both their above and below ground parts throughout their lifetime (Neumann and Römheld, 2007; Kannenberg and Phillips, 2016; Galloway *et al*., 2020). These exudates can come in the form of monomeric compounds such as amino acids, organic acids, sugars, phenolics and an array of secondary metabolites, or as high molecular weight extra-polymeric substances (EPS) containing lipids, polysaccharides and proteins (Bertin *et al*., 2003; Bais *et al*., 2006; Schilmiller *et al*., 2008; Badiri and Vivanco, 2009; Beller *et al*., 2011; Chapman and Ohlrogge, 2012; Vanhercke *et al*., 2014; Alameldin *et al*., 2017; Jimoh *et al*., 2019). Low molecular mass compounds are mostly released passively in the form of diffusion, via ion channels or transport vesicles, while EPS are exuded actively in the form of mucilage (sterile environment) or mucigel (non-sterile environment) from plant surfaces (Rovira *et al*., 1979; Ryan *et al*., 2001; Gang *et al*., 2001; Wagner *et al*. 2004; Weston *et al*., 2012; Huang *et al*., 2014). Plant EPS is a matrix of high molecular weight compounds

which is secreted as a visco-elastic gel that is often polysaccharide-rich (Sasse *et al*., 2018). It is often released by plants as a response mechanism to their immediate environment and may originate from any of the plant parts such as seeds, stem, leaves and roots depending on plant species and environmental condition (Brown *et al*., 2017; Dietz *et al*., 2020). In aboveground plant parts, some EPS such as those found in carnivorous plants, are conspicuous and appear as mucilage on glandular trichomes and used to capture insects as a supplementary source of nitrogen (Huang *et al*., 2015), while others such as those produced by climbers e.g. *Ficus pumila* and English ivy (*Hedera helix*) secrete mucilaginous exudate on tendrils or root surfaces which facilitates vertical climbing on neighbouring surfaces (Groot *et al*., 2003; Melzer *et al*., 2010; Huang *et al*., 2016). In belowground parts of a plant, EPS is less obvious and mostly secreted as mucilage/mucigel at the root surfaces, with the visco-elastic property enabling it to serve as a scaffold for biological interactions in the rhizosphere (Jones *et al*., 2009; Haichar *et al*., 2014; Baetz and Martinoia, 2014). Root exudation is a rhizodeposition process, which serves as a major source of soil organic carbon released into an external environment by intact plant roots (Nguyen, 2003; Thomas, 2014). The composition of root exudate depends on plant species and prevailing abiotic and biotic conditions, and this can influence the community composition of root-associated organisms (Wieland *et al*., 2001; Marschner *et al*., 2004; Micallef *et al*., 2009; Mitchell *et al*., 2010; Lange *et al*., 2015). It is generally assumed that root exudate plays important roles in the rhizosphere dialogue between plants and microbes (Neal *et al*., 2012; Sun *et al*., 2012). The microorganisms in the rhizosphere generally live under conditions of nutrient starvation and are thus constantly seeking out nutrients (Rohrbacher and St-Arnaud, 2016). This leads to competition for space, water and nutrients within the rhizosphere (Narula *et al*., 2009), which is why the study of root exudate in the soil is particularly difficult because of their interactions with soil particles, and the rapid consumption by microorganisms (Todorovic *et al*., 2001; Kuzyakov, 2002). Root exudate not only serves as a carbon source for soil microorganisms (Baudoin *et al*., 2003) but also acts as a signal to attract or repel microbes (Badri *et al*. 2009). The proliferation of microorganisms in the rhizosphere has major impacts on soil processes such as nutrient cycling, the bioavailability of pollutants, and soil health (Curl and Truelove, 1986). Therefore, the release of root exudates is a key issue in the modelling of the growth of microorganisms in the rhizosphere and of the processes mediated by them. Root exudates consist of a complex mixture of organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions, gaseous molecules, enzymes and root border cells (See figure 1.3) (Dakora and Philips, 2002; Bais *et*

al., 2006; Uren, 2007; Shukla *et al*., 2011), which have major direct or indirect effects on the acquisition of mineral nutrients required for plant growth.

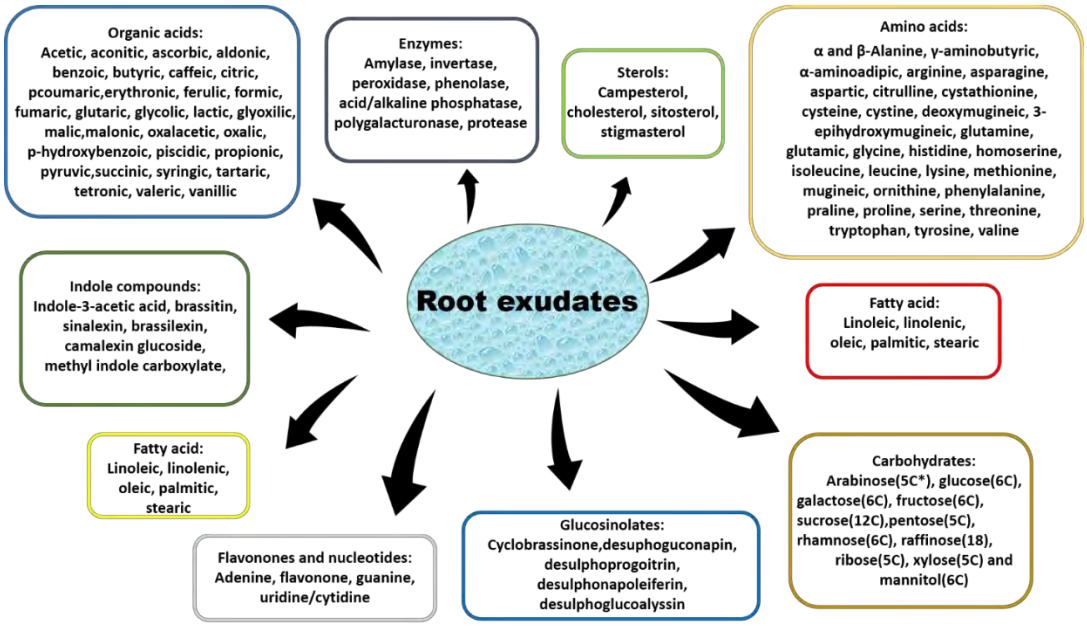


Figure 1.3. Chemical composition of root exudates and some examples of the individual forms of the compounds. Adapted from Badri and Vivanco (2009); Dennis *et al*. (2010); Vranova *et al*. (2013).

1.5.3.2. Role of root exudate in the biodegradation of carbonaceous waste

Root exudates are considered to be one of the most important elements in the mediation of hydrocarbon and carbonaceous waste biodegradation in the rhizosphere (Gao *et al*., 2011; Phillips *et al*., 2012; Martin *et al*., 2014). Generally, an environment contaminated with carbonaceous wastes, particularly coal discard presents harsh environmental conditions for soil microorganisms such as fungi and bacteria to thrive, which in turn affects soil microbial activity as the rate at which biodegradation occurs decreases (Venosa and Zhu, 2003; Rohrbacher and St-Arnaud, 2016). On the other hand, plants exude a wide range of substances via their root surfaces (Figure 1.3), among which are signalling compounds used to repel or recruit beneficial soil microorganisms such as plant growth-promoting bacteria, nitrogen-fixing bacteria or mycorrhizal fungi (Somers *et al*., 2004; Berendsen *et al*., 2012; Huang *et al*., 2014). A good example of the signalling compounds that have been reported is malic acid and various flavonoids (Rudrappa *et al*., 2008; Neal *et al*., 2012). A chemotactic interaction sets the tone for mutualism between plant roots and the soil microorganisms. Also, another possible process which may initiate plant-microorganism mutualism is the production of plant hormones such

as those that promote growth e.g., gibberellin and auxin. Indeed, gibberellins were first identified from a fungus (*Fusarium fujikuroi*) and more recently, both it and auxin have been shown to be produced by plant root-fungi (Gusmiaty and Payangan, 2019), ectomycorrhizal fungi (Kumla *et al*., 2020) and in the phytopathogenic fungus, *Leptosphaeria maculans* (Leontovyčová *et al*., 2020). A study by Spaepen and Vanderleyden (2011), suggested that auxins are sometimes produced by bacteria and act as a signalling molecule which can affect their gene expression. After the soil microorganisms have colonized the rhizosphere of the plant, they quickly begin to feed on the organic acids released from the root surface of the plant, utilizing it as a source of carbon and energy (Gao *et al*., 2011), thus enabling the microorganisms to carry out their metabolic activities effectively (Kuiper *et al*., 2004; Chen *et al*., 2020). During this process, different types of enzymes are released (depending on the organism in question), most of which lead to the degradation of carbonaceous wastes. Specific plants may promote degradation under a particular condition but not under another, and limited information surrounding the bio-mechanisms of phytoremediation hamper attempts at optimization. A study by Phillips *et al*. (2012) on the impact of root exudates from *Elymus angustus* (wildrye) and *Medicago sativa* (alfalfa) on the degradation potential of microbial communities in a weathered hydrocarbon-contaminated soil showed that the root exudates were able to influence certain functional changes in the microbial communities which led to the degradation of the weathered hydrocarbon-contaminated soil. The influence of the root exudates was suggested to be linked to the copy numbers of catechol 2,3 dioxygenase and naphthalene dioxygenase; two genes involved in degradation of polyaromatic hydrocarbons (PAH). Studies on the biodegradation of coal discard have shown that direct contact between coal and metabolically active biocatalysts aids the degradation of coal into humics or humic-like material (Igbinigie *et al*., 2010; Mukasa-Mugerwa *et al*., 2010). In a review by Sekhohola *et al*. (2013) suggested that solubilisation and degradation of coal and other hydrocarbon waste commences upon plant root exudate and ligninolytic microorganism interaction, which in this situation was considered mutualistic, and could include soil bacteria and both mycorrhizal and non-mycorrhizal fungi. Root exudates possess the ability to stimulate the degradative process by increasing the population of rhizosphere microorganisms and their extracellular enzymatic activity, thus leading to the establishment of coal-plant-microorganism interaction in the rhizosphere (Brzostek *et al*., 2013; Sekhohola *et al*., 2013; Rohrbacher and St-Arnaud, 2016). Enzymes such as heme-containing oxidases (manganese peroxidase MnP; EC 1.11.1.13, lignin peroxidase LiP; EC 1.11.1.14, versatile peroxidase VP; EC 1.11.1.16), and phenoloxidase (laccase EC; 1.10.3.2) have been strongly linked to coal biodegradation (Sariaslani, 1989; Gold

et al., 2000; Martinez, 2002; Kersten and Cullen, 2007). Therefore, it will be imperative to understand each of the enzyme metabolic pathways and mode of operation.

1.6. Lignin peroxidases (LiP; EC 1.11.1.14)

Lignin peroxidases [EC 1.11.1.14, 1,2-bis (3,4-dimethoxy phenyl) propane-1,3-diol: hydrogen-peroxide oxidoreductase] were first discovered in N- and C-limited cultures of *Phanerochaete chrysosporium* (Tien and Kirt, 1983; Paszczynski *et al*., 1986), and later in *Trametes versicolor* (Johansson and Nyman, 1993). These enzymes possess the ability to catalyse the H_2O_2-dependent oxidative depolymerization of lignin (Piontek *et al*., 1993; Angel, 2002). The overall reaction is represented by 1,2-bis (3,4-di methoxyphenyl) propane-1,3-diol+H_2O_2⇌3,4-dimethoxybenzaldehyde+1-(3,4-dimethoxy phenyl) ethane-1,2-diol+H_2O (Tien and Kirt, 1983; Hammel *et al*., 1993). Structurally, the isozymes of LiP are made up of glycoproteins in the range of 38–46 kDa and isoelectric points (p*I*) values 3.2–4.0, where the variation of the p*I* depends on the growth medium and nutrient conditions (Wong, 2009; Janusz *et al*., 2017). It also possesses a high redox potential of about (700 to 1,400 mV), and a distinctively low pH of 3 to 4.5 (Piontek *et al*., 1993; Sundaramoorthy *et al*., 1994; Angel, 2002). The high redox potential enables LiPs to oxidize substrates that are not oxidized by other peroxidases (Sigoillot *et al*., 2012).

1.7. Manganese peroxidase (MnP; EC 1.11.1.13)

Manganese peroxidase (MnP; EC 1.11.1.13) is a heme-containing glycoprotein secreted in multiple isoforms and was first detected in the mid-1980's in batch cultures of *Phanerochaete chrysoporium* where expression was reported to be regulated by the presence of Mn (II) in the culture medium (Glenn *et al*., 1983; Kuwahara *et al*., 1984; Paszczynski *et al*., 1986). MnPs play a huge role in the biological degradation of lignin and/or lignin like substances which is a very important process in bioremediation (Bogan *et al*., 1996; Jensen *et al*., 1997; Hofrichter *et al*., 1998; Hofer and Schlosser, 1999; Veitch, 2004; Singh *et al*., 2013). The molecular mass of MnPs ranges from 38 to 62.5 kDa, including 4-18 % glycosylation. Their pI range is from 2.9 to 7.1 and their maximum activity temperature is between 30 and 40 ^{o}C (Martin, 2002; Sigoillot *et al*., 2012). Structurally, MnP consists of two domains with heminic group situated in the middle. The protein molecule contains ten major helices and one minor helix and 5 disulfide bridges, where one of the bridges participates in the Mn bonding sites (Sundaramoorthy *et al*., 1994). A distinct characteristic of Mn-dependent peroxidase is their ability to utilize Mn (II) as a reducing substrate to generate Mn (III), which in turn diffuses

from enzymes into the lignocellulose structure (Paszczynski *et al*., 1986; Janusz *et al*., 2017). The biological oxidation of Mn (II) to form Mn (III) has attracted great interest due to its environmental applications. Mn-oxidizing microorganisms such as fungi and bacteria have been isolated from diverse environments and utilized for metal recovery and bioremediation through biosorption and bio-mineralization (Gadd, 2004; Gadd, 2010). Biosorption is the passive uptake of metals by microbial cells, through either the synthesis and secretion of extrapolymeric substances by microorganisms or through the formation of complexes with microbial cell wall constituents (Gavrilescu, 2004), while bio-mineralization is the organic precipitation of metallic compounds such as oxalates, hydroxides, oxides, sulfides, and carbonates (Gadd, 2004). However, to date, the bio-oxidation of Mn (II) to Mn (III) is still regarded as the most active pathway for Mn-biomineralization (Tebo *et al*., 2005; Das *et al*., 2011; Barboza *et al*., 2015). There are two possible pathways or mechanisms for Mn bio-oxidation which may occur concomitantly (Figure 1.4), the direct pathway which is mediated by cellular components such as enzymes (Brouwers *et al*., 2000; Tebo *et al*., 2004; Tebo *et al*., 2005; Barboza *et al*., 2015), and the indirect pathway where microbial growth and/or metabolites changes the pH and redox conditions of the environment resulting in the bio-oxidation of Mn (II) (Nealson *et al*., 1988; Learman *et al*., 2011;22 and Barboza *et al*., 2015).

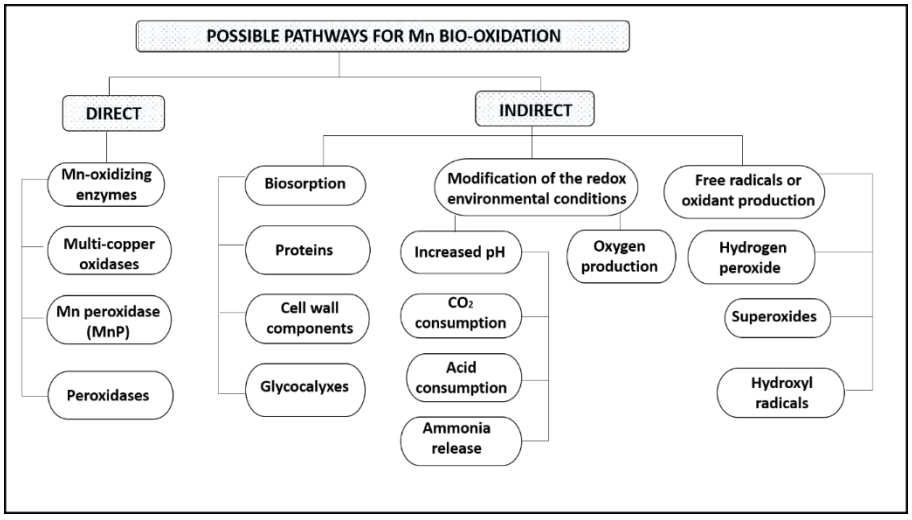


Figure 1.4. A schematic of possible pathways for Mn bio-oxidation (Adapted from Nealson, 2006; Barboza *et al*., 2015). A schematic of possible pathways for Mn bio-oxidation (Adapted from Nealson, 2006; Barboza *et al*., 2015).

1.8. Versatile peroxidase (VP; EC 1.11.1.16)

Versatile peroxidase (VP) belongs to the group of enzymes called ligninases. VPs are extracellularly localized and typically oxidise phenolic and non-phenolic LiP substrates such as veratryl alcohol, methoxybenzenes, and lignin model compounds in the absence of manganese (Wong; 2009; Hofrichter *et al*., 2010). The molecular structure of VP enzymes reveals structures closer to the LiP than to the MnP isozymes of *P. chrysosporium*, (Moreira *et al*., 2005; Ruiz-Duenas *et al*., 2005). The structure of VP includes 11–12 helices, 4 disulfide bonds, 2 structural Ca^{2+} sites, a heme pocket and an Mn^{2+}-binding site like MnP (Perez-Boada *et al*., 2005).

1.9. Laccase (Lac, benzenediol: oxygen oxidoreductases; EC 1.10.3.2)

Laccases (EC 1.10.3.2) are multinuclear copper-containing oxidase enzymes that are widely distributed in plants, fungi, and bacteria, and exhibit various functions, depending on their source organism, physiological and pathological conditions (Dwivedi *et al*., 2011). Laccase was first discovered at the end of the nineteenth century in the latex of the Japanese lacquer tree (*Rhus vernicifera*) by Yoshida (Yoshida, 1883; Leontievsky *et al*. 1997; Wong, 2009; Plácido and Capareda, 2015), and it was reported to be responsible for the darkening of the latex. Since then, several studies have reported the presence of laccases or laccase-like multicopper oxidases (LMCO) genes in plants such as *Zea mays* L. (Caparro´s-Ruiz *et al*., 2006), *Acer pseudoplatanus* (Bligny *et al*., 1986; Sterjiades *et al*., 1992), *Liriodendron tulipifera* (LaFayette *et al*., 1999), *Zinnia elegans* (Liu *et al*., 1994) and *Nicotiana tabacum* (Kiefer-Meyer *et al*., 1996). It has also been discovered in some classes of fungi such as ascomycetes, deuteromycetes, and basidiomycetes, where they were found to be responsible for the depolymerization of complex cell-wall constituents such as lignin (Dwivedi *et al*., 2011). Plant and fungal laccases are extracellularly localized and are glycosylated enzymes, which is useful for the secretion, enzyme activity, copper retention and thermal stability.

1.9.1. Laccase catalytic mechanism

Laccase enzyme catalyses the overall reaction: 4 benzenediol + $O_2 \leftrightarrow$4 benzosemiquinone + $2H_2O$ (Wong, 2009), and in the presence of oxygen, laccase can oxidize a wide variety of ortho- and paraphenols, diamines and amino phenols. The molecular mass of laccase ranges from 58–90 kDa, while their optimal pH and temperature fall within the ranges of 2-10, and 40-65 ºC respectively (Quaratino *et al*., 2007; Zouari-Mechichi *et al*., 2006). Structurally, laccases

possess a primary structure of approximately 500 amino acid residues arranged in three consecutive domains with a Greek key β barrel topology. The first domain contains about 150 initial amino acids, the second domain contains between 150 and 300 residues and the third domain contain 300- 500 amino acid (Plácido and Capareda, 2015). This structure is stabilized by the presence of two disulfide bridges located between domains I and II and between domains I and III (Bertrand *et al*., 2002; Ferraroni et al., 2007; Matera *et al*., 2008). However, some laccases present three disulfide bridges. The interesting characteristics of laccase that makes it a worthy enzyme for bioremediation and biotechnological processes is that it does not require a cofactor and it has a low substrate specificity, instead, it utilizes oxygen as an oxidizing agent and cofactor instead of other expensive cofactors such as pantothenic acid, thiamine, or biotin (Matera *et al*., 2008). This implies that it can easily degrade compounds with phenolic structures (Zouari-Mechichi *et al*., 2006), and its production can be induced by addition of molecules such as copper, dyes, or other recalcitrant compounds (Minussi *et al*., 2007).

1.10. Research scope

The FungCoal biotechnology was developed as a strategy for the rehabilitation of open cast mining spoils and coal wastes or discard sites (Rose *et al*., 2010). This biotechnology exploits coal degrading fungi and plant-fungal mutualism to achieve effective biodegradation of coal spoils to facilitate and promote revegetation (Igbinigie *et al*., 2010; Mukasa-Mugerwa *et al*., 2011; Cowan *et al*., 2016). However, one limiting factor of the FungCoal bioprocess is that it requires either commercially available humics or oxidized weathered coal, with the latter a highly complex and variable resource, as co-substrates to supply carbon for growth and proliferation of the coal degrading microorganisms (Cowan *et al*., 2016).

In a typical plant-fungal-coal rhizosphere (Figure 1.5), it has been hypothesized that plant roots, through the exudates, signal and attract soil fungi into the rhizosphere where they are supplied with readily accessible carbon. In turn, the fungi release ligninolytic enzymes which break down the coal into additional simple carbon compounds and residual humics, and through the hyphae, support the host plant in the uptake of soil nutrients. However, most emerging bioremediation technologies have downplayed the potential of plants, and root exudates from plants, in the bioremediation of hydrocarbons such as coal and biofuels. This is largely due to the complex and variable nature of plants and their exudates, and insufficient understanding of complex microbial interactions (Burns *et al*., 2013). Therefore, it is imperative to understand

the biochemical make-up of plant root exudates and their potential as possible biocatalysts for the effective biodegradation of carbonaceous wastes.

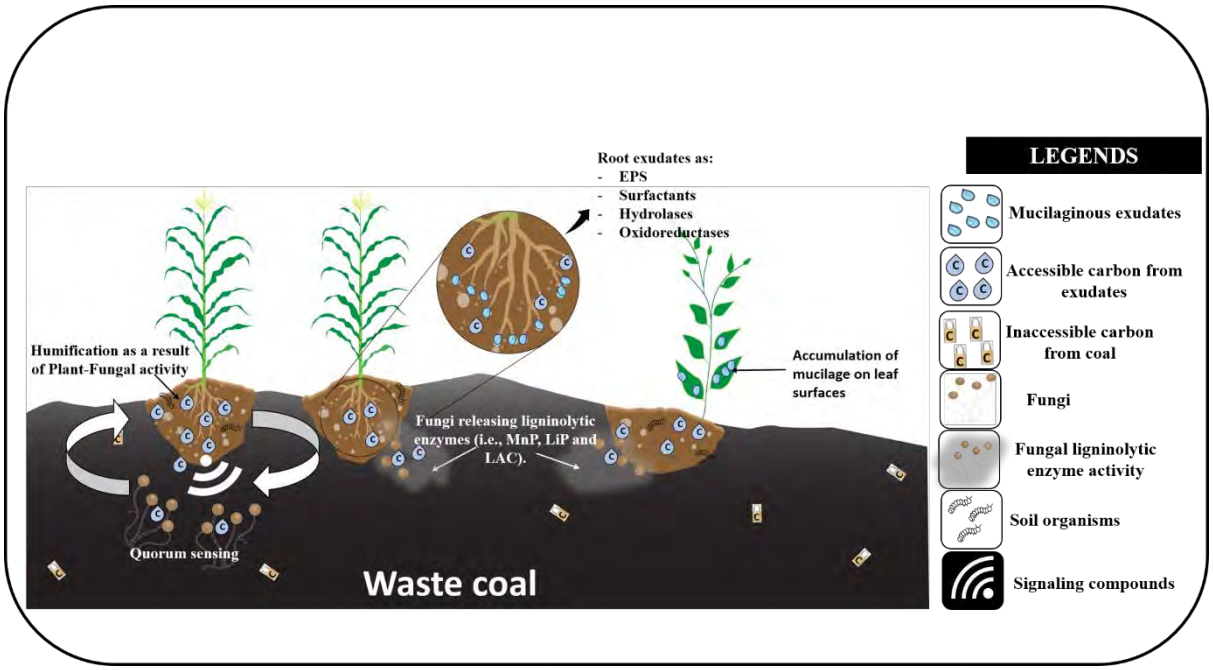


Figure 1.5. Schematic of a typical plant-fungal-coal rhizosphere illustrating the main interactive pathways between ligninolytic microorganisms and root exudates to facilitate plant growth and coal degradation.

1.11. Aims and Objectives.

1.11.1. Aim

This study aimed to investigate the proposed relationship between plant roots, root exudate and coal degrading fungi in more details, to gain insight into the mechanisms underpinning plant-fungal mutualism as a strategy for exploitation in the re-vegetation of coal discard dumps and rehabilitation of hydrocarbon contaminated soil.

1.11.2. Objectives

- Recover and characterise bulk quantities of exudate (extra polymeric substance, EPS) from selected C4 and C3 plants, maize (*Zea mays* L.) and okra (*Abelmoschus esculentus* L. Moench) respectively.
- Confirm the molecular identity of the three coal-degrading fungi, ECCN 84, 225 and 243 used as the biocatalysts in the FungCoal rehabilitation process and possibly to

examine some of the biochemical characteristics that might make these fungi ideal mutualists e.g., produce ligninolytic enzymes etc.

- Investigate the effects of EPS-containing exudates on the ligninolytic enzyme activities of the coal degrading fungi.
- Confirm plant-fungal catalysed biodegradation of waste coal *in situ*.

Chapter Two: Materials and Methods

2.1. Plant material, cultivation, and sampling

Seeds of *Zea mays* L. (maize) and *Abelmoschus esculentus* L. Moench (okra), in hermetically sealed packages, were obtained from Sakata Seed Southern Africa (Pty) Ltd. Seeds were washed in 70 % ethanol for 2 min (Xia *et al*., 2018), then gently stirred for 30 min in a solution containing sodium hypochlorite (12.5 %) and Tween-20 (0.1 % v/v), which was added as a surfactant (Henry *et al*., 2006) to enhance the removal of any adhering preservatives from the surface of the seeds. The seeds were rinsed in Milli-Q water (8 washes) to remove all traces of hypochlorite and other attached chemicals (Delhaize *et al*., 1993; Pino *et al*., 2016) and all floating seeds were regarded as unviable and discarded (Keshinro *et al*., 2017).

A pot-on-beaker system (PoB) was set-up (See figure 2.1.), where five seeds of each of maize and okra were sown in polyvinyl chloride (PVC) pots (7.5 cm i.d.) with a perforated base containing sterilized vermiculite. Pots were placed in a sterile controlled environment at 25 ºC (12/12 h light/dark cycle) under 50 W cool-white-fluorescent tubes (Philips, China) with the radiant energy of 70 - 90 µmol m^{-2} s^{-1}.

The Okra leaf EPS was gently collected from the abaxial surface of leaves of 14 d old okra seedlings with a sterilized spatula into a Petri-dish. For maize, pots containing 4 d old seedlings were transferred to appropriately sized beakers containing Milli-Q water as EPS collecting medium and presence of exudate monitored daily (Figure 2.1).

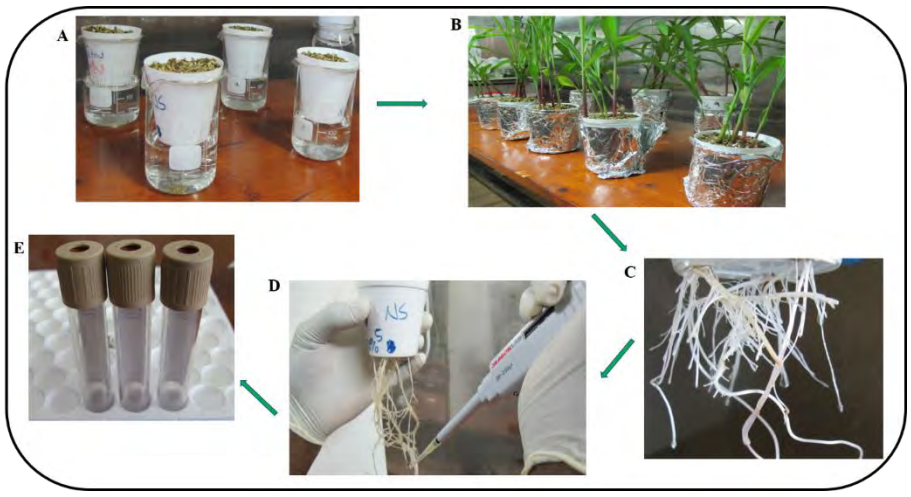


Figure 2.1. Stepwise set-up of the pot-on-beaker (PoB) experiment: (a) Planting pots containing vermiculites placed in beakers; (b) Root region wrapped in foil

upon visible observation of roots; (c) Accumulation of EPS on the root tips of *Zea mays*; (d) Collection of EPS using a pipette; and, (e) EPS collected in sealed tubes and stored at 4 ºC.

2.1.2. Ultrastructural analysis of epidermal features and exudation

Scanning electron microscopy (SEM) was performed using an analytical scanning electron microscope (Vega 3 LMU, TESCAN, Brno, Czech Republic) at 30 kV. Intact plant parts (root tips of maize and leaves of okra) of about 0.5 cm were harvested from a 15-day old plant and cut into smaller sections with a sharp blade. These sections were fixed overnight in 2.5% glutaraldehyde fixative solution, then dehydrated using a graded alcohol series at 30, 50, 70, 80, 90, and 100 %. The samples were further dehydrated using hexamethyldisilazane (HMDS, Sigma Aldrich Chemistry, U.S.A.). The samples were retrieved and mounted on aluminium stubs with double-sided adhesive tape, then gold coated with a gold coater (Quorum Q15ORS, Ashford, Kent, England). Prepared samples were examined and reported.

For the stereomicroscope analysis, EPS samples collected from the abaxial surface of okra leaves and root tips of maize were placed on separate Petri-dishes and examined with a stereomicroscope (Olympus SVX2-ILLT, Tokyo, Japan) at a magnification of 2 mm.

2.1.3. Collection of root and leaf extrapolymeric substances (EPS)-containing exudate.

The EPS from maize was collected from the root tips of 40 seedlings using a pipette (20 – 200 µl) and stored in sterile Eppendorf tubes. This was carried out 5, 10 and 15 days after sowing (DAS) at the 2-, 3- and 4- leaf stage of development. The collected samples were centrifuged (Costar mini centrifuge, U.S.A.) at 3913 x g for 10 min to remove adhering particles such as cell debris and root cap cells, and the supernatant was collected for further analysis. Similarly, the leaf EPS was collected from the abaxial leaf surface of 40 okra seedlings at the 2-, 4-, and 6-leaf stage of development, using a sterile spatula at 10, 15, and 30 DAS and placed into cryotubes for further analysis.

2.1.4. Bulk density of EPS samples

The bulk density of exudates was determined according to the method described by Rayment and Higginson (1992) but with slight modification. The exudate was measured by collecting 1 mL of exudate into a pre-weighed cryotube with a known mass and weighed. Next, the weighed

exudate was removed, and the same cryotube was filled up with I mL Milli-Q water and weighed. The bulk density of the coal was calculated using the following equation:

Weight of exudate = $W_2 - W_1$

Volume of water = V

Bulk density (g/cm^3) = $W_2 - W_1 / V$

Where, W1 = weight of empty bottle (g)

W2 = weight of empty bottle + exudate (g)

V = volume of water needed to fill the empty bottle (mL).

2.1.5. pH of EPS samples

The pH of the exudates was determined according to the method described by Rayment and Higginson (1992). 2 mL of exudate was measured and placed into a test-tube and shaken vigorously for 5 seconds. The pH of the exudate was measured at 25 ^oC without disturbance using a pH meter (HANNA Instruments HI 8314) which had already been calibrated with standard buffer solutions.

2.1.6. Fourier Transformed Infrared Spectroscopy (FT-IR) of EPS samples

The preparation of EPS samples for FT-IR analysis was based on the observable characteristics of both samples. Different freezing methods were employed in the preparation of the EPS samples for FT-IR analysis in order to maintain their integrity. Maize root EPS was frozen in liquid nitrogen and lyophilized using a freeze dryer, and the mass of the residue was determined and stored in a desiccator for FT-IR analysis (Jimoh and Cowan, 2017). For okra leaf EPS, the critical point drying method of Robards (1991) was employed. The collected EPS was carefully placed into a microporous specimen capsule and subjected to critical point drying (Polaron critical point dryer, Watford, England) for 2 h. The mass of the sample was determined and stored in a desiccator for FT-IR analysis.

The FT-IR analysis of sub-samples of the extracted EPS (~1 mg) was carried out using a PerkinElmer Spectrum 100 FT-IR instrument (PerkinElmer, Waltham, MA, USA) with attenuated total reflectance (ATR) accessory eliminating the need for mixing of samples with potassium bromide (KBr). The ATR accessory, fitted with a diamond top-plate, has a spectral range of 25,000 – 100 cm^{-1}, a refractive index of 2.4, and 2.01 μ depth of penetration. FT-IR spectra were recorded in the range of 4,000 – 650 cm^{-1}. Functions were assigned to the peaks by correlating the spectra generated with similar spectra and standards in the relevant literature

(Fan *et al*., 2012; Jonker *et al*., 2012; Maobe and Nyarango, 2013; Garcia *et al*., 2016; Rajashekhar *et al*., 2017; Zhou *et al*., 2019).

2.1.7. Biochemical analysis of EPS samples

2.1.7.1. EPS sample preparation

The root-based EPS was utilized in its aqueous form for the biochemical analysis, while the leaf-based EPS was prepared for biochemical analysis by dissolving 1 mg fresh sample into 1 ml of Milli-Q water.

2.1.7.2. Analysis of Carbohydrate in EPS samples

The carbohydrate content of the extracted EPS was quantified using the phenol-sulphuric acid method described by Dubois *et al*. (1956). Briefly, 50 µL aliquot of a known concentration of the EPS was transferred into a standard well plate to which was added, 150 µL sulphuric acid followed by 30 µL 5 % phenol. The mixture was incubated in a water bath at 90 ^{o}C for 5 min and cooled in another water bath at room temperature. Absorbance at 490 nm was determined using a Microplate reader (SpectraMax® M3 multi-mode microplate reader), and concentration determined by interpolation with a standard curve prepared for D-glucose (Appendix I).

2.1.7.3. Analysis of proteins in EPS samples

The protein content of the extracted EPS was quantified using the protein-dye-binding method of Bradford (1976). To begin, 40 µL Bradford reagent (prepared by dissolving 100 mg of Coomassie Brilliant blue in 50 ml of 95 % ethanol and after addition of 100 ml of 85 % phosphoric acid, the solution was diluted to 1 L and filtered) was added to 160 µL of a known EPS concentration. The mixture was incubated at room temperature for 5 min and absorbance determined at 595 nm in a microplate reader. Concentration was determined by interpolation with a standard curve for bovine serum albumin (BSA) (Appendix II).

2.1.7.4. Analysis of α-Amino nitrogen in EPS samples

Analysis of α-amino nitrogen was carried out according to the ninhydrin method of Lie (1973). To quantify α-amino nitrogen content of extracted EPS, 1 ml of ninhydrin reagent (prepared by dissolving 100 g $Na_2HPO_4.12H_2O$, 60 g anhydrous KH_2PO_4, 5 g ninhydrin, and 3 g fructose in 1 L distilled water, pH 6.7) was added to 2 ml of known EPS concentration in test tubes. The mixture was placed in a boiling water bath for 16 min and transferred immediately to another

water bath at 20 ºC to cool for 20 min. Thereafter, 5 ml of dilution reagent (prepared by dissolving 2 g KIO_3 in 600 ml distilled water, which was then made to 1 L with 96 % ethanol) was added to the tubes and mixed thoroughly. Absorbance was measured at 570 nm within 30 min in a microplate reader. Concentration was determined by interpolation from a standard curve prepared with a known concentration of glycine (Appendix III).

2.1.7.5. Analysis of lipids in EPS samples

The lipid content of the EPS was analysed by the sulfo-phospho-vanillin (SPV) method as described by Cheng *et al*. (2011) and Mishra *et al*. (2014). The method was modified by using hexane to prepare the calibration instead of chloroform. To 100 µL of a known concentration of EPS in microplate wells, 100 µL of concentrated sulfuric acid was added and the mixture incubated at 90 ºC for 20 min in a water bath and cooled on an ice bath for 5 min. 50 µL phospho-vanillin reagent (prepared by dissolving 0.2 mg of vanillin in 1 ml 17 % phosphoric acid) was added to the mixture and incubated at room temperature for 10 min. The absorbance was measured at 540 nm in a microplate reader and concentration interpolated from a standard curve prepared with 2 mg canola oil in 1 ml hexane (Appendix IV). A 100 µL of series of lipid concentration was aliquot in microplate wells and the well plate kept at 60 ºC for about 10 min to evaporate the solvent followed by the SPV reaction as described above.

2.1.7.6. Analysis of total phenolics in EPS samples

The analysis of the total phenolic content of EPS was carried out using the Folin-Ciocalteu-phenol reagent method described by Titto (1985). A 500 µl aliquot of the extract was diluted with 2 ml Milli-Q water. One ml of Folin-Ciocalteu phenol reagent (Sigma Aldrich, South Africa) (which contains sodium molybdate and sodium tungstate, 2.5 and 10 %) was added and the sample shaken vigorously. Immediately, 5 ml of 20 % sodium carbonate solution was added and the mixture made up to 10 ml and shaking thoroughly. After 20 minutes the absorbance was measured at 720 nm without background measurements and the spectrophotometer was set to zero against air. For calculation, a standard curve was prepared using ferulic acid (Appendix V) and the total phenolic content is expressed as ferulic acid equivalents.

2.1.7.7. Analysis of indole/indole-like compounds in EPS samples

EPS samples were centrifuged at 3913 x g for 3 min, and 2 ml of the supernatant was collected and mixed with 2 drops of 10 mM orthophosphoric acid and 4 mL of Salkowski reagent (1 mL

of 0.5 M iron (III) chloride and 50 mL of 35 % perchloric acid) (Gordon and Weber, 1951; Datta *et al*., 2015). After 20 min, colour development (pink) was quantified using a spectrophotometer (Shimadzu UV-1201 spectrophotometer, Japan) at 530 nm. A calibration curve using pure IAA was established for calculating the concentration of the indole-like compounds. 10 mg of pure IAA (Sigma Aldrich, South Africa) was pipetted into a flask containing 10 mL acetone and stirred until dissolved completely. From this stock solution, 50 mg L^{-1} of IAA was then prepared and used for the standard curve (Appendix VI) (Patten and Glick, 2002; Sarker and Al-Rashid, 2013).

2.1.8. Elemental analysis of EPS samples

The Maize and Okra EPS samples were prepared using the freezing and critical point drying methods as described for the FT-IR analysis above. Next, 500 mg of each sample was collected and analysed for carbon, nitrogen, sulphur, and hydrogen contents using an Elemental analyser (PE 2400 CHNS/O, PerkinElmer, Waltham, MA).

2.2. Fungal sample collection and preparation

Fungal isolates with EBRU Culture Collection Number (ECCN) 84, 225 and 243 were previously isolated from the bituminous coal samples collected from *Cynodon dactylon*/coal rhizosphere at Good hope colliery and Excelsior mine, Witbank, Mpumalanga, South Africa (Igbinigie, 2007; Igbinigie *et al*., 2008; Igbinigie *et al*., 2010). ECCN 84 was isolated from the coal samples collected at Good hope colliery while ECCN 225 and 243 were isolated from the coal samples collected from Excelsior mine (See figure 2.2). Pure cultures were in storage as part of the EBRU culture collection of the Institute for Environmental Biotechnology (EBRU), Rhodes University, South Africa.

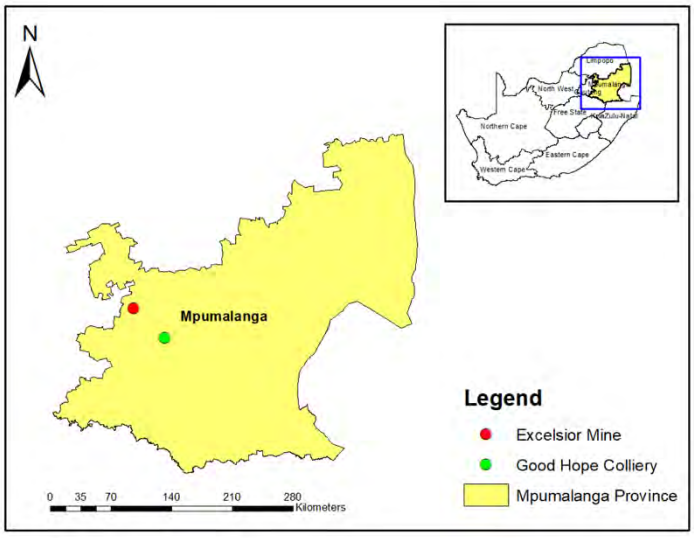


Figure 2.2. The geographical location of the source of fungal samples. The coordinates were taken using Garmin Etrex 20.0 GPS devices. The map was plotted with an ArcGIS 9.3 software.

2.2.1. Morphological characteristics of fungal isolates

Fungal strains ECCN 84, ECCN 225 and ECCN 243 were sourced from EBRU culture collection and maintained on 2.5 % potato dextrose agar (PDA) and stored as mycelial plugs (5×5 mm) in 50 % glycerol (v/v) at -20°C. Each strain was later sub-cultured from stock mycelial plugs and cultivated in Petri-dishes on 2.5% potato dextrose agar (PDA), supplemented with 20 g/L glutamate at 28 °C. The Petri-dishes were unsealed during this process to enable adequate oxygen transfer (Okuda *et al*., 2000). After 7 days of incubation, the microscopic and macroscopic characteristics were observed (Samson *et al*., 2007) and results recorded.

2.2.2. Scanning electron microscopy of fungal spores

For the microscopic characteristics, scanning electron microscopy (SEM) was employed to visualize the presence of conidia from the fungal sclerotia. This was performed using an analytical scanning electron microscope at 30 kV (Vega 3 LMU, TESCAN, Brno, Czech Republic). Sclerotia were collected from the culture media of the three fungal isolates and fixed overnight in 2.5% glutaraldehyde fixative solution. In the next day, the samples were dehydrated using a graded alcohol series at 30, 50, 70, 80, 90%, 100 %, and further dehydrated using HMDS (Aldrich Chemistry, U.S.A.). The samples were retrieved and mounted on

aluminium stubs with double-sided adhesive tape, then gold coated with a gold coater (Quorum Q15ORS, Ashford, Kent, England). Prepared samples were examined and reported.

2.2.3. Extraction and PCR amplification of genomic DNA

Genomic DNA was extracted from 5-day old mycelial cultures using the Quick DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No D6005, USA). Briefly, 60 mg wet weight of each of the fungi mycelia were suspended in 200 µL phosphate buffer solution and then added to a ZR BashingBead™ lysis tube (0.1 mm and 0.5 mm). 750 µL BashingBead™ buffer was added to the tube and vortexed (Vortex-2Gernie G-5608, Scientific industries, USA) at 10,000 rpm for 5 min. Thereafter, the ZR BashingBead™ lysis tube was centrifuged (Costar mini centrifuge MVSS- 07343, USA) at 1000 rpm for 1 min, 400 µL of the supernatant transferred into a Zymo-Spin™ III-F filter in a collection tube and centrifuged at 8000 rpm for 1 min. Exactly 1200 µL of genomic lysis buffer was added to the filtrate and 800 µL of the mixture was centrifuged twice at 10,000 rpm for 1 min. The Zymo-Spin™ IICR column was placed in a new collection tube after which 200 µL of DNA pre-wash buffer and 500 µL gDNA wash buffers were added and centrifuged at 10,000 rpm for 1 minute respectively. The column was transferred into a clean 1.5 ml microcentrifuge tube and 100 µL DNA elution buffer was added directly into the column matrix and centrifuged at 10,000 rpm for 30 secs to elute the DNA. Ultra-pure DNA was stored at -80 ºC for further use.

2.2.4. DNA sequencing and ITS region analysis

Total genomic DNA was extracted from isolates of interest using a DNA isolation kit (Zymo Research), according to the manufacturer's instructions. DNA extracts were sent to Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa for sequencing and identification. Universal primer pair ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990) was used in amplification and amplicons were run on a gel and extracted with Zymoclean™ Gel DNA recovery kit (Zymo Research, catalogue no. D4001). Extracted fragments were sequenced in the forward and reverse direction using Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit and purified using ZR-96 DNA sequencing clean-up kit™, (Zymo research, catalogue no. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied biosystems, Thermofisher Scientific) for each reaction for every sample.

For good quality sequence assurance, ChromasLite version 2.33 software was used for the analysis of chromatograms (sense and antisense) resulting from sequencing reaction. The resulting chromatograms were edited using BioEdit Sequence Alignment Editor (Hall, 2004). After this, the resulting consensus sequences obtained were blasted in the NCBI (http://www.ncbi.nlm.nih.gov) database with the Basic Local Alignment Search Tool (BLAST) for homology to identify the probable organism in question (Altshul *et al.*, 1997), after which the sequences were deposited in the GenBank and their accession numbers were generated. Phylogenetic trees were constructed with neighbour-joining algorithms (Saitou and Nei, 1987) using 1000 bootstraps consensus available in Mega 6.0 software package (Tamura *et al.*, 2013).

2.2.5 Quantification of indole/indole-like compound in fungal isolates

2.2.5.1. Preparation of fungal isolates for the analysis of indoles

To quantify the indole/indole-like compound produced by the fungi ECCN 84, ECCN 225 and ECCN 243, the isolates were grown in an Erlenmeyer flask containing 20 g/L potato dextrose broth (PDB) with 10 g/L of D-glucose and 1 g/L L-tryptophan. The flasks were placed on a shaker which was set at 120 rpm and incubated in the dark at 27 °C, and measurements were taken on the 5^{th} and 7^{th} days of incubation. On each occasion, 30 mL of the cells were collected and pelleted by centrifuging at 3913 x g for 3 min, and 2 mL of the supernatant was collected and used for the analysis.

2.2.5.2. Spectrophotometric quantification of indole/indole-like compound in fungal isolates, ECCN 84, 225 and 243

Thirty millilitres of the cells were collected and pelleted by centrifuging at 3913 x g for 3 min, and 2 mL of the supernatant was collected and used for the spectrophotometric analysis as described in chapter two, section 2.1.8.7.

2.3. Screening of Fungi ECCN 84, 225 and 243 for manganese peroxidase enzyme (MnP) activity

2.3.1. Preparation of fungal isolates

The preparation of fungal isolates ECCN 84, 225 and 243 were same as previously described in Chapter two, section 2.2.1.

2.3.2. Preparation of maize root EPS

The preparation of the EPS obtained from the roots of the maize seedlings was the same as previously described in Chapter Two, Section 2.1.8.1.

2.3.3. Preparation of coal sample

Bituminous coal was obtained from the Navigation Processing Plant, Kleinkopje Mine, Landau Colliery (now Khwezela Colliery), Emalahleni, South Africa (Igbinigie, 2007). Aliquots were powdered using an HP-M 100 Pulverizer (HERZOG Maschinenfabrik GmbH Co., Osnabrück, Germany) and sieved with a 250 microns laboratory test sieve (Endecotts Ltd. London England) to obtain a particle size < 250 microns. The resultant powdered coal (P-coal) sample was sterilized by a freeze-thawing method, using liquid nitrogen at three cycles to eliminate any *in situ* microbial activity. Confirmation of sterilization was achieved by monitoring microbial growth after plating serially diluted aliquots of the waste coal, suspended in sterile Milli-Q water, on nutrient agar and incubated at 28 °C for 48 h.

2.3.4. Colorimetric manganese peroxidase enzyme (MnP) assay

The manganese peroxidase (MnP) assay was modified from the method of Hofrichter *et al.* (1998) and Sekhohola *et al.* (2014). A media containing a mixture of 2.5 % PDA and 1000 µg/mL $MnCl_2$ was prepared and poured into Petri dishes. After few minutes when the PDA-$MnCl_2$ media was fully solidified, a 6 mm cork borer was used to bore three different holes at three different points on the solidified media, and the holes were filled-up with treatments as shown in Figure 2.3 below. After the treatments solidified, the fungal isolates were inoculated on each of these treatment points and incubated at 28 ^{o}C in the dark for 7 days. The formation of a black precipitate (MnO_2) was taken to indicate oxidation of Mn^{2+} to Mn^{3+}.

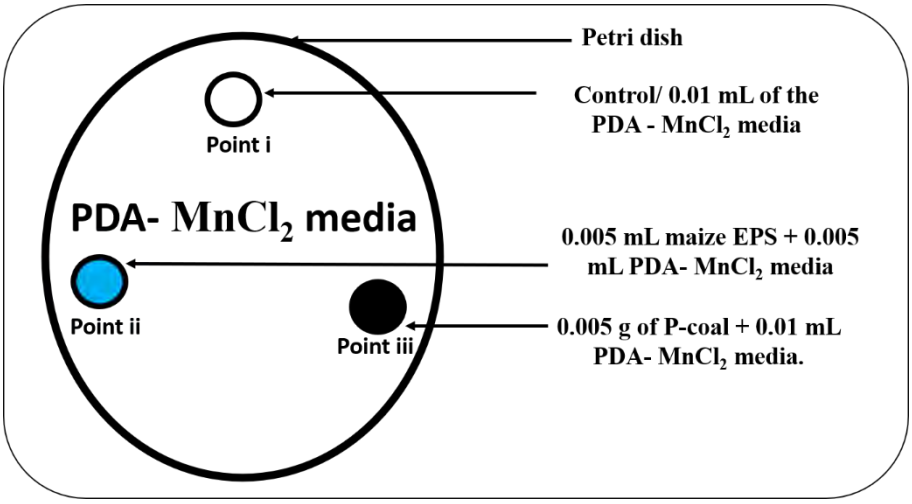


Figure 2.3. A schematic of the manganese peroxidase (MnP) treatments arrangement on Petri dish.

2.3.5. ABTS Assay of fungal strains for extracellular laccase activity (LAC activity)

For analysis of extracellular LAC activity, fungal strains were cultivated in 20 g/L potato dextrose broth (PDB), supplemented with 10 g/L D-glucose, and incubated at 28 ºC for 7 d. For this experiment, 7 d cultures, in a glutamate basal salt medium (GBSM) containing 12.7 g/L KH_2PO_4, 3.0 g/L $NaNO_3$, 3.1 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L KCl and 2.0 g/L glutamate, were used as inoculum for the following treatments:

(a) fungus only

(b) fungus + EPS

(c) fungus + Powdered coal (P-coal)

(d) fungus + EPS + P-coal

After 7 d of incubation, aliquots of each treatment (1.5 mL) were filtered and added to 1.5 mL sodium acetate buffer (1 mM, pH 5), followed by addition of 0.5 mM of 1.5 mL 2.2′[azino-bis-(3-ethylbonzthiazoline-6-sulphonic acid) diammonium salt] (ABTS) as the substrate. The absorbance of the reaction mixture was monitored at intervals (1 min) over 25 min. at 420 nm using a UV-VIS spectrophotometer (Shimadzu UV-1280, Duisburg, Germany). A plot of absorbance vs. time was used to derive the slope and the enzymatic activity was determined by multiplying the slope by the molar extinction coefficient of 36.0 mM^{-1} cm^{-1} for ABTS as described by (Li *et al*., 2008; Sekhohola *et al*., 2014).

2.4. Rhizo-box experimental set-up

The rhizo-boxes, like those used previously by Mukasa-Mugerwa *et al*. (2011), were modified for this study by reducing the height of each of the rhizo-boxes from 150 cm to 50 cm as shown in Figure 2.4. All rhizo-boxes were sterilized using absolute ethanol before use and each has a height of 50 cm, and a width of 12.7 cm respectively (Figure 2.4.).

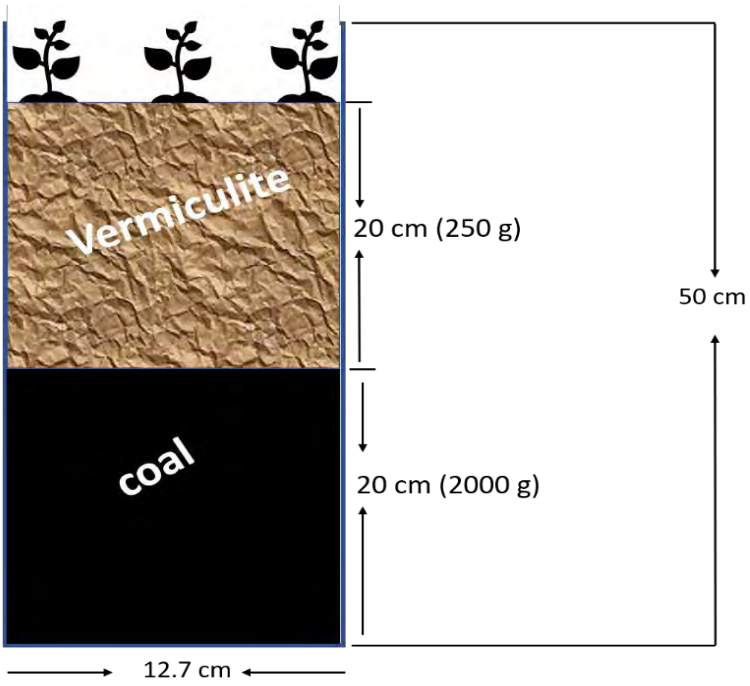


Figure 2.4. A schematic of the rhizo-box set-up

2.4.1. Preparation of P-coal for the rhizo-box experiment

The P-coal used for the experiment was sterilized as previously described in Chapter two, Section 2.3.3. After the sterilization, 2 kg of the P-coal samples were collected from the sterilized, composite, P-coal and poured into each of the rhizo-boxes to create a 20 cm layer, simulating a stockpiled coal environment (i.e., discard dump). A 20 cm layer of 250 g sterilized vermiculite (obtained from Sunnyside Garden Centre, Makhanda, South Africa) was poured onto the coal in the rhizo-box to support seed germination and seedling establishment.

2.4.2. Preparation of fungal inoculum for the rhizo-box experiment

Strain ECCN 84 which has been established by previous research from our laboratory to be an effective coal-degrading fungus (Igbinigie *et al*. 2010; Sekhohola *et al*., 2014) was selected, and cultured on PDA media as described in Chapter two, section 2.2.1., and after the 8th day it

was viewed under a SEM (Vega 3 LMU, TESCAN, Brno, Czech Republic) as described in Chapter two, section 2.2.2. for the production/presence of spores. The fungal spores were harvested by washing the mature fungal lawns with Milli-Q water into a collection beaker, which was later used as the inoculum. For treatments which required the inoculation of fungal spores, 200 ml of the fungal inoculum was applied evenly to each pot containing 2000 g sterilized P-coal samples before the addition of the vermiculite.

2.4.3. Cultivation of seedlings for the rhizo-box experiment

Seeds were sterilized as described in chapter two, section 2.1. Next, they were sown directly into the vermiculite and thinned to 5 per box after two weeks. Five different treatments were used for the experiment, with each having three replicates. Thus, making a total of 15 rhizo-boxes. The series of treatments used are as follows:

1. No biologicals = Control
2. Maize + Okra + Strain ECCN 84 (MOS84) = FungCoal simulated
3. Maize + Strain ECCN 84 (MS84) = FungCoal simulated
4. Strain 84 only = Fungus treatment
5. Maize only = Plant treatment

The treatments were arranged in a completely randomized design (CRD) in a polycarbonate-covered tunnel (Ulma Agricola, Spain), and monitored under ambient conditions (Figure 2.5). The plants were watered with 200 mL of water twice a week.

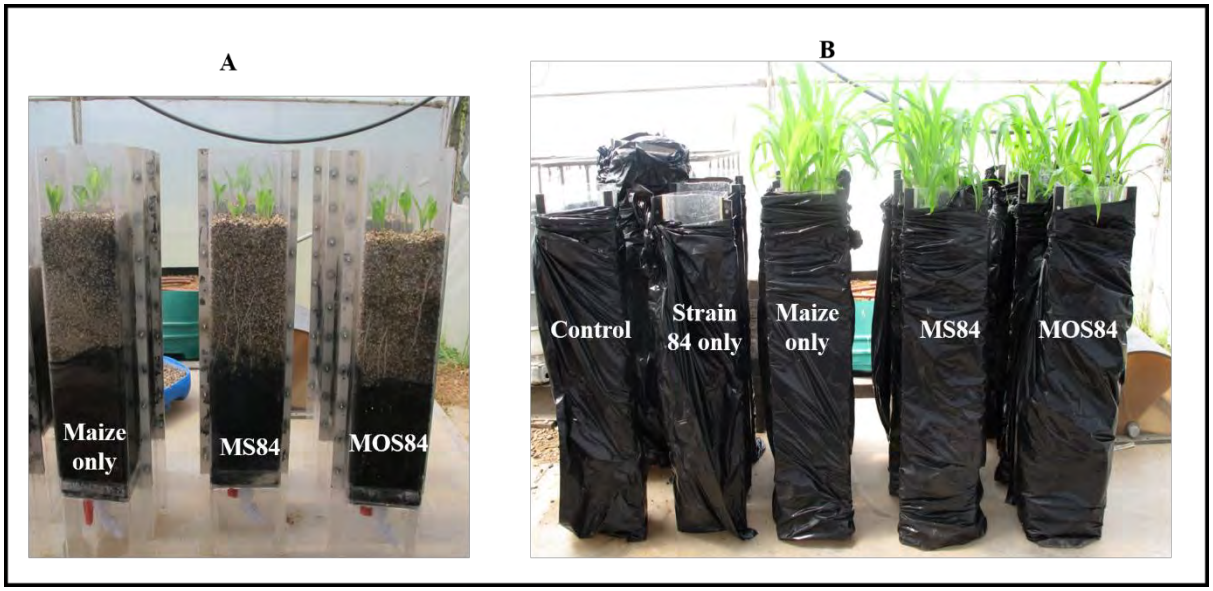


Figure 2.5. A rhizo-box system. (a) Rhizo-box showing the emergence of maize seedlings on the vermiculite-coal substrate and the distinct vermiculite-P-coal layers. (b) Wrapped rhizo-boxes showing the respective treatments and arranged in a polycarbonate-covered tunnel.

2.4.4. Coal sampling

Rhizobox experiment was terminated after the 16th week and each of the boxes was harvested individually. To collect the P-coal samples, each of the rhizo-boxes was opened from the front cover and all the plant materials and vermiculite carefully removed. Next, the resultant P-coal sample was thoroughly mixed with a Heidolph RZR1 stirrer (Germany), after which samples were collected and used for their respective analysis.

2.4.5. Observation of post-harvest P-coal samples for possible fungal colonisation using SEM.

To check for the colonization of P-coal by the fungus ECCN 84 after 16 weeks of cultivation, P-coal were collected and prepared for SEM according to the methods of Cross and Pinchuck (2001). The P-coal were harvested from each of the rhizo-boxes with the fungal treatments (MS84, MOS84 and strain 84 only) and fixed overnight in 2.5 % glutaraldehyde fixative solution prepared from a 25 % glutaraldehyde stock reagent diluted with phosphate buffer (0.1M, pH 7). The glutaraldehyde solution was decanted, and the coal particles were washed twice for 10 minutes with phosphate buffer. P-coal was dehydrated with a series of ethanol concentrations (30, 50, 70, 80, 90, and 100 %) for 15 minutes each. The final dehydration of

samples in absolute ethanol was carried out twice. Samples were then placed in a critical point dryer (Polaron critical point dryer, Watford, England) for 2 hours before being mounted on 12 mm aluminium stubs with a double-sided adhesive tape. The samples were transferred to a gold coater (Quorum Q 15ORS, Ashford, Kent, England) at 80 Millitorr pressure with an applied current of 45 mA and coated for 10 minutes. Prepared samples were examined with a (Vega 3 LMU, TESCAN, Brno, Czech Republic) scanning electron microscope.

2.4.6. Proximate analysis of P-coal samples

Moisture content (M), ash content (A), volatile matter (VM) and fixed carbon (FC) were all determined according to the procedures described by the American Society for Testing and Materials (1999) standard. All analysis was carried out in triplicate and results reported as mean with standard error (mean ± SE).

The moisture content was determined by drying 1 g of P-coal at 105 ^{o}C for 1 h. The samples were allowed to cool in a desiccator at room temperature before the mass was determined. The relative change in mass was reported as the percentage of moisture content.

Ash content was determined following complete combustion of 1 g of coal using a muffle furnace (Carbolite, Bamford, Sheffield, England.) at 750 ^{o}C. The samples were allowed to cool in a desiccator at room temperature, and the difference in mass was measured and reported as the ash content.

The determination of the volatile matter was carried out by placing 1 g of the coal into a platinum crucible which was then heated using a muffle furnace (Carbolite, Bamford, Sheffield, England.) at 950 ^{o}C for 7 minutes. The samples were allowed to cool in a desiccator at room temperature before the mass was measured, and the mass loss was determined and reported as the volatile matter.

The fixed carbon content of the coal was calculated as the carbon left in the coal sample after volatile materials are eliminated.

Below are the detailed equations used for the proximate analysis:

$$\% M = (\text{Weight loss} / \text{Weight of sample}) \times 100$$

$$\% A = (\text{Weight of residue} / \text{Weight of sample}) \times 100$$

$$\% VM = (\text{Weight loss due to VM} / \text{Weight of sample}) \times 100$$

$$\% FC = 100 - (\% M + \% A + \% VM)$$

2.4.7. Ultimate analysis of P-coal

For the ultimate analysis, the elemental content of C, H, N and S of coal was determined using an elemental analyser (PE 2400 CHNS/O, PerkinElmer, Waltham, MA), while % O was determined by the differential method using the formula, 100 - (%C + %H +% N + % S).

2.4.8. Physicochemical analysis of P-coal treatments.

For the physicochemical analysis of P-coal, the bulk density, pH and electrical conductivity (EC) of the P-coal treatments were measured.

2.4.8.1. Determination of the bulk density of P-coal

The bulk density was measured by collecting 80 g of coal from each of the rhizo-boxes and oven-dried at 50 ^{o}C for 2 hours and then allowed to cool. The samples were sieved to a particle size of 0.2 - 0.5 mm diameter with Endecotts laboratory test sieve (Endecotts Ltd. London England). The sieved P-coal were gently transferred into clean beakers and weighed. Next, the weighed P-coal was removed, and the same beakers were filled up with Milli-Q water and the volume of the water was weighed and recorded. The bulk density of the coal was calculated using the following equation:

Weight of coal = $W_2 - W_1$

Volume of water = V

Bulk density (g/cm^3) = $W_2 - W_1 / V$

Where, W1 = weight of empty bottle (g)

W2 = weight of empty bottle + coal (g)

V = volume of water needed to fill the empty bottle (mL).

2.4.8.2. Determination of the pH of P-coal

The pH of the P-coal was determined according to the method described by Rayment and Higginson (1992). 10 g aliquot of P-coal was measured and placed into a 100 mL beaker containing 10 mL of Milli-Q water. The solution was stirred vigorously for 5 seconds, after which the stirrer was removed, and the solution was allowed to stand for exactly 10 min. The pH of the supernatant was measured at 25 ^{o}C without disturbance using a pH meter (HANNA Instruments HI 8314) which had already been calibrated with standard buffer solutions.

2.4.8.3. Determination of the E.C. of P-coal

The electrical conductivity (E.C.) of P-coal was determined according to the method of Rayment and Higginson (1992). 10 g of P-coal was suspended in 50 mL of Milli-Q water and vigorously mixed. The solution was allowed to settle, after which the E.C. was measured using a waterproof E.C. Testr11Dual Range EUTECH conductivity meter (EUTECH Instruments/ Oakton Instruments).

2.4.9. SEM-EDX analysis of P-coal

The energy-dispersive x-ray spectroscopy (EDX) (INCA Penta FETX3) in conjunction with SEM were used to detect the distribution patterns of some elements of interest in the P-coal across the treatments. P-coal preparation was as described for SEM but without gold coating.

2.4.10. Fourier Transformed Infrared Spectroscopy (FT-IR) analysis of P-coal samples.

The P-coal samples were subjected to drying at 105 ^{o}C for 1 hour, then allowed to cool in a desiccator at room temperature, after which they were used for the FT-IR analysis. The FT-IR analysis of the P-coal sub-samples (~1 mg) was carried out using a PerkinElmer Spectrum 100 instrument (PerkinElmer, Waltham, MA, USA) with attenuated total reflectance (ATR) accessory eliminating the need for mixing of samples with potassium bromide (KBr). The ATR accessory, fitted with a diamond top-plate, has a spectral range of 25,000-100 cm^{-1}, a refractive index of 2.4, and 2.01 µ depth of penetration. FT-IR spectra were recorded in the range of 4,000–650 cm^{-1}.

2.4.11. Biochemical analysis of P-coal samples

2.4.11.1. Estimation of humic/humic-like substances from P-coal samples

The estimation of humic/humic-like substances from P-coal samples was carried out according to the Lowry assay described by Redmile-Gordon *et al.* (2013) and Redmile-Gordon *et al.* (2014). The humic/humic-like substances were extracted from the P-coal by adding 8 mL of 20 mM sodium citrate to 1 g of P-coal and autoclaved at 121 ^{o}C for 30 min. Solutions were cooled on ice immediately after autoclaving and centrifuged at 3500 *g* for 20 min. The supernatant (extracts) was decanted and stored at 4 ^{o}C prior for humic/humic-like substances estimation. To 50 µL of P-coal extracts in two separate wells (well A and B), 50 µL Milli-Q water was added and 100 µL of reagent. A solution containing 3.5 % copper sulphate, 7 %

sodium potassium tartrate and 7 % anhydrous sodium carbonate in 0.35 N NaOH, in a ratio 1:1:100 respectively was prepared and added to well A. An equal volume of reagent B, which was prepared as A, except for the substitution of copper sulphate with Milli-Q water was also added to well B and both wells were mixed thoroughly and incubated at room temperature for 10 min. A 10-fold dilution of 2N Folin-Ciocalteu's phenol reagent (Sigma Aldrich, South Africa) was prepared and 100 µL added to both wells before further incubation for 30 min in the dark at room temperature. The absorbance of both wells was measured at 750 nm in a SpectraMax M3 multimode microplate reader, China, and the actual absorbance for estimation of humic acid was derived from the mathematical expressions as shown below. Humic/humic-like substances concentration was determined by interpolation from a standard curve prepared using commercially available humic acid (Sigma Aldrich, South Africa) (Appendix VII).

$A_{Protein} = 1.25 (A_a – A_b)$ …… ……Eq 2.1

$A_{Humic} = A_b – 0.2(A_{Protein})$ …….. Eq 2.2

Where, $A_{Protein}$ and A_{Humic} are the new absorbances derived for estimation of protein and humic acid content of P-coal treatments respectively from absorbances measured at 750 nm for well A (A_a) and well B (A_b).

2.4.11.2. Determination of total phenolic content

The extraction of total phenolic compounds from P-coal was the same as stated for humic acid. The total phenolic content of P-coal was carried out using the method described by Titto (1985). A 500 µl aliquot of the supernatant collected from the P-coal was diluted with 2 mL Milli-Q water. One mL of Folin-Ciocalteu phenol reagent (2.5 % sodium molybdate and 10 % sodium tungstate) was added and the sample shaken vigorously. Immediately, 5 ml of 20 % sodium carbonate solution was added and the mixture made up to 10 ml and shaken thoroughly. After 20 minutes the absorbance was measured at 720 nm using Shimadzu UV-1201 spectrophotometer, Japan. This was done without background measurements and the spectrophotometer was set to zero against air. For calculation, a standard curve was prepared using ferulic acid and the total phenolic content expressed as its equivalents (Appendix VIII).

2.4.11.3. Determination of indole/indole-like compounds from P-coal treatments

The P-coal samples were centrifuged at 3913 x *g* for 3 min, and 2 ml of the supernatant was collected and used for the indole analysis as described in chapter two, section 2.1.8.7.

2.5. Statistical analysis

All sampling, experiments and analyses were carried out in triplicate and data obtained were entered into Microsoft Excel 2016 software and imported into IBM SPSS 20.0 software for analysis. One-way ANOVA test was used to investigate the differences in means for the group of treatments (independent variables) used during the experiment. Post HOC analysis using Duncan Multiple Range Test (DMRT) was also used to separate means that are significantly associated with dependent variables. Significance was determined at 95 % probability levels. Sigma Plot version 13.0 (SPSS Inc., Chicago, IL) was used for the construction of graphs.

Chapter Three: Characterization of the extrapolymeric exudate produced by maize (*Zea mays* L.) and okra (*Abelmoschus esculentus* L. Moench)

3.1 Introduction

Plants play a major role in the biogeochemical function of the environment and do so by exuding a range of organic compounds into the phyllosphere from aerial plant parts and into the rhizosphere typically from roots throughout their lifetime (Neumann and Römheld, 2007; Kannenberg and Phillips, 2016; Galloway *et al*., 2020). These exudates can be in the form of monomeric compounds such as amino acids, organic acids, sugars, phenolics and an array of secondary metabolites, or as polymeric high molecular weight compounds termed, extrapolymeric substances (EPS) (Bertin *et al*., 2003; Bais *et al*., 2006; Badiri and Vivanco, 2009; Jimoh *et al*., 2019; Shen *et al*., 2020). Low molecular mass compounds are mostly released as volatiles through passive diffusion, while EPS are exuded passively in the form of mucilage (sterile environment) or mucigel (non-sterile environment) from plant surfaces (Rovira *et al*., 1979). Plant EPS is a matrix of high molecular weight compounds which are secreted as a visco-elastic gel that is often polysaccharide-rich (Sasse *et al*., 2018). They are typically released by plants as a response mechanism to environmental conditions and may originate from any of the plant parts such as seeds, stem, leaves and roots depending on plant species and prevalence of abiotic/biotic factors (Brown *et al*., 2017). In aboveground plant parts, some EPS such as those found in carnivorous plants, are conspicuous as mucilage on glandular trichomes and appear to be used to capture insects which are a supplementary source of nitrogen (Huang *et al*., 2015), while others such as those produced by climbers e.g., *Ficus pumila* and English ivy (*Hedera helix*) are secreted on tendrils, which enables attachment to adjacent surfaces (Groot *et al*., 2003; Melzer *et al*., 2010; Huang *et al*., 2016). For belowground plant parts, EPS is less obvious and mostly secreted as mucilage/mucigel from the surface near the root tip, with the visco-elastic property enabling it to serve as a scaffold for biological interactions in the rhizosphere (Jones *et al*., 2009; Haichar *et al*., 2014; Baetz and Martinoia, 2014).

Just over a decade ago, studies were initiated in our laboratory to derive a passive biological rehabilitation process to replace topsoil utilization in the rehabilitation of land disturbed by coal mining (Igbinigie *et al*., 2010; Rose *et al*., 2010; Mukasa-Mugerwa *et al*., 2011), These studies identified plant-fungal mutualism as a potential strategy for the development of a

bioprocess with which to revegetate coal discard dumps and restore contaminated soils (Igbinigie *et al*., 2008; Rose *et al*., 2010). In brief, studies examined the influence of a plant-fungal relationship on the biodegradation of coal discard (Igbinigie *et al*., 2010; Mukasa-Mugerwa *et al*., 2011). Reports from this study showed that a certain coal-degrading fungus, identified as *Neosartorya fischeri* (ECCN 84) inhabiting in the rhizosphere of *Cynodon dactylon* (Bermuda grass) was involved in the biological conversion of waste coal to humics or humic-like substances (Igbinigie *et al*., 2008; Rose *et al*., 2010). Further research by Sekhohola *et al*. (2014) showed that colonization of waste coal by *N. fischeri* ECCN 84 was associated with the formation of sclerotia-like structures, a time-dependent decline in weight percentage of elemental carbon and an increase in elemental oxygen, proliferation of hyphal peroxisomes, and increased extracellular laccase activity. This accumulated evidence was used to derive an integrated model to explain interactions in the rhizosphere that transform waste coal to a soil-like humic substance (HS)-rich medium capable of supporting the establishment of healthy vegetation (Sekhohola *et al*., 2013). Confirmation of this outcome was later realized following in situ hectare-scale trials on discard dumps (Cowan e*t al*., 2016) and from controlled pot trial studies (Sekhohola and Cowan, 2017). Thus, the proposal that mutualism between rhizospheric fungi and members of the *Poaceae* and in particular C4 grasses such as *Cynodon dactylon* (Bermuda grass), *Pennisetum clandestinum* (Kikuyu grass), *Eragrostis tef* (Teff grass), and *Zea mays* L. (maize) may together with appropriate saprophytic microbes serve as the catalysts for bioconversion of the polluting substrate. A question that still persists is: what mechanism underpins plant-fungal mutualism to ensure successful plant growth and revegetation in such a harsh environment?

As stated earlier, plant roots release several primary metabolites such as sugars, amino acids, and organic acids that are believed to be passively lost from the root and assimilated by rhizosphere-dwelling microbes. While many studies have attempted to link exudation with soil mobilization, the contribution of exudate and how the process of exudation is controlled and the circumstances that lead to benefit of exudate to plants remain to be elucidated (Canarini *et al*., 2019). As a first step, it seemed pertinent therefore to have a supply of exudate of known origin and composition for (later) use in an appropriate bioassay. For the present work, *Zea mays* L. (maize) and *Abelmoschus esculentus* L. Moench (okra) were selected as model species from which a supply of exudate could be sourced. *Zea mays* L. is an annual, C4 grass, and was selected due to its well-known high extra-polymeric substance (EPS)/mucilage secreting capability, while *Abelmoschus esculentus* L. Moench is unique in that this dicotyledonous

species produces EPS much of which accumulates in the fruits, stems and sometimes on leaf surfaces (Ameena *et al*., 2010; Durazzo *et al*., 2018).

To date, little is known about the contribution of root exudate to the process of biodegradation of soil contaminants and recalcitrant pollutants such as waste coal. Also, due to the difficulty in collecting bulk quantities of plant root exudates, more recently, researchers have devised the use of artificial exudates or cocktails, which is a combination of the most common sugars, organic acids, and amino acids found in root exudates. As an example, Steinauer *et al*. (2016) used artificial exudate or cocktails to test how root exudates enhance soil microbial biomass and properties. In 2017, Lu *et al*. (2017) also studied the effect and mechanisms of root exudates on the rhizoremediation of pyrene-contaminated soil using root exudate cocktail. However, despite the positive output and insight from the aforementioned studies, it is pertinent to note that plant exudates are a complex substance that varies from plant to plant.

This study, therefore, describes the development of an exudate collection protocol and investigates the structure and chemical composition of root exudate from maize and leaf exudate from okra.

3.1.1 Objectives

- To develop a protocol suitable for the cultivation of *Zea mays* L. (Maize) and *Abelmoschus esculentus* L. Moench (Okra) for bulk collection of exudates.
- Physico-chemical characterisation of the scaffold extra-polymeric substance (EPS) material of the exudates.

3.2. Results

3.2.1. Characteristics and microscopic analysis of EPS and EPS-secretory regions

Microscopic examination of the epidermal surfaces of both maize and okra considered to be responsible for exudate secretion are shown in Figure 3.1. Regions containing exudate secretions on the abaxial leaf surface of okra showed that it was pubescent with distinct non-glandular trichomes and presence of stomatal opening (Figure 3.1 A & B). By comparison, the examination of the root tip region of maize revealed some secretory pores and the presence of patches of mucilage attached directly to the surface despite the series of alcohol treatment used for its SEM preparation (Chapter two, section 2.1.2), suggesting the possible involvement of extracellular vesicles in production or release of EPS (Figure 3.1 C & D). Taken together,

results from this analysis show that maize EPS has a slimy texture while the okra EPS appear as a gel-like enveloped mucilage whose content is protected by a lipid-like membrane that is neither soluble in water nor ethanol except when crushed (Figure 3.2).

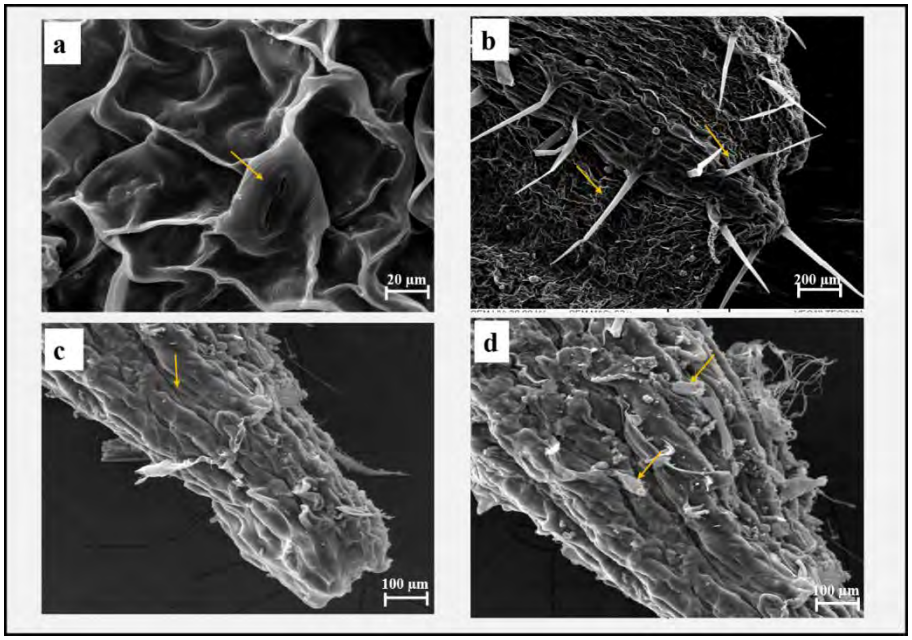


Figure 3.1. SEM of the regions of okra leaf and maize root surfaces associated with the presence of exudate. Tissue samples were prepared from 4 d old seedlings and analysed using SEM. (a) Abaxial leaf surface of okra with an arrow to indicate the location of stomata on the surface. (b) Non-glandular trichomes on the leaf surface of 4 d old seedlings of okra. (c) Root tip of 4 d old maize seedlings showing secretory pores. (d) Cap cells of maize roots from 4 d old seedlings, with arrows indicating patches of mucilage attached to the root surface.

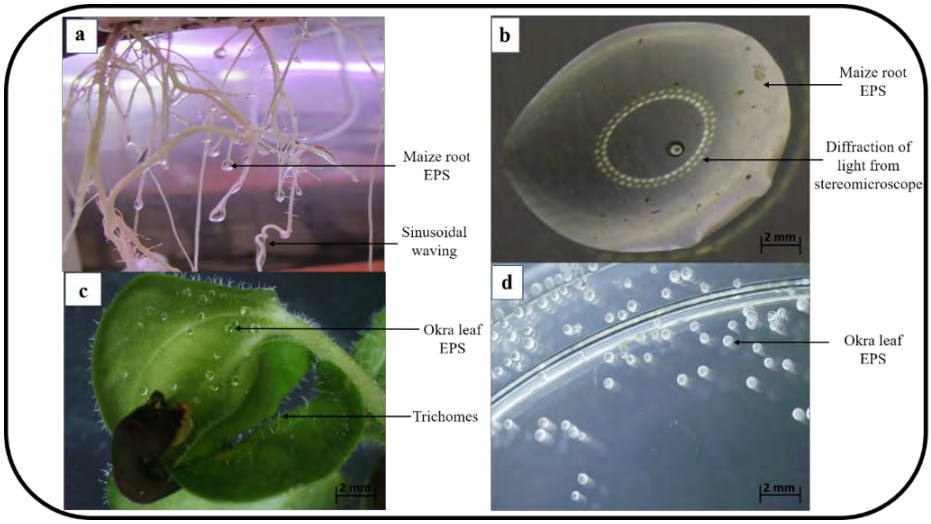


Figure 3.2. Stereomicrograph of the root and leaf EPS and EPS secreting regions (a.) accumulation of slimy EPS on the root tips of the maize plant (b) root EPS as seen on a Petri-dish. (c) accumulation of EPS on leaves of okra (d) leaf EPS as seen on a Petri dish.

3.2.2. Collection of root and leaf EPS-containing exudate

The collection of EPS from the roots and leaves of maize and okra respectively was carried out based on observable EPS accumulation at the root and leaves region, which was made possible with the use of the PoB system (See figure 3.3). On the 5th day after sowing (DAS) (2-leaf stage of the maize seedling), accumulation of EPS was observed at the root tips of the maize root, and 0.4 mL of EPS sample was collected from 40 maize seedlings (Figure 3.3). The root region was monitored daily and on the 10th DAS (3-leaf stage of the maize seedling), more EPS had accumulated at the root tips, and 0.9 mL was collected. Finally, on the 15th DAS (4-leaf stage of the maize seedling), 0.2 mL was collected from the root tips of the 40 maize seedlings and afterwards no secretion of EPS was observed, and seedlings gradually wilted (Figure 3.3). For the okra leaf EPS, accumulation of EPS on the abaxial surface of the leaves was first observed and collected on the 10th DAS (2-leaf stage of the okra seedling). A total of 480 mg EPS was collected from 40 okra seedlings, after which the next secretion was observed on the 15th DAS (4-leaf stage of the okra seedling) and 360 mg EPS was collected. Lastly, at 30th DAS (6-leaf stage of the okra seedling), the final secretion of EPS was observed, and 420 mg EPS was collected (Figure 3.3), after this, there was no visible secretion of EPS on the leaves.

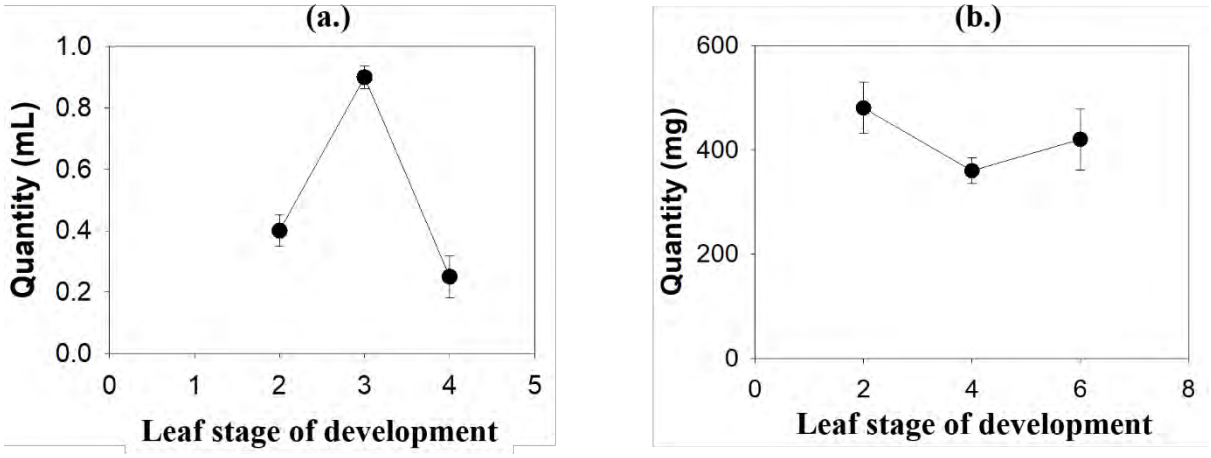


Figure 3.3. Analysis of root and leaf EPS collected from 40 maize and okra seedlings respectively at three different growth stages. (a) Maize EPS (mL) at 2-, 3-, and 4- leaf stages of development. (b) Okra EPS (mg) at 2-, 4- and 6- leaf stages. (Values and error bars are Mean ± SE of triplicates samples).

3.2.3. Bulk density of EPS

The results from the bulk density analysis showed that the maize EPS has a higher density (0.88 g/cm^3), compared to the okra EPS which was 0.16 g/cm^3 (Table 3.1).

3.2.4. pH of EPS

The results from the pH analysis showed that both the maize and okra EPS have a slightly acidic to neutral pH of 6.99 and 6.97 respectively (Table 3.1), which implies that they can also provide a more habitable environment for coal degrading fungi by secreting EPS into a naturally harsh and acidic coal environment.

Table 3.1. Characteristics of maize and Okra EPS

Samples	EPS Character	EPS origin	Bulk density	pH
Maize EPS	Slimy texture	Root tip	0.88 g/cm^3	6.99
Okra EPS	Gel-like texture	Abaxial leaf surface	0.16 g/cm^3	6.97

3.2.5. FT-IR spectroscopy of maize root and okra leaf EPS

FTIR spectra of both exudates are shown in Figure 3.4 A and B. The interpretation of FT-IR absorption frequencies was carried out using an online IR Wizard 2019^{beta} platform (St. Thomas, 2019), and Infrared and Raman characteristic group frequencies: tables and charts (Socrates, 2004). The FT-IR spectra of both the maize and okra EPS revealed a broad absorption peak at wave number 3366 $cm^{-1}$and 3372 cm^{-1}respectively, corresponding to carboxylic acids, which are due to the O-H stretching vibrations (Mohan, 2004) (Figure 3.4, Table 3.2). While the absorption peaks present at 1461 cm^{-1} of both spectra represent the region of C-H alkanes (Maobe and Nyarango, 2013). There were also noticeable peaks at wavenumber 842 cm^{-1} and 844 cm^{-}, which are due to stretching of C-H in the aromatic region of the spectra. The okra EPS spectrum showed further visible peaks of C-H in alkane at 2919 cm^{-1}, 2847 cm^{-1} and C-H in aromatics at 1973 cm^{-1}, with the former due to medium stretching and the latter due to weak stretching (Figure 3.4, Table 3.2). The peaks at wavenumber 1734 cm^{-1} and 1242 cm^{-1} on the okra EPS spectrum indicated the presence of C=O (ester group) and P=O (phosphate group) respectively, which are the closely related to the functional groups of lipids (Yokota *et al*., 2012; Lu *et al*., 2017). The presence of amines was also revealed by both spectra as medium stretching of N-H and C-N of amines at 1628 cm^{-1} and 1040 cm^{-1} respectively (Figure 3.4, Table 3.2), which are characteristic functional groups of proteins (Barth, 2007; Jonker *et al*., 2012). The C=C peaks observed at 1500 cm^{-1} and 1504 cm^{-1} in both EPS spectra is due to symmetrical stretching of aromatic compounds (Fan *et al*., 2012), while the peaks on the wavelengths 2160 cm^{-1} and 2029 cm^{-1} of the okra EPS spectrum corresponds to C≡C in alkynes (Zhou *et al*., 2019) (Table 3.2).

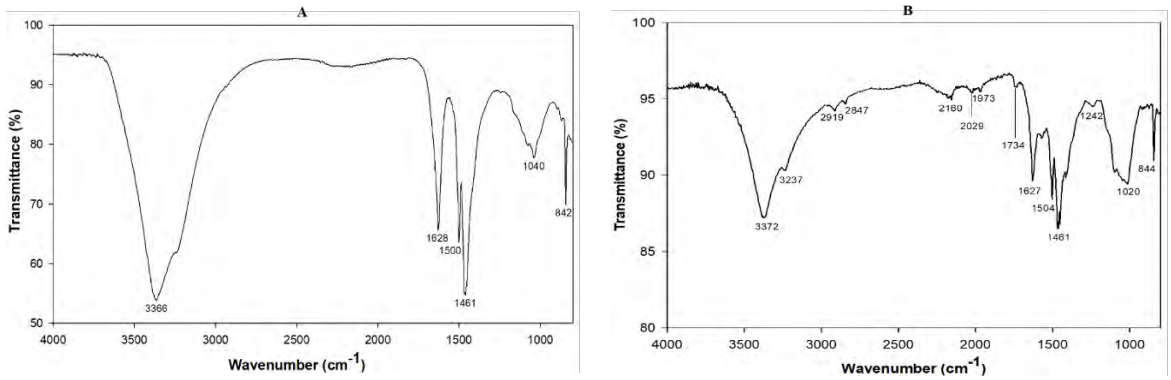


Figure 3.4. FT-IR spectra of exudates (A) Maize EPS spectrum (B) Okra EPS spectrum.

Table 3.2. FTIR absorption bands and assignments for maize and okra EPS (Socrates, 2004; St. Thomas, 2019).

Maize EPS wavenumber/frequency	Okra EPS wavenumber/frequency	Frequency (cm^{-1})	Assignments
3366	3372 and 3237	3550 – 3200	O-H stretching (carboxylic acids)
–	2919 and 2847	3000 – 2840	C-H medium stretching (alkane)
–	2160 and 2029	2250 –2000	C≡C (alkyne)
–	1973	2000 – 1650	C-H weak (aromatics)
–	1734	1740 - 1730	C=O (ester group)
1628	1627	1650 – 1580	N-H medium (amine)
1500	1504	1550 – 1500	C=C symmetrical stretching (aromatics)

1461	1461	1461 – 1450	C-H (alkane)
–	1242	1280 – 1200	P=O (phosphate group)
1040	1020	1170 – 950	C-N medium Stretching(amines)
842	844	900 – 600	C-H stretching (aromatics)

3.2.6. Biochemical composition of maize and okra EPS

The biochemical composition of the maize and okra EPS elucidates the result revealed by the FT-IR and elemental analysis above. The maize EPS contained 88.24 ± 24.08 µg/mL of carbohydrate, 40.23 ± 17.27 µg/mL of protein, 16.15 ± 1.15 µg/mL of lipid and 1.12 ± 0.01 µg/mL of α-amino N, while okra EPS contain 404.61 ± 59.06 µg/mL of carbohydrate, 50.76 ± 8.11 µg/mL of protein, 35.92 ± 0.68 µg/mL of lipid and 21.50 ± 0.42 µg/mL of α-amino N (Table 3.4). Overall, it appeared that while the okra EPS is richer in carbohydrate and protein, the maize EPS contains more of the phenolics and indole/indole-like compounds, which are very essential signalling compounds responsible for the initiation of biological interactions in the rhizosphere.

Table 3.3. Biochemical composition of maize and okra EPS

Samples	Carbohydrate (µg/mL)	Protein (µg/mL)	Lipid (µg/mL)	α- Amino N (µg/mL)	Total Phenolics (µg/mL)	Indole/ indole-like compounds (µg/mL)
Maize EPS	88.24 ± 24.08	40.23 ± 17.27	16.15 ± 1.15	1.12 ± 0.01	0.91 ± 0.19	0.77 ± 0.05
Okra EPS	404.61 ± 59.06	50.76 ± 8.11	35.92 ± 0.68	21.50 ± 0.42	0.61 ± 0.08	0.15 ± 0.02

Data are presented as the mean ± SE of three analyses.

3.2.7. Elemental analysis of exudate from maize roots and okra leaves

The results from the elemental analysis study (Table 3.4) showed that the isolated EPS had, in descending order, 24.17 % carbon of EPS, 1.32 % nitrogen of EPS, 1.01 % sulphur of EPS and 0.29 % hydrogen of EPS, for the maize EPS, while for the okra EPS, 35.16 % carbon of EPS, 4.32 % nitrogen of EPS, 2.78 % sulphur of EPS and 0.09 % hydrogen of EPS was recorded. This result shows that relative to the nitrogen, sulphur and hydrogen, the EPS of both the maize and okra are both carbon-rich and therefore, corroborates the results of the functional groups reported in the afore-mentioned FT-IR spectra. Also, the carbon-hydrogen ratio also reflects the polymeric status of both EPS samples.

Table 3.4. Elemental analysis of Percentage C, H, N, and S of maize and okra EPS

Samples	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulphur (%)
Maize EPS	24.17	0.29	1.32	1.01
Okra EPS	35.16	0.09	4.32	2.78

3.2.8. Summary

- An easy-to-use pot-on-beaker (PoB) protocol was developed that was robust and reproducible and could be adopted for the collection of EPS from EPS-secreting grasses such as *Zea mays* L. for laboratory studies.
- The EPS collected using the PoB method was characteristically visco-elastic with slightly acidic to neutral pH, and composed mainly of carbohydrates, proteins, lipids and alpha-amino acids which shows that they can possibly serve as the scaffold for myco-phytoremediation in contaminated soils.
- This study shows that both maize and okra EPS provide soil organisms, particularly fungi, with accessible carbon from the alkanes, alkenes, alkynes as an alternative to the poly-aromatics which are naturally locked-up in a recalcitrant coal material. This will, in turn, facilitate metabolic processes by the fungi and other soil organisms.
- EPS samples contained low amounts of phenolics and indole or indole-like compounds which are considered to be important secondary metabolites for root growth and signalling.

Chapter Four: Molecular identification of coal-degrading fungi and effect of maize root exudate on ligninolytic enzyme activity

4.1. Introduction

Coal is made up of lignin-like polymers, presumably derived from plant origin, which was believed to be recalcitrant to degradation. However, in recent years it has been discovered that many fungi and bacteria produce a variety of extracellular oxidoreductase enzymes that can effectively biodegrade coal (Breen and Singleton, 1999; Singh and Kumar, 2010; Bugg *et al*., 2011; Huang *et al*., 2013; Li *et al*., 2020). Indeed, initial studies using fungi isolated from the rhizosphere of grasses growing on coal discard dumps in South Africa resulted in the isolation and identification of the "Deuteromycete", *Neosartorya fischeri* (Igbinigie *et al*., 2008). In this study, Igbinigie *et al*. (2008) simulated a coal dump environment with a perfusion fixed-bed bioreactor and was able to show that *N. fischeri* was capable of degrading waste coal. Further study revealed some detail of a possible mutualistic interaction between plant roots of the C4 grass *Cynodon dactylon* and the saprophytic fungi, *Aspergillus* I (ECCN 84), *Aspergillus* II (ECCN 98), *Ulocladium* (ECCN 88), *Alternaria* (ECCN 105), and *Penicillium* (ECCN 115) species (Igbinigie *et al*., 2010). Results demonstrated enhanced bioconversion of coal facilitated by low molecular weight organics and initiated by the introduction of nitrogen moieties. The authors claimed that this supported phyto-bioconversion of coal and the resultant promotion of growth of *C. dactylon*. Further exploration seemingly revealed an increase in coal biodegradation and production of humic acid as a consequence of *Cynodon dactylon* growth in association with arbuscular mycorrhizal fungi and *Neosartorya fischeri* (Mukasa-Mugerwa *et al*., 2011). Indeed, it was this interaction between *Cynodon dactylon*, arbuscular mycorrhizal fungi, *Neosartorya fischeri* and other coal-degrading rhizosphere fungi coupled to apparent in situ degradation of coal that was proposed to underpin the application of these organisms to discard dumps as a novel method of coal dump rehabilitation (Rose *et al*., 2010). Other fungi that have also been widely reported to depolymerise and mineralise the lignin-like components of coal include fungi such as *Trichoderma atroviride, Penicillium* spp., and white-rot fungus *Phanerochaete chrysosporium* currently known as *Phanerodontia chrysosporium* and many of these studies predate the above observations (Ralph and Catcheside, 1994; Blanchette, 1995; Silva-Stenico *et al*., 2007; Oboirien *et al*., 2008). Even so, the precise mechanisms utilised by these fungi in the bioconversion/degradation of coal remained obscure until the publication of

details of fungal colonisation and biodegradation (Sekhohola *et al*., 2014). This study revealed that fungal colonization of coal was associated with the formation of sclerotia-like structures, a time-dependent decline in weight percentage of elemental carbon, an increase in elemental oxygen, proliferation of peroxisomes in hyphae attached to coal particles and an increase in extracellular laccase activity. Support for this as a major mechanism in fungal degradation of coal has been confirmed and the major candidate enzymes seem to be laccase. Although, other enzymes such as lignin peroxidase (LiP, E.C. 1.11.1.14) and Mn-dependent peroxidase (MnP, E.C. 1.11.1.13) have also been reported to contribute to the possible biodegradation of coal (Fakoussa and Hofrichter, 1999; Igbinigie *et al*., 2010; Sekhohola *et al*., 2014), laccase remain the leading enzyme responsible for the biodegradation of coal. Further studies have also shown that LAC, LiP and MnP enzymes can either act individually or synergistically with one another to affect the degradation of lignin, hard coal, discard coal, xenobiotics, and humification (Thurston, 1994; Bourbonnais *et al*., 1995; Youn *et al*., 1995; Igbinigie *et al*., 2008; Mot *et al*., 2012; Sekhohola *et al*., 2013). Recently, a heterologous expression of *Fusarium oxysporum* laccase in *Pichia pastoris* was shown to depolymerise and liquefy solubilised brown coal with the release of humic and fulvic acids (Kwaitos *et al*., 2018; Kwaitos *et al*., 2020). In other studies, lignite depolymerization by *Penicillium decumbens* strain P6 increased the content of humic acids (HAs) and water-soluble humic substances (HS) while the molecular mass of HA decreased (Dong *et al*., 2006). In this example, depolymerization is believed to be a consequence of an esterase (Yang *et al*., 2018). Much of the work outlined above, made use of a fungal strain termed ECCN 84 as the biocatalyst. While no details or description of source site were provided, ECCN 84 originated from the Hope Colliery, Witbank, South Africa (Chapter two, section 2.2.0) and was identified by PCR as *Neosartorya fischeri* (Igbinigie *et al*., 2008). Oddly, the deposit of the sequences of this important coal-degrading strain was seemingly never carried out. It is important to note that the taxonomic designation for *Neosartorya fischeri* is: Kingdom *Fungi*, Sub-kingdom *Dikarya*, Division *Ascomycota* (ascomycetes or sac fungi), Subdivision *Pezizomycotina*, Class *Eurotiomycetes*, Subclass *Eurotiomycetidae*, Order *Eurotiales*, Family *Trichocomaceae*, Genus *Neosartorya*, Species *Neosartorya fischeri*. So why did the original authors refer to this fungus as a member of the "Deuteromycetes" and not "Ascomycetes"?

Another, interesting observation from most of the above-mentioned studies is that while many studies have focused on the potential and contribution of rhizospheric fungi to degrade coal, not much attention has been given to the contribution of the associated plant(s). This is

surprising given that it is the rhizosphere in which most of these alleged coal-degrading fungi have been found. A study by Cowan *et al.* (2016), reported that successful vegetation facilitates the biodegradation of carbonaceous material such as discard coal, and suggested that to achieve this, metabolically active catalysts need to be brought in contact with the coal substrates. Materials such as low-rank coal (Klein *et al*., 2014), discard coal (Sekhohola *et al*., 2013) and weathered coal (Cowan *et al*., 2016) are examples of substrates that have been reported and adopted in many studies. Interestingly, several studies have also hypothesized that root exudates from certain plants could serve as a biocatalyst for an efficient fungal enzymatic activity and overall biodegradation of coal (Burns *et al*., 2013; Oboirien *et al*., 2013; Sekhohola *et al*., 2013). Plants, particularly C4 plants, release a wide variety of substances (exudates) through their roots, into their rhizosphere which serves as signalling compounds, a carbon source, or an auxiliary substrate to enhance enzymatic activity and productivity in the rhizosphere (Pilon-Smits, 2005). In lieu of this, the contribution of root exudates to the stimulation of the enzymatic activity of fungi needs further elucidation.

4.2 Objectives

- The objective of this study was to characterize the three coal-degrading fungal isolates using standard molecular procedures, generate the respective accession numbers, and successfully deposit these in GenBank for easy identification and accessibility.
- Investigate the effects of root exudate from maize seedlings on the ligninolytic enzyme activities of the fungal isolates.

4.3 Results

4.3.1 Morphological characteristics of fungal isolates

In order to determine the morphological characteristics of the three fungal strains, organisms were cultured in PDA-glutamate agar and incubated at 28°C for 7 d and the results are presented in Figure 4.1. For ECCN 84, colonies appeared plain on agar plates, sclerotia appeared white on the obverse and reverse of the plates; the texture is velutinous, and no form of exudate was seen (Figure 4.1 a & b). The same characteristic was seen for ECCN 225 except that the mycelia and sclerotia were not as white as that of the former (Figure 4.1 c & d). For the morphological characteristics of ECCN 243; Colonies were nearly circular or irregular, sporulation dense; sclerotia greyish green obverse and cream on the reverse; exudates absent

(Figure 4.1 e & f). The SEM results also showed the presence of conidia in all the fungal samples analysed as seen in Figure 4.1 g-i.

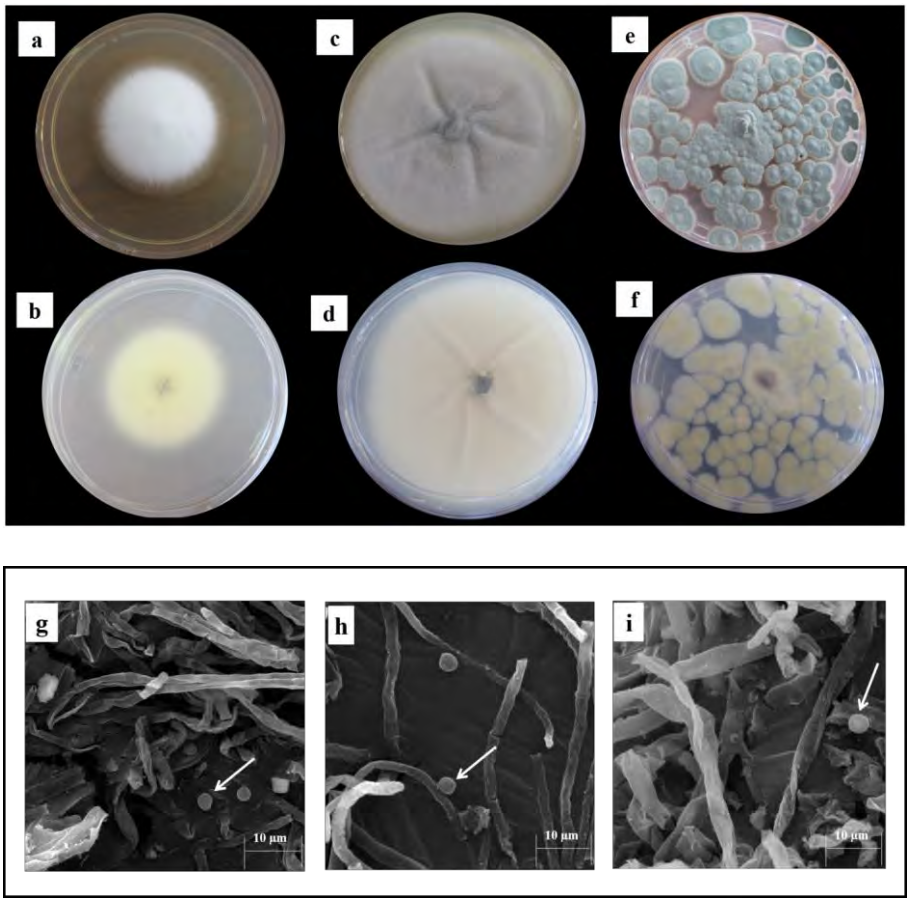


Figure 4.1. Morphological characteristics of fungal strains. Obverse and reverse of ECCN 84 (a & b), ECCN 225 (c & d), and ECCN 243 (e & f) grown on PDA-glutamate agar at 28°C for 7 d. SEM micrographs showing the conidia of the respective isolates (g-i).

4.3.2. Taxonomic identification and phylogenetic analysis of isolates

Polymerase chain reaction (PCR) amplification of ITS 1 and ITS 4 of the three fungal isolates yielded nucleic acid fragments of approximately 600 bp (Figure 4.2). Thus, to establish the phylogenetic position of each isolate, the 16S rDNA sequences (Appendix IX) were compared with related species isolated mostly from coal mines and soil environments, retrieved from NCBI database (See Table 4.1 and Figure 4.3). The BLASTn results revealed that the Genus *Aspergillus* dominated the ECCN 84 and ECCN 225 samples, while Genus *Penicillium* dominated the ECCN 243 samples, with the former having a percentage identification ranging

from 99.16 – 100 % and the latter from 99.49 – 100 % (Figure 4.3). The phylogenetic analysis revealed that isolates ECCN 84 clustered with *Aspergillus* sp. (KF367532) and *Aspergillus felis* (FR733865) with 100 % percentage identification, and then with *Aspergillus felis* (HE578063), *Neosartorya fischeri* (AB674772) and *Neosartorya spinosa* (AB674770) with percentage 99.83 %, 99.5 % and 99.32 % homology respectively. ECCN 225 clustered with *Aspergillus* sp. (KM051386), *Aspergillus* sp. (MH550493), *A. fumigatus* (JN246062) and Uncultured fungus (KU931404), all with 100 % percentage identification. While others such as *Neosartorya fischeri* (AB369900), *A. oerlinghausenensis* (KT359601) and *A. oerlinghausenensis* (NR_138362) clustered with 99.5 %, 99.16 % and 99.16 % homology respectively. Interestingly, ECCN 84 and ECCN 225 both share a percentage identity of 98.67 % to each other. ECCN 243 clustered with *Penicillium* sp. (KF367505), *Penicillium* sp. (KF367508), *P. crustosum* (MK578185) and *Fungal* sp. (KY607733) all with percentage identification of 100 %. While other related isolates such as *Uncultured penicillium* (KM062087), *P. chrysogenum* (HQ380757) and *P. commune* Strain F (KF028371) clustered with 99.83 %, *P. commune* (MK580820), *P. camemberti* (EU664481) with 99.66 % and *P. discolor* (MG490868), *P. discolor* (MG490869), *P. discolor* (MG490877), *P. discolor* (MG490880) clustered with 99.49 % percentage identification. On this basis, isolates were identified as new 'type' strains and deposited in NCBI database as shown in Table 4.1.

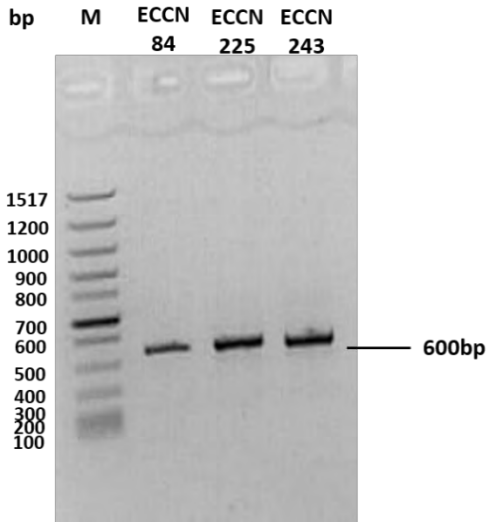


Figure 4.2. Photographic image of an agarose gel indicating the fragments generated by the ITS1-ITS4 PCR for ECCN 84, 225 and 243 respectively.

Table 4.1. Blast prediction and GenBank accession numbers of ECCN 84, 225 and 243 nucleotide sequences.

Fungal isolates	Blast prediction	Accession Number
ECCN 84	*Aspergillus* sp.	MT239561
ECCN 225	*Aspergillus* sp.	MT239564
ECCN 243	*Penicillium* sp.	MT239576

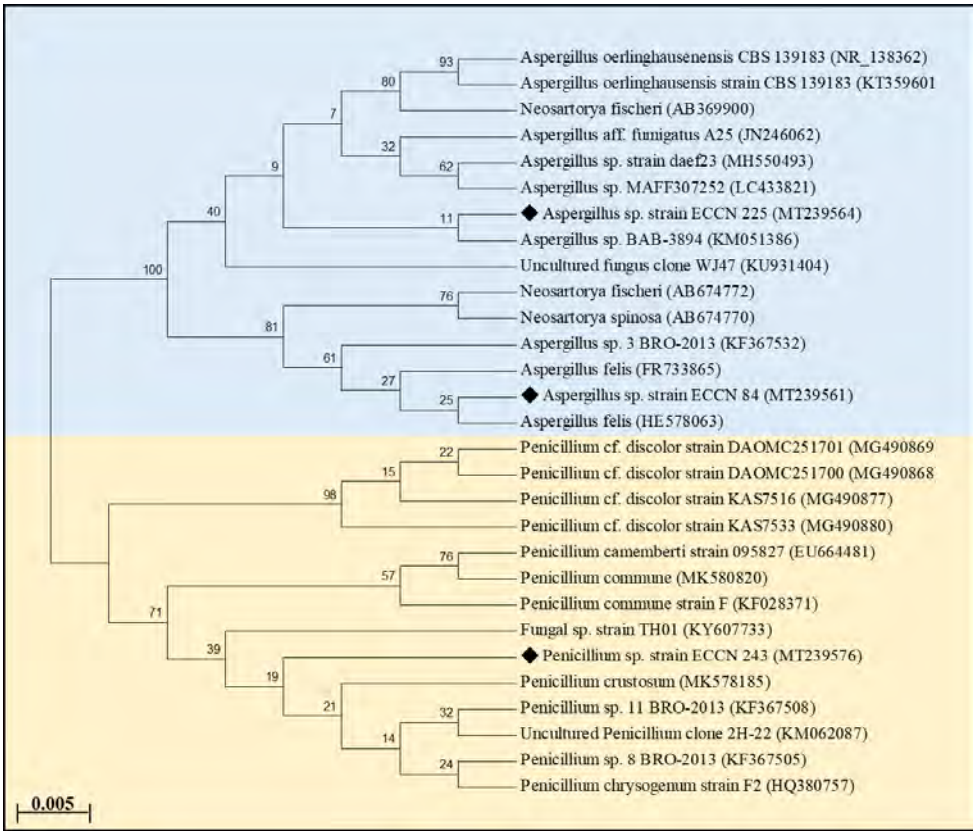


Figure 4.3. Neighbour-joining tree constructed based on the 16S rDNA gene sequencing of *Aspergillus* sp. ECCN 225, *Aspergillus* sp. ECCN 84, and *Penicillium* sp. ECCN 243. The phylogenetic analysis was carried out on MEGA software version 6.0 using 1000 bootstrap.

4.3.2. Production of indole/indole-like compounds in fungal isolates ECCN 84, 225 and 243

The results from the analysis of variance (ANOVA) show that after five days of inoculation, *Aspergillus* sp. strain ECCN 225 produced 2.26 ± 0.58 µg mL^{-1} of indole/indole-like compounds which were the highest produced among the three fungal isolates and statistically different at $P < 0.05$ (Table 4.2). When re-evaluated after 7 days, the indole/indole-like compounds produced in *Aspergillus* sp. strain ECCN 225 had dropped to 1.39 ± 0.12 µg mL^{-1}, while *Aspergillus* sp. strain ECCN 84 and *Penicillium* sp. strain ECCN 243 increased from 0.92 ± 0.11 µg mL^{-1} to 1.49 ± 0.09 µg mL^{-1} and 1.23 ± 0.38 µg mL^{-1} to 1.75 ± 0.21 µg mL^{-1} respectively. However, despite the observed increase in the indole/indole-like compounds produced by *Aspergillus* sp. strain ECCN 84 and *Penicillium* sp. strain ECCN 243 after 7th day of inoculation, there was no apparent significant difference when compared with that produced by *Aspergillus* sp. strain ECCN 225 (Table 4.2).

Table 4.2. Production of indole/indole-like compounds in *Aspergillus* spp. and *Penicillium* sp. after the 5th and 7th days of inoculation.

Treatments	Production of indole/indole-like compounds Mean ± S.E. (95 % C.I.)	
	5-day culture (µg/mL)	7-day culture (µg/mL)
Aspergillus sp. (ECCN 84)	0.92 ± 0.11 (0.43 – 1.40)^{b}	1.49 ± 0.09 (1.10 – 1.88)^{b}
Aspergillus sp. (ECCN 225)	2.26 ± 0.58 (0.81 – 3.71)^{c}	1.39 ± 0.12 (0.89 – 1.89)^{b}
Penicillium sp. (ECCN 243)	1.23 ± 0.38 (0.28 – 2.17)^{b}	1.75 ± 0.21 (0.86 – 2.64)^{b}
Control	0.00 ± 0.00 (0.00 – 0.00)^{a}	0.00 ± 0.00 (0.00 – 0.00)^{a}
F-ratio, p-value	19.84, 0.00	38.56, 0.00

*S.E. = Standard Error; C.I. = Confidence Interval

Mean values with the same superscript across column are not significantly different at 95 % probability levels.

4.3.3. Manganese peroxidase activity

The result of the colorimetric manganese peroxidase (MnP) activity on the three strains of fungi, *Aspergillus* sp. ECCN 84, *Aspergillus* sp. ECCN 225 and *Penicillium* sp. ECCN 243

after 7 d of incubation showed that the two *Aspergillus* strains were able to oxidize Mn^{2+} to Mn^{3+} by the formation/presence of black halo around the fungal colony with or without the substrates EPS or P-coal (Figure 4.4 and 4.5).

On the ECCN 84 plates (Figure 4.4.), not much difference was observed between the EPS and P-coal treatments which were inoculated in point ii and iii, respectively. However, in the ECCN 225 plates (Figure 4.5), the black halo was more intense in the EPS (point ii) and P-coal (point iii) treatments, than the control (point i). Although, the most intense halo was observed in the coal treatment making it a good substrate for MnP oxidation. The *Penicillium* sp. ECCN 243 (Figure 4.6) showed a negative Mn peroxidase activity, as no sign of black halo was evident. Although, it does show some form of decolorization from its natural cream colour to a deep brown colour which shows there may be some other ongoing activities (Figure 4.6).

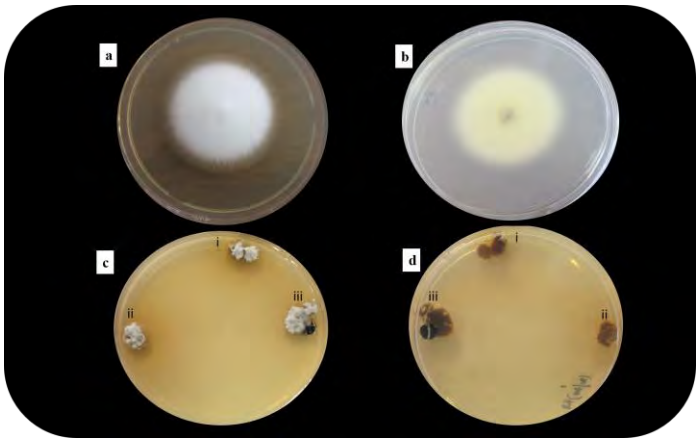


Figure 4.4. 7 d cultures of *Aspergillus* sp. (ECCN 84) showing the formation of black halos (MnO_2) around the fungal colony, indicating the oxidation of Mn^{2+} to Mn^{3+}. (a-b) obverse and reverse side of *Aspergillus* sp. (ECCN 84) on PDA medium without any form of treatment. (c-d) obverse and reverse side of the fungal ECCN 84 on; (i) control (PDA-$MnCl_2$ media (2.5 % PDA + 1 mg/mL $MnCl_2$) (ii) 0.005 mL of EPS + 0.005 mL PDA-$MnCl_2$ media (iii) 0.005 g of P-coal + 0.01mL PDA-$MnCl_2$ media.

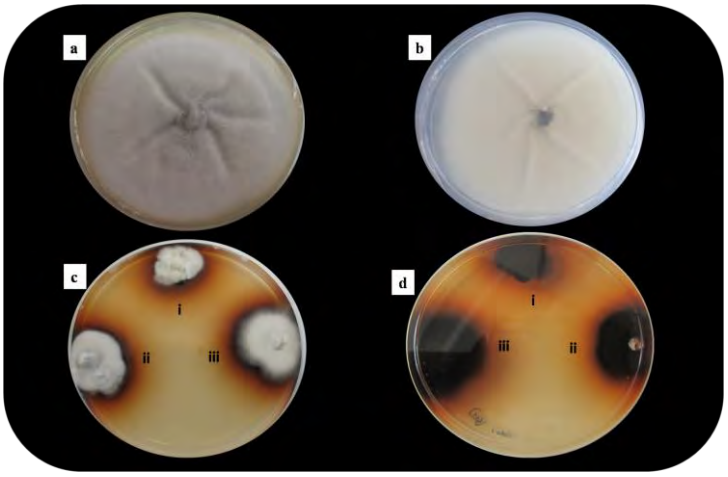


Figure 4.5. **7 d cultures of *Aspergillus* sp. (ECCN 225) showing the formation of black halos (MnO_2) around the fungal colony, indicating the oxidation of Mn^{2+} to Mn^{3+}. (a-b) obverse and reverse side of *Aspergillus* sp. (ECCN 225) on PDA medium. (c-d) obverse and reverse side of the fungal ECCN 225 on; (i) control (PDA-$MnCl_2$ media (2.5 % PDA + 1 mg/mL $MnCl_2$) (ii) 0.005 mL of EPS + 0.005 mL PDA-$MnCl_2$ media (iii) 0.005 g of P-coal + 0.01mL PDA-$MnCl_2$ media.**

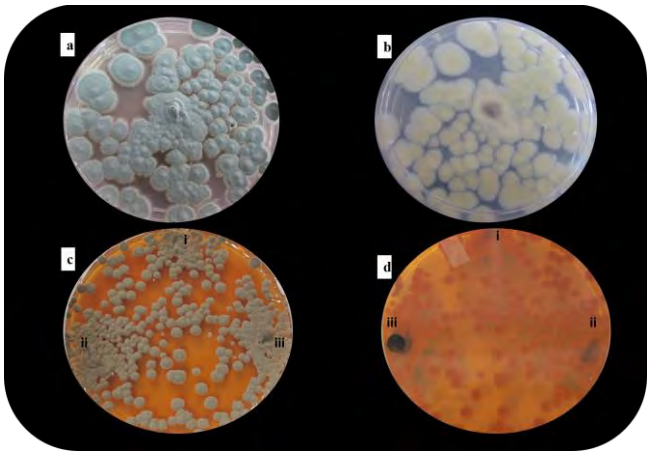


Figure 4.6. **7 d cultures of *Penicillium* sp. (ECCN 243) showing the no visible sign of black halo (MnO_2) around the fungal colony, indicating no oxidation (a-b) obverse and reverse side of the fungal culture on PDA medium. (c-d) obverse and reverse side of the fungal ECCN 243 on; (i) control (PDA-$MnCl_2$ media (2.5 % PDA + 1 mg/mL $MnCl_2$) (ii) 0.005 mL of EPS + 0.005 mL PDA-$MnCl_2$ media (iii) 0.005 g of P-coal + 0.01mL PDA-$MnCl_2$ media.**

4.3.4. ABTS Assay of fungal strains for LAC activity

The absorbance of the ABTS reaction mixture was monitored at intervals (1 min) over 25 min and a graph of the absorbances was plotted against time (Figure 4.7). The result revealed a little or no activity in the three fungi investigated when P-coal was added to the GBSM media, while an increased activity was observed in all the fungal treatment with the addition of EPS (Figure 4.7). Interestingly, all the fungal treatments containing EPS and P-coal also showed improved activities when compared to those with the P-coal treatments only (Figure 4.7). After carefully observing the pattern of the activities displayed by these fungi, their slopes were obtained from the graphs (Appendix X) and multiplied by the molar extinction coefficient of 36.0 mM^{-1} cm^{-1} as described in Chapter two; section 2.3.5., to calculate the LAC activity.

The results from the LAC activity revealed that *Penicillium* sp. strain ECCN 243 showed an extracellular LAC activity of 9 $nmol.min^{-1}mL^{-1}$ with the addition of P-coal (ECCN 243 + P-coal treatment), and 808.2 $nmol.min^{-1}mL^{-1}$ with the addition of EPS (ECCN 243 + EPS treatment), as compared to the control which was negative with -72 $nmol.min^{-1}.mL^{-1}$ (Figure 4.8). However, when EPS was added to the P-coal treatment (ECCN 243 + EPS + P-coal), the LAC activity of fungus *Penicillium* sp. strain ECCN 243 increased from 9 $nmol.min^{-1}.mL^{-1}$ to 415.8 $nmol.min^{-1}.mL^{-1}$ (Figure 4.8). On the other hand, *Aspergillus* sp. strain ECCN 225 elicited an extracellular LAC activity of 18 $nmol.min^{-1}mL^{-1}$ with the addition of P-coal (ECCN 225 + P-coal), and 302.4 $nmol.min^{-1}.mL^{-1}$ with the addition of EPS (ECCN 225 + EPS). However, the addition of EPS to P-coal (ECCN 225 + EPS + P-coal) showed an improved LAC activity of 496.8 $nmol.min^{-1}.mL^{-1}$ which outperformed all other treatments (Figure 4.8). Although *Aspergillus* sp. strain ECCN 84 showed a pattern akin to the previous two fungi, an extracellular LAC activity of 946.8 $nmol.min^{-1}mL^{-1}$ was recorded in the ECCN 84 + EPS treatment, while a negative LAC activity of -3.6 $nmol.min^{-1}.mL^{-1}$ was recorded in the ECCN 84 + P-coal treatment (Figure 4.8). However, a tremendous increase was observed when the EPS was added to the PCS (ECCN 84 + EPS + P-coal), as it showed an extracellular LAC activity of 536.4 $nmol.min^{-1}.mL^{-1}$ (Figure 4.8).

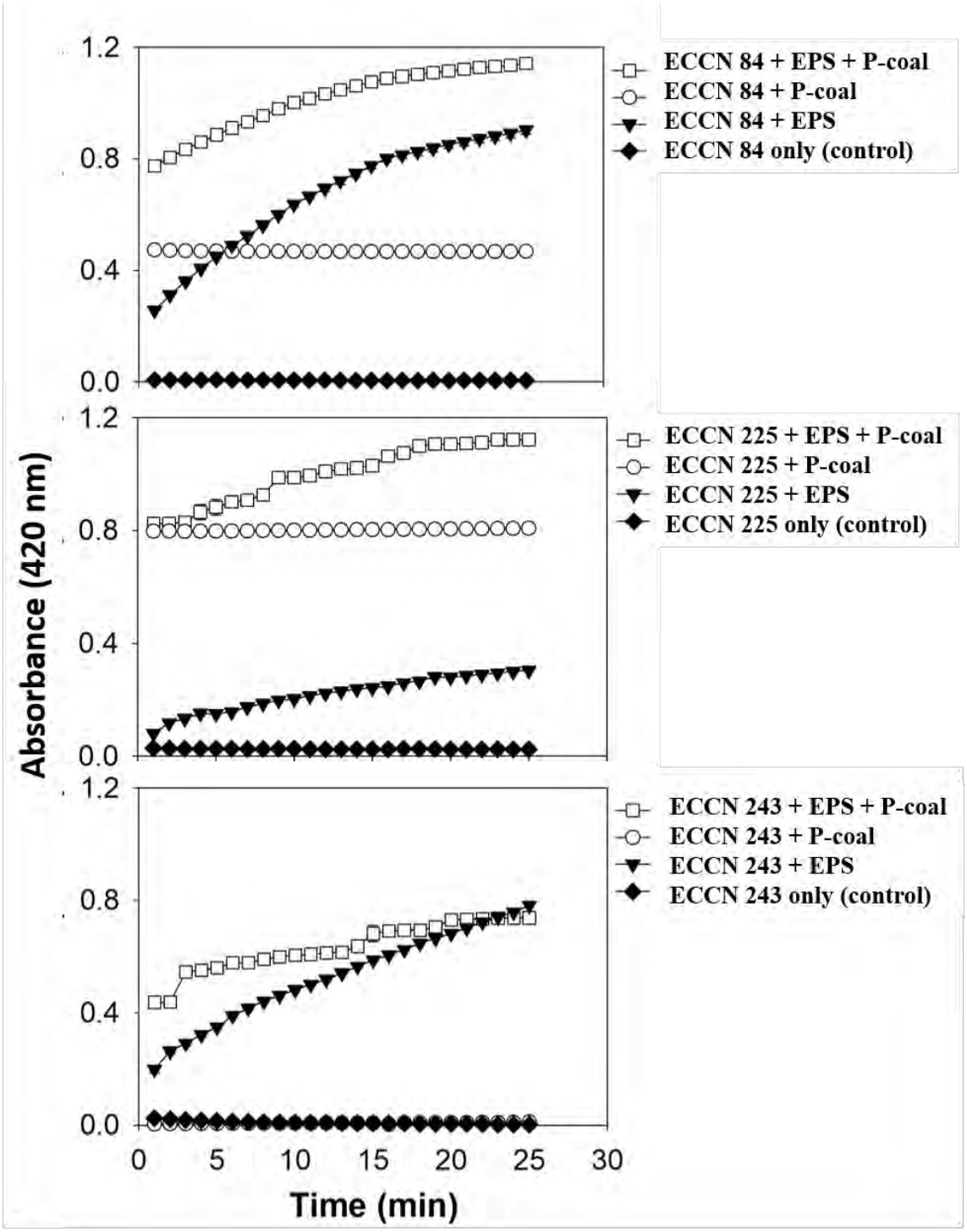


Figure 4.7. Absorbance of the ABTS reaction mixture over 25 min period.

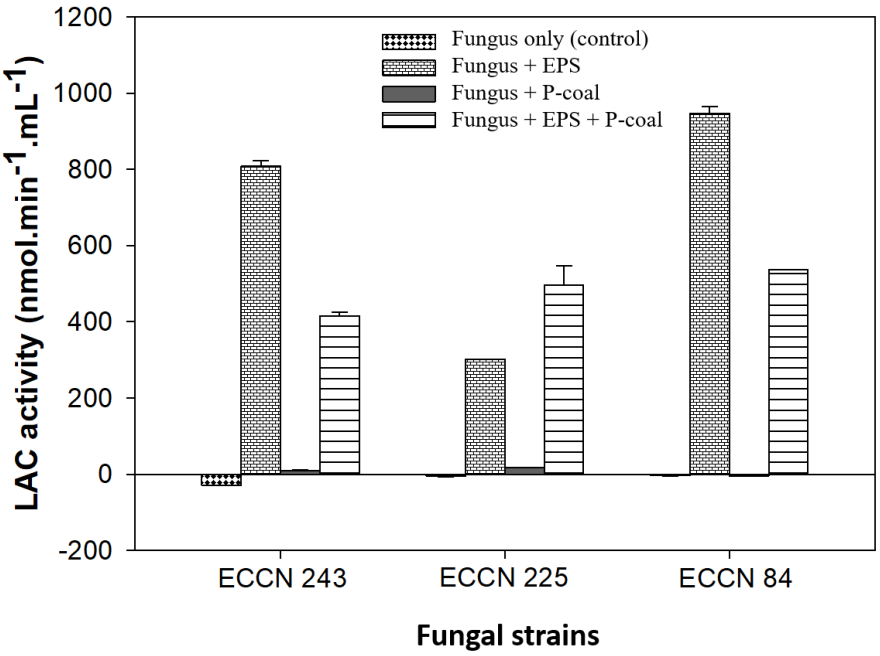


Figure 4.8. Contributory effects of EPS on the extracellular LAC activity of *Aspergillus* spp. (strains ECCN 84 and ECCN 225), and *Penicillium* sp. strain ECCN 243.

4.4. Summary

- The three coal degrading fungi, ECCN 84, 225 and 243, previously isolated from coal samples purportedly collected from *Cynodon dactylon*/coal rhizosphere were identified molecularly as *Aspergillus* sp. strains ECCN 84, *Aspergillus* sp. strain ECCN 225 and *Penicillium* sp. strain ECCN 243 respectively.
- The fungus, strain ECCN 84 was confirmed to belong to the Genus – *Aspergillus* and the BLAST result showed that it has 100 % similarity when compared to related sequences in the NCBI database. This contrasts with an earlier study in which the strain was identified as Genus *Neosartorya* and Species *fischeri*.
- Accession numbers for *Aspergillus* sp. strain ECCN 84 - **MT239561**; *Aspergillus* sp. strain ECCN 225 - **MT239564**; and *Penicillium* sp. strain ECCN 243 - **MT239576** were issued following deposit of the nucleotide sequences in GenBank.
- *Aspergillus* strain ECCN 225, *Aspergillus* strain ECCN 84 and *Penicillium* strain ECCN 243 all produced indoles and/or indole-like compounds.

- *Aspergillus* spp. (strains ECCN 84 and ECCN 225) showed positive MnP activity with or without the EPS or P-coal substrates whereas *Penicillium* sp. strain ECCN 243 showed no MnP activity on the plates after 7d incubation.
- EPS-containing exudates produced from the root tips of maize enhanced LAC activity by all the three fungal strains.

Chapter Five: Assessment of the impacts of plant-fungal activity on the colonization and biodegradation of bituminous coal

5.1 Introduction

The removal of soil pollutants from the environment using natural processes such as vegetation (phytoremediation), fungi (mycoremediation) or bacteria (bactoremediation) is gradually replacing more traditional engineering approaches which are of high cost and low efficiency (Yoshitomi and Shann, 2001; Hussain *et al*., 2009; Cowan *et al*., 2016; Olawale *et al*., 2020; Smolenova *et al*., 2020). It has long been speculated that bioremediation processes could be of huge potential in the biodegradation of complex material such as coal (Schnoor *et al*., 1995; Vidali, 2001). Early work carried out in our laboratory showed, although under defined conditions, that a symbiotic relationship between the fungus ECCN 84 and the roots of *Cynodon dactylon*, resulted in the effective degradation of waste coal and the formation of humic substances (Igbinigie *et al*., 2010; Mukasa-Mugerwa *et al*., 2011; Sekhohola *et al*. 2014). Furthermore, using long-term pot trials, it was shown that biological oxidative degradation of coal discard leads to increased humic-like substance concentration and formation of a soil-like material (Sekhohola and Cowan, 2017). Even so, while most of these studies focused on contributions by fungi to the coal degrading process, contributions from the plant and in particular root exudate have not yet been considered. However, other studies although sparse, indicate that individual plant species contribute substantially to the soil microflora including soil microbial biomass and activity increased abundance of fungal and bacteria-derived fatty acids, and overall soil fertility (Innes *et al*., 2004; Jones *et al*., 2004; Bengough *et al*., 2011; Tan *et al*., 2015; Williams and de Vries, 2019). Thus, it might be anticipated that the combined effect of plant and fungal action would be the establishment of conditions within the rhizosphere that facilitate enhanced biological activity and consequently, degradation or conversion of waste coal into a soil-like material. Indeed, this was the observed outcome in both greenhouse and hectare-scale trials (Cowan *et al*., 2016; Sekhohola and Cowan, 2017). Most recent studies seem to suggest that coal degrading fungi can produce a suite of catalysts that allow for bioconversion/degradation of recalcitrant materials like coal. The major products seem to be a mixture of humic and fulvic acids and other organic compounds. Several processes are thought to contribute products of solubilisation and these include, release or formation of alkali substances, biosurfactants, chelators, and enzymes (Hambrick *et al*., 1980; Ghani *et al*., 2015). Substantial support for the involvement of enzymes was recently obtained following

successful heterologous expression of a *Fusarium oxysporum* laccase in *Pichia pastoris* which was then shown to depolymerise and liquefy solubilised brown coal with the release of humic and fulvic acids (Kwaitos *et al*., 2018). This, coupled with earlier evidence supporting the involvement of an extracellular LAC in coal biosolubilization (Sekhohola *et al*., 2014) and the very tentative but positive response of LAC in the coal degrading strains, ECCN 84, 225 and 243, especially with the addition of maize EPS, (see Chapter 4) indicate their involvement in the biodegradation of waste coal or coal.

To this effect, this study set out to investigate the impact of the *Zea mays*-ECCN 84 interaction on colonisation and biodegradation of discard coal. To achieve this, a rhizobox was constructed to allow for simulation of a 'natural' coal environment within the laboratory. A series of these rhizoboxes containing coal, with or without fungal spores from *Aspergillus* sp. ECCN 84, were seeded with or without *Zea mays* and the change in physicochemical properties of the coal substrate analysed after 16-weeks of cultivation.

5.2 Results

5.2.1 Colonisation of P-coal by fungus *Aspergillus* sp. ECCN 84

Scanning electron microscopy (SEM) was used to determine the extent of colonisation of P-coal substrate by the fungus *Aspergillus* sp. ECCN 84 at the *Zea mays*-coal rhizosphere at 16 weeks after sowing (WAS) and the results are shown in Figure 5.1. From these SEM micrographs, attachment of the fungal hyphae to the surface of the P-coal can be easily seen (see arrows), confirming the proliferation and colonisation of the coal substrate by the fungus *Aspergillus* sp. Strain ECCN 84.

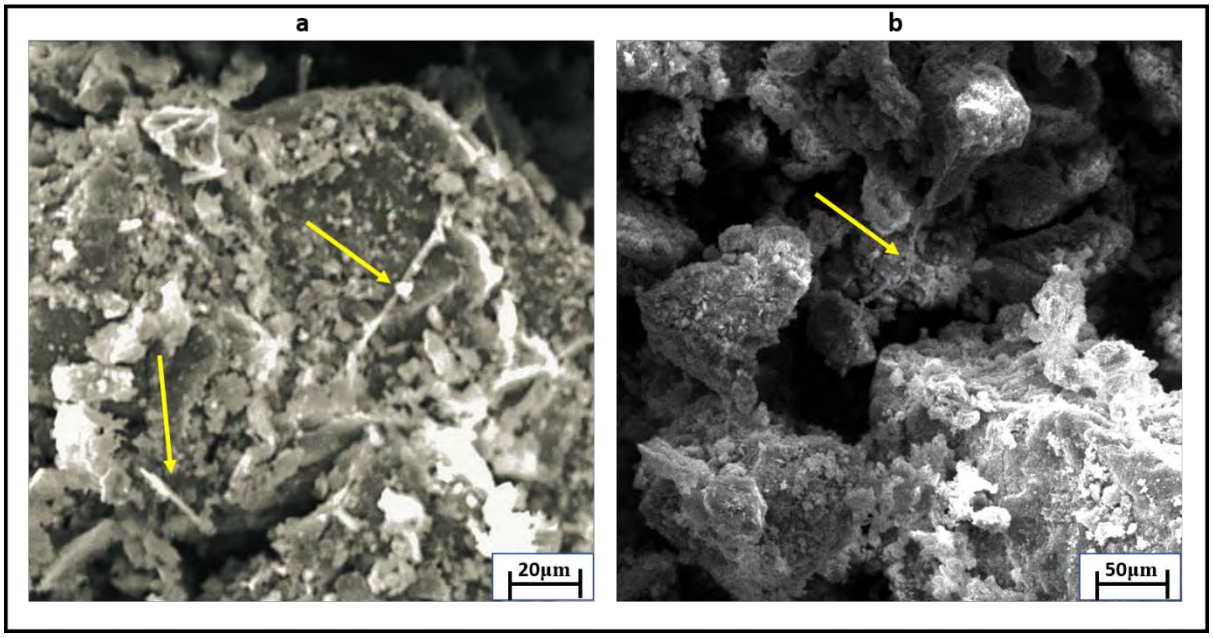


Figure 5.1. SEM micrographs; arrows showing the proliferation and attachment of fungal hyphae to P-coal substrate; (a) P-coal sample collected from MS84 treatment after 16 weeks of sowing (WAS); (b) P-coal sample collected from MOS84 treatment after 16 WAS.

5.2.2 Physicochemical analysis of P-coal substrates at 16 weeks after sowing (WAS)

5.2.2.1. Bulk density

The result from the bulk density analysis revealed that there was not much change in the compactness of the substrate at 16 WAS. There was no significant difference ($p < 0.05$) across the treatments according to the analysis of variance (ANOVA), except for MOS84 treatment which was significantly higher 0.84 ± 0.02 g/cm^3 than both Strain 84 only and the control treatments, 0.72 ± 0.03 g/cm^3 and 0.73 ± 0.03 g/cm^3 respectively (Table 5.1; Appendix XI).

5.2.2.2. pH

Although the pH of all the treatments remained in the acidic range at 16 WAS, there were significant changes in their respective pH values (Appendix XI). All the treatments containing plants such as MOS84, MS84 and maize only, all have a slightly acidic pH of 6.02 ± 0.97, 5.75 ± 0.16, and 5.32 ± 0.33 respectively, which was significantly different from the control and Strain 84 only treatments with high acidic pH values of 3.84 ± 0.08 and 3.34 ± 0.03 (Table 5.1).

5.2.2.3. Electrical Conductivity (EC)

An ascending increase in EC was observed across the treatments; control-Strain 84 only -maize only-MS84-MOS84. However, the result from the statistical analysis showed that MOS84 and MS84 both have EC values of 62.33 ± 98.21 mS/m and 43.67 ± 52.39 mS/m which makes them statistically different from maize only, S and C treatments with EC values of 24.67± 48.07 mS/m, 13.67 ± 21.86 mS/m and 12.00 ± 10.00 mS/m respectively (Table 5.1). It was also observed that the standard error (S.E.) seems to be higher than the mean in all the samples and this could be largely due to the huge variations in values that were used in calculating the mean for each treatment.

Table 5.1. Physicochemical analysis of P-coal substrates after 16 WAS.

Treatments	Mean ± S.E. (95 % C.I.)		
	pH	**EC (mS/m)**	**Bulk density (g/cm^3)**
MOS84	6.02 ± 0.97 (5.60 – 6.44) [a]	62.33 ± 98.21 (200.79 – 1045.88) [a]	0.84 ± 0.02 (0.77 – 0.91) [a]
MS84	5.75 ± 0.16 (5.08 – 6.42) [ab]	43.67 ± 52.39 (211.26 – 662.07) [b]	0.81 ± 0.04 (0.63 – 0.10) [ab]
Maize only	5.32 ± 0.33 (3.91 – 6.73) [b]	24.67± 48.07 (39.82 – 453.51) [c]	0.80 ± 0.02 (0.70 – 0.90) [ab]
Strain 84 only	3.34 ± 0.03 (3.21 – 3.48) [c]	13.67 ± 21.86 (42.62 – 230.71) [c]	0.72 ± 0.03 (0.61 – 0.84) [b]
Control	3.84 ± 0.08 (3.52 – 4.16) [c]	12.00 ± 10.00 (76.97 – 163.03) [c]	0.73 ± 0.03 (0.60 – 0.86) [b]
F-ratio, p-value	48.38, 0.00	15.08, 0.00	3.13, 0.65

*S.E. = Standard Error; C.I. = Confidence Interval; E.C. = Electrical Conductivity

Mean values with the same superscript across column are not significantly different at 95 % probability levels.

5.2.3. Proximate analysis

The proximate analysis was carried out based on the moisture, ash content, volatile matter and fixed carbon (percentage) of all the P-coal treatments at 16 WAS. For the moisture content, the lowest was recorded in the MOS84 treatment with 2.67 ± 0.33 %, while there was not much difference between the percentage moisture of MS84 and maize only treatment, as both recorded 4.33 ± 0.88 % and 4.67 ± 1.20 % respectively. The highest moisture contents were found in the Strain 84 only and control treatments, both with 6.00 ± 1.53 % and 5.67 ± 1.20 % respectively. There were not many changes in the ash content and volatile matter of all the treatments, but there were notable changes in the fixed carbon content as all the treatments had higher fixed carbon than the control (7.67 ± 1.20 %). The highest percentage for fixed carbon was recorded in the MS84 treatment (12.33 ± 1.20 %), closely followed by Strain 84 only treatment (10.33 ± 2.85 %), maize only (9.67 ± 1.33 %) and MOS84 (8.00 ± 1.00 %) (Table 5.2).

5.2.4. Ultimate analysis

The ultimate analysis of the P-coal treatments revealed that there was a decrease in the carbon (C) content of all the treatments when compared with the control. The C content which was about 44.35 ± 3.44 % in the control treatment, reduced after 16 weeks of planting to 41.54 ± 2.33 % in MOS84, 37.72 ± 1.43 % in maize only, 33.56 ± 5.65 % in MS84 and 32.39 ± 7.23 % in Strain 84 only. On the other hand, an increase was observed in the oxygen content (O) of the P-coal across the treatments, with the MS84 treatment recording the highest at 56.58 ± 7.31 % followed closely by maize only with 56.01 ± 2.89 %, and Strain 84 only with 54.38 ± 8.12 %, as compared to the control which recorded a lower O content with 53.03 ± 3.65 % (Table 5.3). Only MOS84 treatment recorded a lower O content than the control which may be due to the high level of competition for minerals and organic acids among the fungus, *Zea mays* L. and okra plant in the rhizobox. There were not many changes in the % H and % N of all the treatments as they all recorded < 1 and ~ 1 respectively (Table 5.3).

Table 5.2. Proximate analysis of P-coal substrates at 16 weeks after sowing (WAS)

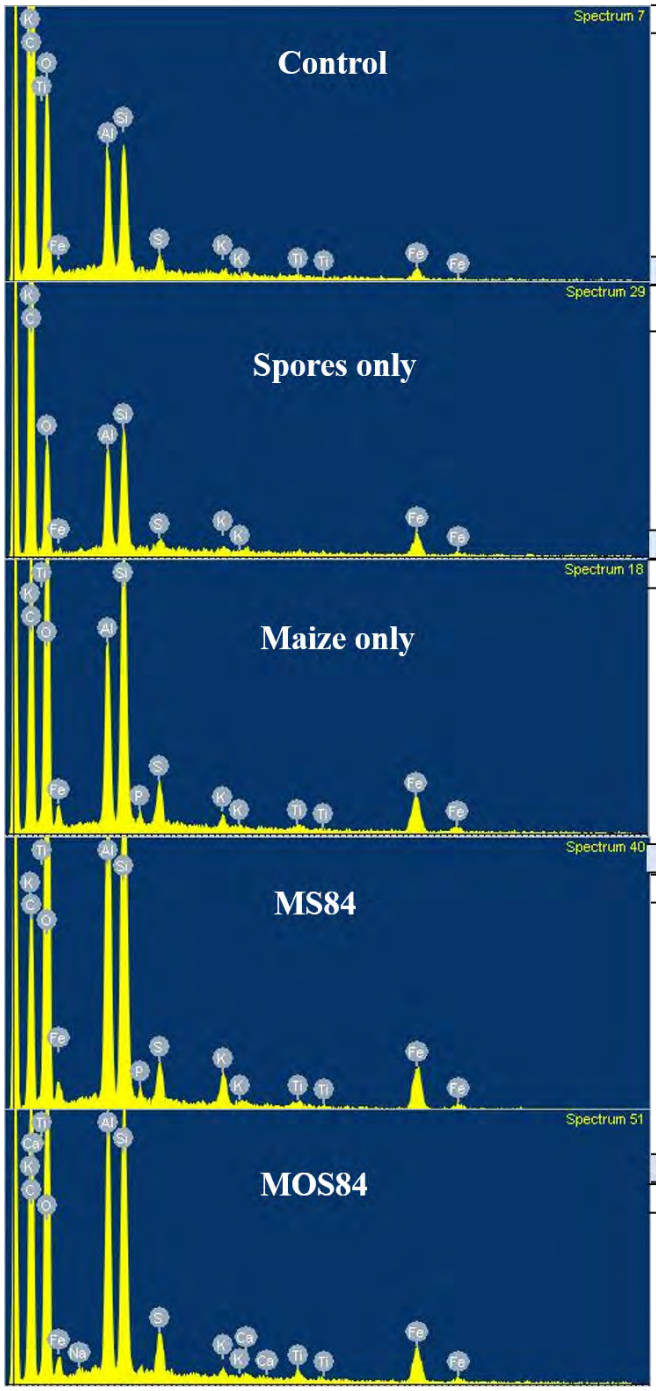
Treatment	Moisture (%)	Ash Content (%)	Volatile Matter (%)	Fixed Carbon (%)
MOS84	2.67 ± 0.33	43.00 ± 1.00	46.33 ± 0.33	8.00 ± 1.00
MS84	4.33 ± 0.88	40.00 ± 1.00	43.33 ± 0.88	12.33 ± 1.20
Maize only	4.67 ± 12.0	40.67 ± 0.88	45.00 ± 1.53	9.67 ± 1.33
Strain 84 only	6.00 ± 1.53	39.00 ± 2.08	44.67 ± 2.40	10.33 ± 2.85
Control	5.67 ± 1.20	39.67 ± 0.88	47.00 ± 1.53	7.67 ± 1.20

Table 5.3. Ultimate analysis of P-coal substrates at 16 weeks after sowing (WAS)

Treatment	C (%)	H (%)	N (%)	S (%)	O (%)
MOS84	41.54 ± 2.33	0.32 ± 0.19	1.09 ± 0.02	8.39 ± 2.07	48.65 ± 3.73
MS84	33.56 ± 5.65	0.28 ± 0.15	1.07 ± 0.03	8.50 ± 1.91	56.58 ± 7.31
Maize only	37.72 ± 1.43	0.74 ± 0.10	1.23 ± 0.06	4.29 ± 1.49	56.01 ± 2.89
Strain 84 only	32.39 ± 7.23	0.43 ± 0.10	1.12 ± 0.07	11.67 ± 0.86	54.38 ± 8.12
Control	44.35 ± 3.44	0.78 ± 0.18	1.29 ± 0.08	0.55 ± 0.05	53.03 ± 3.65

5.2.5 SEM-EDX Analysis

Energy dispersive spectroscopy (EDS) of the P-coal treatments at 16 WAS was carried out and the result was compared to the control (P-coal without treatment). The result revealed a gradual increase in the carbon to- oxygen- ratio (C:O) from the control which is composed of (71.79 %C; 22.94 %O), to treatment Strain 84 only (64.94 %C; 24.18 %O), maize only (42.45 %C; 41.06 %O), and MOS84 (45.94 %C; 38.15 %O) respectively. However, in the MS84 treatment, the oxygen (O) was higher than the carbon (C) with 30.34 %C and 47.43 %O (Figure 5.2). The increase in the oxygen content could be due to the oxidation of P-coal by the oxidoreductase enzymes released because of plant-fungal activities in the rhizosphere. Other elements such as silicon (S), iron (Fe), sulphur (S), potassium (K) and aluminium (Al) were all observed across the treatments but in little proportions (Figure 5.2).



Element	Weight%	Atomic%
C	71.79	78.84
O	22.94	18.91
Al	1.79	0.87
Si	2.09	0.98
S	0.32	0.13
K	0.10	0.03
Ti	0.09	0.02
Fe	0.89	0.21
Total	100.00	

Element	Weight%	Atomic%
C	64.94	74.62
O	24.18	20.86
Al	2.92	1.50
Si	3.97	1.95
S	0.38	0.16
K	0.21	0.07
Fe	3.41	0.84
Total	100.00	

Element	Weight%	Atomic%
C	42.45	53.48
O	41.06	38.83
Al	3.87	2.17
Si	6.56	3.53
P	0.23	0.11
S	1.22	0.58
K	0.37	0.14
Ti	0.17	0.05
Fe	4.08	1.11
Totals	100.00	

Element	Weight%	Atomic%
C	30.34	40.73
O	47.43	47.80
Al	6.85	4.09
Si	9.01	5.17
P	0.17	0.09
S	0.93	0.47
K	0.95	0.39
Ti	0.24	0.08
Fe	4.07	1.18
Totals	100.00	

Element	Weight%	Atomic%
C	45.94	57.01
O	38.15	35.54
Na	0.19	0.12
Al	4.70	2.60
Si	5.82	3.09
S	1.05	0.49
K	0.19	0.07
Ca	0.16	0.06
Ti	0.33	0.10
Fe	3.46	0.92
Total	100.00	

Figure 5.2. Energy-dispersive X-ray (EDX) analysis showing internal elemental distribution in P-coal treatments after 16 weeks of growth of *Zea mays* seedlings.

5.2.6. FT-IR analysis of P-coal after 16 weeks of growth of *Zea mays* seedlings.

In the obtained Fourier transform infrared (FT-IR) spectra of all the treatments, there was an observable similar absorption frequency at 3683 cm $^{-1}$, 3614 cm $^{-1}$, 1592 cm $^{-1}$, 1004 cm $^{-1}$, 910 cm $^{-1}$, 785 cm $^{-1}$, 747 cm $^{-1}$ and 686 cm $^{-1}$ region of the spectra, while some distinct absorption frequencies 1735 cm $^{-1}$, 1363 cm $^{-1}$ and 1214 cm $^{-1}$ were seen on the FT-IR spectra of MS84 and MOS84, which was very similar to some vibrations observed in the commercial humic acid spectrum (Figure 5.3; Table 5.4). In details, there was an observed absorbance peak at 3683 cm $^{-1}$ and 3614 cm $^{-1}$ region on the spectra of all the treatments, corresponding to the functional groups of alcohols, phenols and organic acids which is due to O-H stretching (de la Puente *et al*., 1998; Mohan, 2004). Absorption peaks were also observed at the 1735 cm $^{-1}$ region of the MS84 and MOS84 spectra which corresponds to the aldehyde and esters functional groups and this may be due to the 6- atom rings saturated esters or the small stretching of the C=O (Larboda *et al*., 1999; Yuan *et al*., 2006).

Another absorption peak was observed at the 1591 cm $^{-1}$ region on the spectra of all the treatments, corresponding to the functional group of nitro compounds, and this is largely due to the asymmetrical medium stretching of the N=O bands (Pavia *et al*., 2001; Alvarez *et al*., 2003; Elbeyi *et al*., 2006). A distinct absorption peak was visible at the 1363 cm $^{-1}$ region of the MS84 and MOS84 spectra, corresponding to the S=O functional group, which may imply the presence of sulfoxides, sulfones, sulfonyl, sulfates, or sulfonamides (Yakota *et al*., 2012) (Table 5.4). Furthermore, absorption peaks were noticed at the 1214 cm $^{-1}$ region of the MS84 and MOS84 spectra, corresponding to the functional groups of acyl and phenyl which are due to the medium stretching C-O peak. There was an observed absorbance peak at the 1004 cm $^{-1}$ region on the spectra of all the treatments, corresponding to the functional groups of carboxylic acids, alcohol, ester and anhydride and this is due to the strong stretching of the C-O peaks (Figure 5.3). Finally, absorption peaks were observed at the 910 cm $^{-1}$, 785 cm $^{-1}$, 747 cm $^{-1}$, and 686 cm $^{-1}$ regions of all the treatments which correspond to the functional group of alkenes and this was mainly due to the SP^2 C-H bending patterns (Maobe and Nyarango, 2013) (Figure 5.3; Table 5.4).

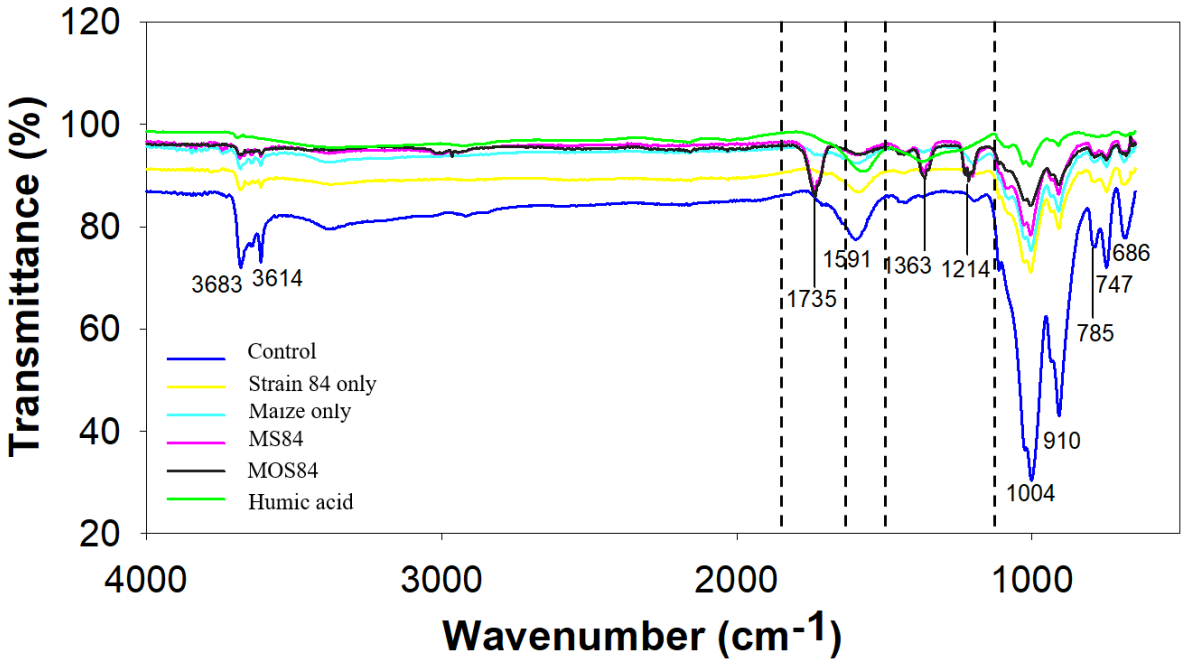


Figure 5.3. FT-IR spectra of P-coal treatments after 16 weeks of planting *Zea mays*.

Table 5.4. The absorption bands of Fourier transform infrared (FT-IR) spectra (Mozgawa *et al*., 2006; Soco *et al*., 2020)

Absorption bands (cm $^{-1}$)	Assignments
3850 – 3600	Stretching (-OH) and (N-H) vibrations
1740 – 1720	Stretching (C=O) aldehyde or esters
1600 – 1500	Asymmetric stretching (N=O) Nitro compounds
1375 – 1300	S=O (sulfoxides, sulfones, sulfonyl, sulfates, sulfonamides)
1350 – 1000	C-N (amines)
1300 – 1000	C-O (alcohol ether, ester, carboxylic acid, anhydride alcohol)
1000 – 650	SP^2 (C-H) alkenes

5.2.7 Humic/Humic-like substances

The quantification of humic/humic-like substances from the P-coal substrates after 16 weeks of planting revealed that maize only treatment recorded the highest amount of humic/humic-like substances with 209.70 ± 23.59 µg/mL, followed by MS84 treatment 166.70 ± 8.46 µg/mL, Strain 84 only with (148.70 ± 16.25 µg/mL) and MOS84 with (139.03 ± 5.63 µg/mL) (Table 5.5). However, even though there was some form of humic/humic-like substances in the aforementioned treatments, statistically, there was no significant difference across the treatments as shown in (Table 5.5; Appendix XII).

5.2.8 Total Phenolics

Results from the analysis of total phenolics in p-coal treatments after 16 WAS revealed that the highest amount of total phenolics was recorded in maize only (18.01 ± 3.20 µg/mL), followed by MS84 (14.87 ± 0.59 µg/mL), MOS84 (14.04 ± 0.91 µg/mL) and Strain 84 only (13.26 ± 0.87 µg/mL). Although all the treatments have higher total phenolic contents than the control (12.55 ± 1.00 µg/mL), there was no significant difference across all the treatments after 16 WAS (Table 5.5; Appendix XIII).

5.2.9 Indole/indole-like compounds

The MS84 and maize only treatments produced the highest indole/indole-like compounds with 1.80 ± 0.16 µg/mL and 1.76 ± 0.18 µg/mL, respectively. However, no significant difference was observed in the indole/indole-like compounds produced when compared with all other treatments (Appendix XIV), as the MOS84, Strain 84 only and control all produced 1.41 ± 0.07 µg/mL, 1.60 ± 0.25 µg/mL, and 1.66 ± 0.14 µg/mL respectively (Table 5.5).

Table 5.5. Analysis of humics, total phenolics and indoles produced from P-coal at 16 WAS of *Zea mays*

Treatments	Mean ± S.E. (95 % C.I.)		
	Indole/indole-like compounds (µg/mL)	**Total Phenolics (µg/mL)**	**Humic/humic-like substances (µg/mL)**
MOS84	1.41 ± 0.07 (1.10 – 1.72) [a]	14.04 ± 0.91 (10.12 – 17.96) [a]	139.03 ± 5.63 (114.81 – 163.26) [b]
MS84	1.80 ± 0.16 (1.11 – 2.49) [a]	14.87 ± 0.59 (12.35 – 17.40) [a]	166.70 ± 8.46 (130.30 – 203.10) [ab]
Maize only	1.76 ± 0.18 (0.99 – 2.53) [a]	18.01 ± 3.20 (4.25 – 31.76) [a]	209.70 ± 23.59 (108.22 – 311.19) [a]
Strain 84 only	1.60 ± 0.25 (0.51 – 2.70) [a]	13.26 ± 0.87 (9.50 – 17.02) [a]	148.70 ± 16.25 (78.78 – 218.62) [b]
Control	1.66 ± 0.14 (1.04 – 2.28) [a]	12.55 ± 1.00 (8.25 – 16.85) [a]	160.87 ± 16.95 (87.92 – 233.82) [ab]
F-ratio, p-value	0.81, 0.55	1.71, 0.22	3.05, 0.07

*S.E.= Standard Error: C.I.= Confidence Interval; WAS = Weeks after sowing

Mean values with the same superscript across column are not significantly different at 95 % probability levels.

5.3 Summary

- *Zea mays* L. seedling growth together with root-produced EPS increased E.C. and alkalinised the coal substrate.
- Proliferation of *Aspergillus* sp. ECCN 84, and colonisation of the coal substrate by this fungus was confirmed.
- Functional groups similar to those found in commercial humics such as amines, carboxylic acids, esters, alkenes, sulphates were observed and confirmed in the P-coal substrates collected from MOS84 and MS84 treatments at 16 WAS.
- Reduction in elemental carbon and a concomitant increase in elemental oxygen in all treatments containing *Zea mays* such as MOS84, MS84 and maize only was observed at 16 WAS.

Chapter Six: General Discussion and Conclusion

6.1. General Discussion

The quest for green technologies for the successful remedy and rehabilitation of contaminated mining environments in South Africa has long been an iterative process. In recent years there has been a shift in paradigm from the conventional, unsustainable, engineering-based remediation approach to a much more phytoremediation-based approach (Salt *et al*., 1998; Cowan *et al*., 2016). In the conventional approach, topsoil is excavated and transported over vast distances to the areas requiring rehabilitation. Although it does vary from mine to mine, a layer of 0.5-1 m of this topsoil is deposited over the waste coal or spoil which is typically treated with lime, the topsoil then seeded with natural grasses and the 'reclaimed' area irrigated. Cladding of discard dumps with perennial vegetation is assumed to bind and stabilise the substrate thereby reducing dust, reducing the ingress of oxygen and percolation of water to minimise erosion and both acidic leachate formation and sediment loss (Truter *et al*., 2009). In reality, it does precisely what the term 'clad' might be expected to do; it covers or clothes the surface to mask the underlying problem. However, rehabilitation strategies must go beyond planting a new landscape by considering the land as an integrated system with function both above and below the ground. Any rehabilitation protocol must therefore include strategies that rebuild soil structure, stimulate soil microbial populations, and re-establish nutrient cycles if this system is to reassemble and become self-sustaining. It is therefore critical that novel, more sustainable and passive rehabilitation processes be developed for use by the mining sector.

Current studies suggest that plant-mycorrhizal associations could be a viable option to help return disturbed coal mined/soil environment to some original state (Broeckling *et al*., 2008; Igbinigie *et al*., 2010; Cowan *et al*., 2016; Akthar and Mannan, 2020). This hypothesis has continued to receive increasing attention in the past few years and an example of a technology that has emerged from this school of thought is the patented FungCoal bioprocess. FungCoal is based on the proposed mutualism that is believed to exist between plants and mycorrhizal fungi, in particular C4 grasses, and considered responsible for bioremediation of coal-contaminated soils. However, one limiting factor of the FungCoal bioprocess is that it is apparently dependent upon an oxidized weathered coal co-substrate, which is a highly complex and variable resource, to supply carbon for growth and proliferation of arbuscular mycorrhizal fungi (AMFs) and the saprophytic coal degrading microorganisms. An apparent omission in

development of the FungCoal biotechnology was that the contribution of root exudates to this bioprocess was either not or only partially considered. Since weathered coal (equivalent in structure and texture to Leonardite, a product of atmospheric oxidation of lignite or brown coal) is a scarce resource in South Africa, it was important to look at another contributing source of carbon that effectively sustains and potentially enhances the growth and activity of the AMFs and coal-degrading microorganisms. A recent review has elaborated the many studies that have shown that gramineous plants secrete heterogenous, monomeric, and polymeric compounds, which enables them to shape their rhizobiomes (Sasse *et al*., 2018). To this effect, the contribution of root exudate to plant-strain 84 interaction and their role in the biodegradation and depolymerization of hydrocarbon-containing pollutants such as coal discard needs to be further elucidated. This study aimed to investigate the proposed relationship between plant roots, root exudate and coal degrading fungi in more detail, and to gain insight into the likely mechanisms underpinning plant-fungal mutualism as a strategy for exploitation in the re-vegetation of coal discard dumps and rehabilitation of coal-contaminated soil. The results confirm that these EPS-rich exudates make a distinct contribution and seem to be key in fungal-mediated biodegradation of waste coal and/or coal discard by triggering the involvement of ligninolytic enzymes such as laccases and peroxidases.

A PoB method was developed for the easy cultivation and collection of exudates from *Zea mays* L. (maize) and *Abelmoschus esculentus* (okra) as a supply for use as scaffold EPS material. In this method, vermiculite was used as the growth medium because it mimics the soil structure to a reasonable extent and creates the needed mechanical impedance suitable for root penetration (Haase *et al*., 2007; Neumann *et al*., 2009; Vranova *et al*., 2013).

Milli-Q water was selected as the exudate collecting medium to avoid confounding and unnecessary interference from salts-containing nutrient solutions and microbes. Milli-Q water is often used for collection of root exudates for specific purposes such as assessing effects on the sporulation and growth of microorganisms (Broeckling *et al*., 2008; Li *et al*., 2009), mycorrhizal establishment (Vierheilig *et al*., 2003), or biodegradation of hydrocarbon in the soil (Yoshitomi and Shann, 2001; Toyama *et al*., 2011; Phillips *et al*., 2012).

First evidence for exudation appeared a few days after emergence of the leaves. The most observable difference between maize and okra EPS was in physical appearance as revealed by stereomicroscopy. Maize root EPS appeared slime-like while okra leaf EPS was bead-like with what appeared to be a protective membrane. In an effort to preserve these structural

characteristics, freeze-drying (FD) was adopted for samples of maize EPS while critical point drying (CPD) was used for samples of okra EPS. The reason for choice of the latter was because CPD preserves surface structures that might otherwise be ruptured due to breakage of surface tension during the sublimation process, and also to avoid leakage of any encapsulated materials (Ingvarsson *et al*., 2011). This is further supported by previous studies by Nordestgaard and Rostgaard (1985) who compared CPD and FD methods as preparative procedures for scanning electron microscopy of isolated hepatocytes cells. The study reported that micrographs of freeze dried, isolated hepatocytes cells showed a ruptured ultrastructure and surface structure caused by internal and external ice crystals respectively, while micrographs of the critical-point dried, isolated hepatocytes cells showed an intact, well-preserved ultrastructure and surface structure. This is in line with the FT-IR analysis results which revealed the presence of esters and phosphate groups in the okra leaf EPS spectrum, both of which are considered to be representatives of the polar head of phospholipids as found in liposomes (Yokota *et al*., 2012). Liposomes are thermodynamically stable and versatile bilayered vesicles, and in recent years, most membrane enclosed vesicular structures released by (plant) cells are typically regarded as extracellular vesicles (Stremersch *et al*., 2016; Woith and Melzig, 2019; Gangadaran and Ahn, 2020).

In plants, extracellular vesicles are typically made up of lipids, proteins, carbohydrates, and nucleic acids which are largely derived from the parent cells (Rutter and Innes, 2017), and are responsible for a variety of functions such as intra and inter-cellular communication, and the transportation of various compounds and proteins into extracellular space (An *et al*., 2006; Rutter and Innes, 2017). This corresponds with the observable characteristics in the okra leaf EPS, where the EPS exuded from the abaxial part of the leaves contains copious amounts of lipids, proteins, and carbohydrates. Thus, enriching the soil with vesicular loads of compounds, thereby making them a sought-after material for agricultural and biotechnological purposes. According to Chen *et al*. (2008) organic acids may promote desorption of metals by dissolving minerals through chelation, which may influence a change in the microbial structure of plant's root, and the physicochemical properties of the soil.

For the maize root exudate, the slime-like EPS was soluble. From results obtained in this work, exudation or secretion only persisted for 15 DAS after which it ceased, the root became discoloured, and the plant gradually wilted and died. The reason for this outcome can be related to results from earlier studies carried out by Jones and Moore (1973) who demonstrated that when the supply of carbohydrate to the Golgi apparatus of maize roots became depleted,

formation of secretory glands ceased, and exudation was truncated. Cytological and physiological studies have shown, though not explicitly stated, that root EPS from most gramineous plants is secreted from the secretory Golgi apparatus located at the root tips of the plant (Jones and Moore, 1973; Sechet *et al*., 2018; Voiniciuc *et al*., 2018; Galloway *et al*., 2020). According to Voiniciuc *et al*. (2018), these secretions are largely composed of polysaccharides which are produced either via cytosolic monosaccharide synthesis or within the Golgi apparatus by glycosyl-transferase enzymes. Although the presence of Golgi apparatus was not examined in this experiment, the biochemical analysis confirmed that maize root EPS contains a copious amount of carbohydrates, lipids and proteins which are likely to have originated during the formation of the exuded material. The carbohydrates typically occur in the form of polymers comprised of monosaccharides such as glucose, fructose, arabinose, xylose, mannose, ribose etc. (Vranova *et al*., 2013). Regarding the presence of proteins in EPS, positive detection of proteins using the Bradford dye-binding assay coupled with the small amount of α-amino-nitrogen detected may indicate that polypeptides, smaller peptides and free amino acids are components of the exuded EPS. Whether these can be further analysed by PAGE deserves further attention. Nevertheless, when taken together, small molecules typically provide an easily accessible source of carbon and nitrogen to soil microorganisms and also serve as an easier source of accessible nutrient and alternative to the poly-aromatics which are naturally locked-up in a recalcitrant coal. Together, these substances are important for the stimulation of growth and proliferation of soil microorganisms such as fungi and bacteria which may in turn aid biodegradation of recalcitrant substrates and pollutants and may also fulfil a signalling role between fungi and plants (Malcolm and Vaughan, 1979; Canellas *et al*., 2008; Al-Othman *et al*., 2011; Karst *et al*., 2016; Zhou *et al*., 2019). By way of example, Phillips *et al*. (2012) reported that exudates from the roots of *Elymus angustus* (wildrye) and *Medicago sativa* (alfalfa) were able to stimulate and influence the microbial communities in the rhizosphere which led to the biodegradation of weathered hydrocarbon-contaminated soil.

Also, FT-IR analysis of the maize EPS showed broad stretching peaks of hydroxyl and carbonyl groups, suggesting the likely presence of carbohydrate in the EPS sample, which is a major component in the biological activities of a plant system (Tablin *et al*., 2001; Pritchard *et al*., 2002; Nguyen, 2003; Wolkers *et al*., 2004; Chen *et al*., 2015; Karst *et al*., 2016). An earlier study by Azaizeh *et al*. (1995) also reported that root exudate from maize (cv. Alize) is mainly composed of sugars (70 %), phenolics (18 %), organic acids (7 %), and amino acids (3 %). Further analysis of the maize EPS by FT-IR confirmed vibrations at the carboxylic and

hydroxylic group regions of the spectrum, both of which have been suggested to play an important role in the determination of the activities of humic substances (Malcolm and Vaughan, 1979; Canellas *et al*., 2008; Lu *et al*.,2017; Zhou *et al*., 2019). This can be supported with previous studies by Haichar *et al*. (2012) who reported that aminocyclopropane-1-carboxylic acid (ACC) exuded from roots of *Arabidopsis thaliana* can be used as a source of carbon and nitrogen by plant growth promoting rhizobacteria (PGPR).

Another interesting finding was the presence of indole or indole-like compounds and phenolics in the maize root EPS. Although only indirect spectrophotometric analysis of crude extracts was used, the results of these secondary metabolites nevertheless indicate a potential of these complex polymer to house signalling molecules. Similar findings have been reported for exudate of *Oryza sativa* (Singh *et al*., 2016) and *A. esculentus* (Ray *et al*., 2018). Studies have also shown that the phenolics of root extrapolymeric substance not only serve as chemotactic signals for soil microbiota (Perret *et al*., 2000) but also help in the mineralization of soil nitrogen and in humification (Halvorson *et al*., 2009). And, a study by Prusty *et al.* (2004) reported that the auxin, indole-3-acetic acid, promoted growth of the filamentous *Saccharomyces cerevisiae* and invasion by the yeast, supporting the role for IAA as a signalling molecule.

Several of the coal degrading fungal isolates viz, ECCN 84, ECCN 225 and ECCN 243, used in earlier studies, were identified using standard molecular techniques. Molecular methods are an important and necessary addition to conventional identification techniques and help to avoid unusual or erroneous phenotypic or biochemical profiles. For example, strain ECCN 84, was previously referred to as a member of the *Deuteromycetes* or fungi imperfecti (Igbinigie *et al*., 2008). However, this no longer formally accepted as a taxon and many of the fungi included in the Deuteromycota have either been reclassified or have yet to find a place in modern fungal classification (Gam, 1995; Taylor, 1995). Even so, using conventional PCR technique it was shown that the coal degrading fungi previously referred to as Deuteromycetes were in fact closely related to and confirmed as members of the *Ascomycetes*. While these findings put to rest any doubts about the original published data (Igbinigie *et al*., 2008), it must be remembered that *Neosartorya* is the sexual state (teleomorph) of *Aspergillus* species, notably the *Aspergillus fumigatus* group among others. The teleomorph is the sexual reproductive stage, typically a fruiting body, of the fungus whereas the anamorph is the asexual reproductive stage (morph) and is often mould-like in appearance (Horn *et al*., 2009; Lee *et al*., 2010; Dyer and O'Gorman, 2012; Varga *et al*., 2014). In this study, the mould-like morphs of ECCN 84, 225 and 243 were

characterized and identified as *Aspergillus* sp. ECCN 84, *Aspergillus* sp. ECCN 225, *Penicillium* sp. ECCN 243, respectively.

Although, some members of the *Aspergillus* have been reported to be pathogenic and could pose a threat to human health such as *A. fumigatus*, *A. flavus*, *A. niger* (Balajee *et al*., 2009) and *A. terreus* (Lass-Florl, 2012), current studies have shown that they also play an important role in the bioremediation of hydrocarbon polluted soils. Asemoloye *et al*. (2020) demonstrated that *Aspergillus oryzae* and *Mucor irregularis* isolated from Nigerian crude oil-polluted sites were able to degrade hydrocarbons through a series of enzymatic activities. The authors identified laccase and manganese peroxidase as the major enzyme catalysts responsible for hydrocarbon degradation. Another study by Uribe-Alvarez (2011) demonstrated that *Neosartorya fischeri* was able to metabolize and mineralize asphaltenes (a recalcitrant petroleum fraction) through the production of extracellular laccase. This agrees with the reports by Mester and Field (1997), Mukasa-Mugerwa *et al*. (2011) and Sekhohola *et al*. (2014) which suggest that some coal-degrading fungi tend to possess an ability to release extracellular oxidative enzymes such as laccases and peroxidases which enables them to effectively degrade hydrocarbon pollutants such as coal. However, in most cases, soil microorganisms often require a substrate or co-substrates to stimulate or facilitate these enzymatic activities (Cowan *et al*., 2016). For example, studies by Mester and Field (1997) showed that the addition of organic acids such as glycolate, malonate, glucuronate, 2-hydroxybutyrate to a culture medium can effectively stimulate the production of MnP by the white-rot fungus, *Bjerkandera* sp. strain BOS55. Therefore, in this study, the effect of maize root exudates on the ligninolytic enzyme activities, specifically manganese peroxidase enzymes (MnP) and laccase enzyme (LAC), of the three coal-degrading fungi was analysed and reported.

Maize root EPS contributed massively to LAC activity of strains ECCN 84, ECCN 225 and ECCN 243, particularly in the presence of coal. Since LAC activity was proposed as a major contributing enzyme responsible for the biodegradation of coal by strain ECCN 84 (Sekhohola *et al*., 2014; 2017), it seems reasonable to suggest that EPS from maize root exudate either stimulated or enhanced the laccase activity of fungi in a recalcitrant coal rhizosphere.

A role for fungal laccases in the biodegradation of coal has now been confirmed by an elegant series of studies by Kwaitos *et al*. (2018; 2020). These authors demonstrated the heterologous expression of a laccase from the ascomycete *Fusarium oxysporum*, in *Pichia pastoris* and showed that it catalysed the depolymerisation and liquefaction of solubilised brown coal with

the release of humic and fulvic acids. Although fungal laccases may be the primary enzyme responsible for the biodegradation of hydrocarbon such as coal, it is pertinent to note that more than one enzyme is likely responsible for the microbial degradation of a complex substrate such as coal (Fakoussa and Hofrichter, 1999). The many enzymes that have been proposed as participants included laccases, peroxidases, polyphenol oxidases, esterases etc., and many are often produced simultaneously by coal-degrading microorganisms (Xiao *et al*., 2018). In most cases, coal-degrading microorganism release signalling molecules which are often in the form of secondary metabolites such as indoles and phenolics to guide their path towards the soil zones where they can easily access readily available carbon for their growth and subsequent activities.

All of the fungal isolates used in the present study were sourced from the rhizosphere of grasses on waste coal dumps and appeared capable of producing some form of indole or indole-like compound suggesting that these might play a role in modification of plant-fungi rhizosphere. Phylogenetic studies carried out by Fu *et al*. (2015) suggest that the biosynthesis of indoles evolved independently in fungi and that fungal indole is often used as a physiological code in plant-microorganism interaction which would seem to confirm the ubiquity of this trait. Numponsak *et al*. (2018) also reported that *Colletotrichum fructicola* CMU-A109, isolated from *Coffea arabica* plants in Northern Thailand, produced indole-3-acetic acid when cultivated in liquid medium and demonstrated that the crude fungal IAA could stimulate plant growth. This is in line with other related studies that likewise suggest that fungal-produced IAA stimulates roots, and root hair formation (Fu *et al*., 2015; Kamla *et al*., 2020; Turbat *et al*., 2020). This is essential as it will increase the channels of root exudations and also enhance the exudation process.

The impact of *Zea mays*-ECCN 84 interaction on the *in situ* colonisation and biodegradation of coal was assessed using constructed soil columns or rhizoboxes after a 16-week cultivation period. Overall, the use of rhizoboxes has been shown to be a very effective tool for rhizospheric studies. Mukasa-Mugerwa *et al*. (2011) used rhizoboxes to simulate a coal dump environment which was effectively used to demonstrate plant-fungal interaction in the rhizosphere. Also, rhizobox studies have been shown to be effective in studying/monitoring the rhizosphere of young or annual plants (Boukcim *et al*., 2006), or even older trees (Aviani *et al*., 2006).

Analysis by SEM confirmed the proliferation and colonisation of the coal substrate in treatments cultivated with maize seedlings and containing either maize plus okra exudate (MOS84) or only maize exudate (MS84) and fungal spores. Establishment of contact between the fungus and the substrate coal is the first step in the biodegradation process and was readily observable using SEM. This finding agreed with previous studies by Sekhohola *et al*. (2013) and Cowan *et al*. (2016) who suggested that for successful biodegradation to occur, there must be direct contact between the substrate and the coal degrading microorganisms. Further analysis confirmed an increase in the amount of oxygen in the coal substrate after the 16-week experimental period which must have arisen as a result of ligninolytic activities by the fungus. A very good example is the laccase enzymatic activity whose mode of action is to reduce a molecule of dioxygen to two molecules of water while performing one-electron oxidation of a range of aromatic compounds which in turn results in an oxygen-centred free radical (Thurston, 1994).

FT-IR analysis of the coal substrate from each treatment confirmed vibrations which peaked at regions of the alkanes, carboxylic acids, amides, esters, anhydrides, sulphates, sulfonyl, and sulphonamides, functional groups, indicating the presence of easily accessible sources of carbon to the fungus. Current studies have shown that the presence of carboxylic acids, amides, alkanes, and anhydrides provide reactive sites for chemical reactions which leads to the removal of phytotoxins and increase in pH buffering capability of the soil (Akimbekov *et al*., 2020). More so, esters produced extracellularly have been reported to be linked to the depolymerization of coal (Crawford and Gupta, 1992). This is supported by the results from the humic acid analysis which showed that coal samples from MOS84, MS84 and maize only treatments, all contained some form of humic or humic-like substance at 16 WAS, indicating that humification of the waste coal substrate had occurred. This is a very crucial and positive step in bioremediation, as humics enhances soil penetration and contribute to the retention of important soil minerals and the overall transportation of nutrients from soil to plant cells (Chabbi *et al*., 1998; Chabbi 1999; Tahir *et al*., 2011; Akinbekov *et al*., 2020).

Apart from humic acid quantification, another good indicator for mined soil is pH. According to the “Land Rehabilitation Guidelines for Surface Coal Mines”, which was released by Coaltech and the Mineral Council of South Africa in May 2019, the pH of mined soil ideal for establishment of vegetation must be in the range of 6.0 and 7.5. As long ago as the 1980s it was reported that application of the Actinobacterium *Streptomyces* to coal resulted in an

increase in medium pH (Gupta *et al*., 1988). Similarly, alkaline filtrates of culture medium in which the fungus *Polyporus versicolor* (now *Trametes versicolor* – also known as *Coriolus versicolor*) was grown were shown to solubilize coal (Cohen *et al*., 1987). And since that time, numerous studies using coal degrading microorganisms have confirmed that alkalinisation is an essential early step and indicator of the microbial biodegradation of coal (Igbinigie *et al*., 2008; Sekhohola *et al*., 2014; Olawale *et al*., 2020). Thus, it was most encouraging that the simulated coal environment within the rhizobox demonstrated medium alkalisation in response to MOS84, MS84 and maize only treatments after only 16-weeks.

Also, soil EC, much like pH, is a good overall indicator of soil fertility. While the substrate pH reflects the mineral balance, an increase in substrate EC indicates an increase in the quantity of available minerals or nutrients (Cowan *et al*., 2016; Olawale *et al*., 2020). Thus, results from the rhizobox experiment confirmed that treatments containing both *Z. mays* and ECCN 84 spores had the highest EC, which apparently indicates an increase in more negatively charged sites which in turn implies that more cations are being held within the coal substrate. Further analysis by EDS of the substrate following the 16-w cultivation period confirmed the presence of beneficial cations such as sodium (Na^{+}), potassium (K^{+}), calcium (Ca^{2+}), iron (Fe^{2+}), and aluminium (Al^{3+}).

These observations indicated better rhizosphere colonisation and, substrate biodegradation and humification in response to plant-strain 84 interaction. Therefore, root exudate appears to play a significant role in the coordination of soil microorganisms within the rhizosphere and likely serves both as a scaffold for rhizospheric interactions by providing microorganisms with accessible carbon and as a likely ‘trigger’ for induction of coal-degrading enzymes such as fungal LAC for mobilisation of recalcitrant carbon.

6.2. Conclusion

The work described in this thesis has demonstrated that plant-strain 84 interaction support mutualism between plants and mycorrhizal fungi through supply of nutrients to support phytoremediation of hydrocarbon (e.g., coal and other fossil fuels) polluted soils. This study has shown that EPS exuded from roots of *Zea mays* together with coal degrading fungi such as *Aspergillus* strain ECCN 84 can alkalinise the substrate, facilitate introduction of oxygen possibly as a result of increased laccase activity, and increase the availability of nutrients (as indicated by higher EC) in a coal-polluted rhizosphere thereby providing plants and microorganisms a more mesic environment for sustained biodegradation activity and with enhanced rehabilitation potential. It was also shown that root-produced EPS contains small molecules that may fulfil a signalling function (e.g., phenolics and indoles), and increases activity of ligninolytic enzymes, particularly laccase. Therefore, root-produced EPS could serve as both a scaffold and as a substrate or co-substrate for coal-degrading fungi in a bioremediation technology such as FungCoal, where a highly complex and variable product such as weathered coal/oxidized weathered coal is being used. At the end of the study, the results confirmed that indeed, there is a distinct contribution of extrapolymeric substances (EPS) in the plant-fungal interaction which underpins the biodegradation of waste coal or coal and which may support continued phytoremediation and rehabilitation of soils containing carbonaceous pollutants.

Future studies should focus on harnessing the potentials of exudates as revealed in this study for effective bioremediation study. Commercial-scale field trials should be carried out on open-cast mined soil using perennial grasses such as (*Cynodon dactylon*) and the root-produced EPS from maize as substrates or co-substrates for the FungCoal rehabilitation bioprocess. Other biotechnological approach such as transgenic method should also be considered for investigation for possible contribution to the self-cladding technology.

6.3. Challenges

The major challenge encountered during the final year of study was the impact of the global Covid-19 pandemic. The University was in a mandated state of lockdown for a long period and facilities and some major equipment were not readily accessible. Therefore, other intended analysis such as Gas chromatography-mass spectrometry (GC-MS) and Transmission Electron Microscopy (TEM) could not be carried out.

References

Achi, O. K. (1994) Growth and coal-solubilizing activity of *Penicillium simplicissimum* on coal-related aromatic compounds. *Bioresource Technology* **48,** 53-57.

Adeniyi, M., Titilawo, Y., Oluduro, A., Odeyemi, O., Nakin, M. and Okoh, A. I. (2018) Molecular identification of some wild Nigerian mushrooms using internal transcribed spacer: polymerase chain reaction. *AMB Express* **8,**148 https://doi.org/10.1186/s13568-018-0661-9.

Akcil, A. and Koldas, K. (2006) Acid mine drainage (AMD): causes, treatment and case studies. *Journal of Cleaner Production* **14,** 1139-1146. DOI: 10.1016/j.jclepro.2004. 09.006.

Akhtara, N. and Mannan, M. A. (2020) Mycoremediation: Expunging environmental pollutants. *Biotechnology Reports* **26**(2020), e00452.

Akimbekov, N., Digel, I., Abdieva, G., Ualieva, P. and Tastambek, K. (2020) Lignite biosolubilization and bioconversion by *Bacillus* sp.: the collation of analytical data. *Biofuels.* DOI: 10.1080/17597269.2020.1753936.

Alameldin, H., Izardi-Drbandi, A., Smith, S. A., Balan, V., Jones, A. D. and Sticklen, M. (2017). Production of Seed-Like Storage Lipids and Increase in Oil Bodies in Corn (Maize; *Zea mays* L.) Vegetative Biomass. *Industrial Crops & Products* **108**, 526-534.

Alkorta, I., Hernández-Allica, J., Becerril, J. M., Amezaga, I., Albizu, I. and Garbisu, C. (2004) Recent findings on the phytoremediation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic. *Reviews in Environmental Science and Biotechnology* **3,** 71-90.

Al-Othman, Z. A., Hashem, A., Habila, M. A. (2011). Kinetic, equilibrium and thermodynamic studies of cadmium (II) adsorption by modified agricultural wastes. *Molecules* **16**, 10443-10456. https://doi.org/10.3390/molecules161210443.

Altshul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman,

D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389-3402.

Ameena, K., Dilip, C., Saraswathi, R., Krishnan, P. N., Sankar, C., and Simi, S. P. (2010) Isolation of the mucilages from *Hibiscus rosa-sinensis* Linn. and Okra (*Abelmoschus esculentus* Linn.) and studies of the binding effects of the mucilages. *Asian Pacific Journal of Tropical Medicine* **3**, (7), 539-543. https://doi.org/10.1016/S1995-7645(10) 60130-7.

American Society for Testing and Materials (1999) Coal and coke, *in* Annual book of ASTM standards, section 5, Petroleum products, lubricants, and fossil fuels, v. 05.05, Gaseous fuels; Coal and coke: Philadelphia, Pa., American Society for Testing and Materials, p. **155**-584.

An, Q., Ehlers, K., Kogel, K. H., van Bel, A. J, and Hückelhoven, R. (2006) Multivesicular compartments proliferate in susceptible and resistant MLA12-barley leaves in response to infection by the biotrophic powdery mildew fungus. *New Phytologists* **172,** 563-576.

Angel, T. M. (2002) Molecular biology and structure-function of lignin degrading heme peroxidases. *Enzyme and Microbial Technology* **30,** 425-444. doi:10.1016/S0141-0229(01)0 0521-X.

Aprill, W. and Sims, R. C. (1990) Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. *Chemosphere* **20,** 253-65.

Aremu, B. R. and Babalola, O. O. (2015) Construction of specific primers for rapid detection of South African exportable vegetable macergens. *International Journal of Environmental Research and Public Health* **12**(10),12356 -12370.

Arsene, M. A. Bilba, K., Savastano, H. and Ghavami, K. (2013) Treatments of non-wood plant fibers used as reinforcement in composite materials. *Materials Research* **16**(4), 903-23.

Asemoloye, M. D., Tosi, S., Daccò, C., Wang, X., Xu, S., Marchisio, M. A., Gao, W., Jonathan,

S. G. and Pecoraro, L. (2020) Hydrocarbon degradation and enzyme activities of *Aspergillus oryzae* and *Mucor irregularis* isolated from Nigerian crude oil-polluted sites. *Microorganisms* **8,** 1912. doi:10.3390/microorganisms8121912.

Aviani, I., Laor, Y. and Raviv, M. (2006) Limitations and potential of in situ rhizobox sampling for assessing microbial activity in fruit tree rhizosphere. *Plant Soil* **279,** 327-332. doi:10.1007/s11104-005-2189-4

Azaizeh, H. A., Marschner, H., Römheld, V. and Wittenmayer, L. (1995) Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. *Mycorrhiza* **5,** 321-327. https://doi.org/10.1007/BF00207404

Badri, D. V. and Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant, Cell and Environment* **32**, 666-681.

Badri, D. V., Weir, T. L., van der Lelie, D., and Vivanco, J. M. (2009) Rhizosphere chemical dialogues: plant-microbe interactions. *Current Opinion in Biotechnology* **20,** 642-650

Baetz, U. and Martinoia, E. (2014) Root exudates: the hidden part of plant defense. *Trends in Plant Science* **19,** 90- 98.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S. and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Reviews of Plant Biology* **57**, 233-266.

Balajee, S. A., Kano, R., Baddley, J. W., Moser, S. A., Marr, K. A., Alexander, B. D., Andes, D., Kontoyiannis, D. P., Perrone, G., Peterson, S., Brandt, M. E., Pappas, P. G. and Chiller, T. (2009) Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *Journal of Clinical Microbiology* **47**(10),3138-41. doi: 10.1128/JCM.01070-09.

Banuelos, G. S. and Meek, D. W. (1990) Accumulation of selenium in plants grown on selenium treated soil. *Journal of Environmental Quality* **19,** 772–77.

Barboza, N. R., Guerra-Sá, R. and Leão, V. A. (2016) Mechanisms of manganese

bioremediation by microbes: an overview. *Journal of Chemical Technology & Biotechnology* **91**, 2733–2739. DOI 10.1002/jctb.4997.

Barth, A. (2007) Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta* (BBA) – *Bioenergetics* **1767** (9), 1073-101.

Baudoin, E., Benizri, E., and Guckert, A. (2003). Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biology and Biochemistry* **35,** 1183–1192.

Belcarz, A., Ginalska, G. and Kornillowicz-Kowalska, T. (2005) Extracellular enzyme activities of Bjerkandera adusta R59 soil strain, capable of daunomycin and humic acids degradation. *Applied Microbiology and Biotechnology* **68,** 686-694.

Beller, M., Thiel, K., Thul, P. J. and Jäckle, H., (2011) Lipid droplets: a dynamic organelle moves into focus. *FEBS Letters* **584**, 2176-2182.

Bengough, A. G., McKenzie, B. M., Hallett, P. D. and Valentine, T. A. (2011). Root elongation, water stress, and mechanical impedance: A review of limiting stresses and beneficial root tip traits. *Journal of Experimental Botany* **62** (1), 59-68.

Berendsen, R. L., Pieterse, C. M. J. and Bakker, P. A. H. M. (2012) The rhizosphere microbiome and plant health. *Trends Plant Science* **17,** 478-486.

Berthet, S., Demont-Caulet, N., Pollet, B., Bidzinski, P., Cézard, L. and Le Bris, P. (2011). Disruption of LACCASE4 and 17 results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems. *Plant Cell* **23,** 1124-1137. doi:10.1105/tpc.110. 082792.

Bertin, C., Yang, X. and Weston, L. (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* **256**: 67-83.

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C. and Mougin, C. (2002) Crystal structure of a four-copper laccase complexed with an Arylamine: insights into substrate recognition and correlation with kinetics. *Biochemistry* **41,** 7325-7333. doi:10.1021/bi0201318.

Blanchette, R. A. (1995) Degradation of the lignocellulose complex in wood. *Canadian*

Journal of Botany **73**, 999 -1010.

Blaylock, M. J. and Huang, J. W. (2000) Phytoextraction of metals. In *Phytoremediation of Toxic Metals. Using Plants to Clean up the Environment*, ed. Raskin, I. and Ensley, B. D., pp. **53**-70. New York: Wiley.

Bligny, R., Gaillard, J. and Douce, R. (1986) Excretion of laccase by sycamore (*Acer pseudoplatanus* L.) cells. Effects of a copper deficiency. *The Biochemical Journal* **237**(2), 583-588. DOI: 10.1042/bj2370583.

Bogan, B. W., Lamar, R. T. and Hammel, K. E. (1996). Fluorene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. *Applied and environmental microbiology* **62**(5),1788-1792. https://doi.org/10.1128/AEM.62.5.1788-1792.1996.

Boukcim, H., Pages, L. and Mousain, D. (2006) Local NO_3^- or NH_4^+ supply modifies the root system architecture of *Cedrus atlantica* seedlings grown in a split-root device. *Journal of Plant Physiology* **163,** 1293-1304. doi:10.1016/j.jplph.2005.08.011

Bourbonnais, R., Paice, M. G., Freiermuth, B., Bodie, E. and Borneman, S. (1997) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Applied and Environmental Microbiology* **63,** 4627–4632.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Breen, A. and Singleton, F. L. (1999) Fungi in lignocellulose breakdown and biopulping. *Current Opinion in Biotechnology* **10**, 252-8.

Broeckling, C. D., Broz, A. K., Bergelson, J., Manter, D. K. and Vivanco, J. M. (2008) Root Exudates Regulate Soil Fungal Community Composition and Diversity. *Applied and Environmental Microbiology* **738,** 744. doi:10.1128/AEM.02188-07.

Brouwers, G. J., Vijgenboom, E., Corstjens, P. L. A. M., de Vrind, J. P. M. and de Vrind-de

Jong, E. W. (2000) Bacterial Mn^{2+} oxidizing systems and multicopper oxidases: an overview of mechanisms and functions. *Geomicrobiology Journal* **17**, 1-24.

Brown, L. K., George, T. S., Neugebauer, K. and White, P. J. (2017) The rhizosheath – a potential trait for future agricultural sustainability occurs in orders throughout the angiosperms. *Plant and Soil* **418,** 115-128.

Brzostek, E. R., Greco, A., Drake, J. E. and Finzi, A. C. (2013) Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. *Biogeochemistry* **115,** 65-76. https://doi.org/10.1007/s10533-012-9818-9.

Bugg, T. D., Ahmad, M., Hardiman, E. M. and Rahmanpour, R. (2011) Pathways for degradation of lignin in bacteria and fungi. *Natural Product Reports* **28**, 1883-96.

Burken, J. G. and Schnoor, J. L. (1997) Uptake and metabolism of atrazine by poplar trees. *Environmental Science & Technology* **31,** 1399-406.

Burns, R. G., Deforest, J. L., Marxsen, J., Sinsabaugh, R. L., Stromberger, M. E., Wallenstein, M. D., Weintraub, M. N. and Zoppini, A. (2013) Soil enzymes in a changing environment: current knowledge and future directions. *Soil Biology and Chemistry* **58**, 216-234.

Bustin, R. M. (1998) Coal: Origin and diagenesis. In: Geochemistry. Encyclopedia of Earth Science. Springer, Dordrecht. https://doi.org/10.1007/1-4020-4496-8_58.

Canarini, A., Kaiser, C., Merchant, A., Richter, A. and Wanek, W. (2019) Root exudation of primary metabolites: Mechanisms and their roles in plant responses to environmental stimuli. *Frontiers in Plant Science* **21,** 10-157. doi: 10.3389/fpls.2019.00157. PMID: 30881364; PMCID: PMC6407669.

Canellas, L. P., Zandonadi, D. B., Busato, J. G., Baldotto, M. A., Simões, M. L. and Martin-Neto L., (2008). Bioactivity and chemical characteristics of humic acids from tropical soils sequence. *Soil Science* **173**, 624- 637.

Caparro´s-Ruiz, D., Fornale´, S., Civardi, L., Puigdomenech, P. and Rigau, J. (2006) Isolation and characterisation of a family of laccases in maize. *Plant Science* **171,** 217-225.

Chabbi, A., Pietsch, W., Wiehe, W. and Huttl, R. F. (1998**)** *Juncus bulbosus* L.: Strategies of survival under extreme phytotoxic conditions in acid mine lakes in the Lusatian mining district,Germany. *International Journal of Ecology and Environmental Sciences* **24**, 271-292.

Chabbi, A. (1999) *Juncus bulbosus* as a pioneer species in acidic lignite mining lakes: interactions, mechanism, and survival strategies. *New Phytologist* **144**, 133-142.

Chang, L. K. Ibrahim, D. Omar, I. C. (2011). A laboratory scale bioremediation of Tapis crude oil contaminated soil by bioaugmentation of *Acinetobacter baumannii. African Journal of Microbiology* **5** (18), 2609-2615.

Chanoca, A., de Vries, L. and Boerjan, W. (2019) Lignin engineering in forest trees. *Frontiers in Plant Science* **10,** 912. doi: 10.3389/fpls.2019.00912

Chapman, K. D. and Ohlrogge, J. B. (2012). Compartmentation of triacylglycerol accumulation in plants. *Journal of Biological Chemistry* **287**, 2288–2294.

Chen, C., Li, Z., Li, S., Deng, N. and Mei, P. (2020) Effects of root exudates on the activation and remediation of cadmium ion in contaminated soils. *Environmental Science and Pollution Research* **27,** 2926-2934. https://doi.org/10.1007/s11356-019-07263-8.

Chen, L., Lin, W., Qu, X., Sosso, D., McFarlane, H. E., Londono, A., Samuels, A. L., and Frommer, W. B. (2015). A cascade of sequentially expressed sucrose transporters in the seed coat and the endosperm provides nutrition for the Arabidopsis embryo. *The plant cell* **27**, 607- 619.

Chen, Z., Kim, K., Owens, G. and Naidu, R. (2008) Determination of carboxylic acids from plant root exudates by Ion Exclusion Chromatography with ESI-MS. *Chromatographia* **67,** 113-117.

Cheng, Y., Zheng, Y. and Vander-Gheynst, J. S. (2011). Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. *Lipids* **46**, 95-103. DOI 10.1007/s11745-010-3494-0.

Cohen, M. S., Bowers, W. C., Aronson, H. and Gray, E. T. (1987) Cell-free solubilization of coal by *Polyporus versicolor*. *Applied and Environmental Microbiology* **53,** 2840-2843.

Coppin, N. J. (2013) 'A framework for success criteria for mine closure, reclamation and post-mining regeneration', in M Tibbett, AB Fourie & C Digby (eds), *Proceedings of the Eighth International Seminar on Mine Closure*, *Australian Centre for Geomechanics*, Cornwall, pp. 485-493, https://doi.org/10.36487/ACG_rep/1352_41_Coppin

Cowan, A. K. Lodewijks, H. M. Sekhohola, L. M. and Edeki, O. G. (2016) In situ bioremediation of South African coal discard dumps. In, Fourie, A. B. and Tibbett, M. (eds). Mine closure 2016, Proceedings, **pp 501**-509. *Australian Centre for Geomechanics, Perth*. ISBN 978-0-99248104-9.

Crawford, D. L. and Gupta, R. K. (1992) Microbial depolymerization of coal, in microbial transformation of low rank coals. **pp. 171**-211. CRC Press, Boca Raton, Florida.

Curl, E. A. and Truelove, B. (1986). The rhizosphere. In: Bommer, D. F. R., Sabey, B. R., Thomas, G. W., Vaadia, Y. and Van Vleck, L. D. eds. Advanced series in agricultural.

Dakora, F. D. and Philips, D. A. (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments, *Plant and Soil* **245,** 35- 47.

Das, A., Sukla, L. B., Pradhan, N. and Nayak, S. (2011) Manganese biomining: A review. *Bioresource technology* **102** (16), 7381-7. DOI:10.1016/j.biortech.2011.05.018.

Datta, A., Singh, R. K. and Tabassum, S. (2015) Isolation, Characterisation, and Growth of Rhizobium strains under Optimum Conditions for Effective Bio-fertiliser production. *International Journal of Pharmaceutical Sciences* **32** (1), 199-208.

Delhaize, E., Ryan, P. R., and Randall, P. J. (1993). Aluminum tolerance in wheat (*Triticum aestivuml*.). II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiology* **103**, 695-702.

Dennis, P. G., Miller, A. J. and Hirsch, P. R. (2010) Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities, *FEMS Microbiology Ecology* **72,** 313–327.

de Oliveira, P. L., Duarte, M. C., Ponezi, A. N. and Durrant, L. R. (2009). Purification and

Partial characterization of manganese peroxidase from *Bacillus pumilus* AND *Paenibacillus* sp. *Brazilian journal of microbiology* **40**(4),818-826.

Diamantidis, G., Effosse, A., Potier, P. and Bally, R. (2000) Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum*. *Soil Biology & Biochemistry* **32**, 919-27.

Dietz, S., Herz, K., Gorzolka, K., Jandt, U., Bruelheide, H. and Schee, D. (2020) Root exudate composition of grass and forb species in natural grasslands. *Scientific Reports* **10,** 10691. https://doi.org/10.1038/s41598-019-54309-5.

Dong, L., Yuan, Q. and Yuan, H. (2006) Changes of chemical properties of humic acids from crude and fungal transformed lignite. *Fuel* **85**(2006), 2402–2407.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28** (3), 350-356.

Dubrovskaya, E., Pozdnyakova, N., Golubev, S., Muratova, A., Grinev, V., Bondarenkova, A. and Turkovskaya, O. (2017) Peroxidases from root exudates of *Medicago sativa* and *Sorghum bicolor*: Catalytic properties and involvement in PAH degradation. *Chemosphere* **169,** 224-232. doi: 10.1016/j.chemosphere.2016.11.027.

Durazzo, A., Lucarini, M., Novellino, E., Souto, E. B., Daliu, P. and Santini, A. (2018) *Abelmoschus esculentus* (L.): Bioactive components' beneficial properties-focused on anti-diabetic role for sustainable health applications. *Molecules* **24,** 38. doi:10.3390/ molecules24010038.

Dwivedi, U. N., Singh, P., Pandey, V. P. and Kumar, A. (2011) Structure–function relationship among bacterial, fungal and plant laccases. *Journal of Molecular Catalysis B: Enzymatic* **68**, 117–128.

Dyer, P. S. and O'Gorman, C. M. (2012) Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. *FEMS Microbiology Reviews* **36,** 165-192.

Engels, C., Neumann, G., Gahoonia, T., George, E. and Schenk, M. (2000). Assessment of

the ability of roots for nutrient acquisition. In: Smit, A. L., Bengough, A. G., Engels, C., Van Noordwijk, M., Pellerin, S., Van de Geijn, S.C. (eds) Root methods. A handbook. Springer, Heidelberg, Germany **pp 403**-459.

Fakoussa, R. M. Hofrichter, M. (1999). Biotechnology and microbiology of coal degradation *Journal of Applied Microbiological Biotechnology* **52,** 25-40.

Fan, M., Dai, D. and Huang, B. (2012). Fourier Transform Infrared Spectroscopy for Natural Fibres, Fourier Transform - Materials Analysis, Dr Salih Salih (Ed.), ISBN: 978-953-51-0594-7, InTech, Available from: http://www.intechopen.com/books/fourier-transform-materials-analysis/fourier-transform-infraredspectroscopy-for-natural-fibres

Ferraroni, M., Myasoedova, N., Schmatchenko, V., Leontievsky, A., Golovleva, L., Scozzafava, A. and Briganti, F. (2007) Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. *BMC Structural Biology* **7**, 60.

Finkelman, R. B., and Brown, R. D. (1991) Coal as a host and as an indicator of mineral resources, *in* Peters, C.C., ed., Geology in coal resource utilization: Fairfax, Va., *Tech Books* **p. 471**- 481.

Fu, S. F., Wei, J. Y., Chen, H. W., Liu, Y. Y., Lu, H. Y. and Chou, J. Y. (2015) Indole-3-acetic acid: a widespread physiological code in interactions of fungi with other organisms. *Plant Signaling & Behavior* **10** (8), e1048052.

Gadd, G. M. (2010) Metals, minerals, and microbes: geomicrobiology and bioremediation. *Microbiology* **156**, 609-643.

Gadd, G. M. (2004) Microbial influence on metal mobility and application for bioremediation. *Geoderma* **122**,109-119.

Galloway, A. F., Pedersen, M. J., Merry, B., Marcus, S. E., Blacker, J., Benning, L. G., Field, K. J. and Knox, J. P. (2018) Xyloglucan is released by plants and promotes soil particle aggregation. *New Phytologist* **217,** 1128–1136.

Galloway, A. F., Knox, P. and Krause, K. (2020) Sticky mucilage and exudates of plants:

putative microenvironmental design elements with biotechnological value. *New Phytologist* **225,** 1461-1469. doi: 10.1111/nph.16144.

Gams, W. (1995) "How natural should anamorph genera be?". *Canadian Journal of Botany* **73** (1), 747-753. doi:10.1139/b95-318.

Gang, D. R., Wang, J., Dudareva, N., Nam, K. H., Simon, J. E., Lewinsohn, E. and Pichersky, E. (2001). An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. *Plant Physiology* **125**, 539-555.

Gangadaran, P. and Ahn, B. (2020) Extracellular vesicle- and extracellular vesicle mimetics-based drug delivery systems: new perspectives, challenges, and clinical developments. *Pharmaceutics* **12,** 442. doi:10.3390/pharmaceutics1205044.

Gao, Y., Yang, Y., Ling, W., Kong, H. and Zhu, X. (2011) Gradient distribution of root exudates and polycyclic aromatic hydrocarbons in rhizosphere soil. *Soil Science Society of American Journal* **75,** 1694–1703.

Garcia, A. C., De Souza, L. G. A., Pereira, M. G., Castro, R. N., Garcı´a-Mina, J. M., Zonta, E., Lisboa, F. J. and Berbara, R. L. (2016) Structure-property-function relationship in humic substances to explain the biological activity in plants. *Scientific Reports* **10,** (6): 20798. doi: 10.1038/srep20798. PMID: 26862010; PMCID: PMC4748406.

Gavrilescu, M. (2004) Removal of heavy metals from the environment by biosorption. *Engineering in Life Sciences* **4**, 219-232.

Ghani, M. J., Rajoka, M. I. and Akhtar, K. (2015) Investigations in fungal solubilization of coal: mechanisms and significance. *Biotechnology and Bioprocess Engineering* **20,** 634-642. DOI 10.1007/s12257-015-0162-5.

Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M. and Gold, M. H. (1983). An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. *Biochemical and Biophysical Research Communications* **114,** 1077-1083.

Global Energy Monitor and Centre for Research on Energy and Clean Air (2020) A new coal

boom in China- New coal plant permitting and proposals accelerate. Briefing: June 2020. https://globalenergymonitor.org/wp-content/uploads/2020/06/China-coal-plant-brief-June-2020v2.pdf.

Gold, H. M., Youngs, H. L. and Sollewijn-Gelpke, M. D. (2000). Manganese peroxidase. metal ions in biological systems, *Medline* **37,** 559-586.

Gordon, S. A. and Weber, R. P. (1951) Colorimetric estimation of indoleacetic acid. *Plant Physiology* **26,** 192-195.

Groot, E. P., Sweeney, E. J. and Rost, T. L. (2003) Development of the adhesive pad on climbing fig (*Ficus pumila*) stems from clusters of adventitious roots. *Plant and Soil* **248,** 85-96.

Gupta, R. K., Spiker, J. K. and Crawford, D. L. (1988) Biotransformation of coal by ligninolytic Streptomyces. *Canadian journal of microbiology* **34**(5), 667-674.

Gusmiaty, M. R. A. and Payangan, R. Y. (2019) Production of IAA (Indole Acetic Acid) of the rhizosphere fungus in the Suren community forest stand. IOP Conference Series: *Earth and Environmental Science* **343,** 012058.

Haase, S., Neumann, G., Kania, A., Kuzyakov, Y., Romheld, V., and Kandeler, E. (2007). Atmospheric CO_2 and the nutritional status modifies nodulation, nodule-carbon supply and root exudation of *Phaseolus vulgaris* L. *Soil Biology and Biochemistry* **39**, 2208-2221.

Haichar, F. E. Z., Roncato, M. A. and Achouak, W. (2012) Stable isotope probing of bacterial community structure and gene expression in the rhizosphere of *Arabidopsis thaliana*. FEMS *Microbiology Ecology* **81,** 291-302. doi: 10.1111/j.1574-6941.2012.01345.x

Haichar, F. Z., Santaella, C., Heulin, T. and Achouak, W. (2014). Root exudates mediated interactions belowground. *Soil Biology & Biochemistry* **77,** 69-80.

Hall, T. (2004) BioEdit. *Ibis Therapeutics*, Carlsbad.

Halvorson, J. J., Gonzalez, J. M., Hagerman, A. E., Smith, J. L. (2009). Sorption of tannin and

related phenolic compounds and effects on soluble-N in soil. *Soil Biology and Biochemistry* **41**, 2002–2010.

Hambrick, G. A., DeLuane, R. D. and Patrick, W. H. (1980) Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation. *Applied and Environmental Microbiology* **40,** 365-369.

Hammel, K. E., Jensen, K. A., Mozuch, M. D., Landucci, L. L., Tien, M. and Pease, E. A. (1993). Ligninolysis by a purified lignin peroxidase. *Journal of Biological Chemistry* **268,** 12274-12281.

Hawes, M. C., Gunawardena, U., Miyasaka, S. and Zhao, X. (2000). The role of root border cells in plant defense. *Trends in plant science* **5**(3), 128-133.

Hazrin-Chong, N. H. Marjo, C. E. Das, T. Rich, A. M. and Manefield, M. (2014) Surface analysis reveals biogenic oxidation of sub-bituminous coal by *Pseudomonas fluorescens. Journal of Applied Microbiology and Biotechnology* **98,** 6443-6452.

Hazrin-Chong, N. H., Das, T. and Manefield, M. (2020) Surface physico-chemistry governing microbial cell attachment and biofilm formation on coal. *International Journal of Coal Geology* **2020**, 103671, ISSN 0166-5162. https://doi.org/10.1016/j.coal.2020.103671.

Henry, A., Doucette, W., Norton, J., Jones, S., Chard, J., and Bugbee, B. (2006). An axenic plant culture system for optimal growth in long-term studies. *Journal of Environmental Quality* **35**, 590-598.

Hodek, W. (1994) The chemical structure of coal in regard to microbiological degradation. *Fuel Processing Technology* **40,** 369-378.

Hofer, C. and Schlosser, D. (1999) Novel enzymatic oxidation of Mn^{2+} to Mn^{3+} catalyzed by a fungal laccase. *FEBS Letters* **451**, 186-190.

Hofrichter, M. (2002) Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology* **30,** 454-466.

Hofrichter, M., Ullrich, R., Pecyna, M. J., Liers, C. and Lundell, T. (2010) New and classic

families of secreted fungal heme peroxidases. *Applied Microbiology Biotechnology* **87**(3), 871-897. doi:10.1007/s00253-010-2633-0.

Hofrichtera, M., Ziegenhagena, D., Varesc, T., Friedrichb, M., JaÎgerb, M. G., Fritschea, W. and Hatakkac, A. (1998) Oxidative decomposition of malonic acid as basis for the action of manganese peroxidase in the absence of hydrogen peroxide. *FEBS Letters* **434,** 362-366.

Horn, B. W., Moore, G. G. and Carbone, I. (2009) Sexual reproduction in *Aspergillus flavus*. *Mycologia* **101,** 423-429.

Horne, A. J. (2000) Phytoremediation by constructed wetlands. In *Phytoremediation of Contaminated Soil and Water*, ed. Terry, N, and Banuelos, G., **pp.13**-40. Boca Raton: Lewis.

Huang, X. F., Santhanam, N., Badri, D. V., Hunter, W. J., Manter, D. K., Decker, S. R., Vivanco, J. M. and Reardon, K. F. (2013) Isolation and characterization of lignin-degrading bacteria from rainforest soils. *Biotechnology and Bioengineering* **110**, 1616-26.

Huang, X. F., Chaparro, J. M., Reardon, K. F., Zhang, R. F., Shen, Q. R. and Vivanco, J. M. (2014). Rhizosphere interactions: Root exudates, microbes, and microbial communities. *Botany* **92,** 267-275.

Huang, Y., Wang, Y., Sun, L., Agrawal, R. and Zhang, M. (2015) Sundew adhesive: a naturally occurring hydrogel. *Interface* **12**. doi: 10.1098/rsif.2015.0226.

Huang, Y., Wang, Y., Tan, L., Sun, L., Petrosino, J., Cui, M. Z., Hao, F., Zhang, M. (2016) Nanospherical arabinogalactan proteins are a key component of the high-strength adhesive secreted by English ivy. *Proceedings of the National Academy of Science, USA* 113: 3193–23102.

Hussain, S., Siddique, T., Arshad, M. and Saleem, M. (2009) Bioremediation and Phytoremediation of Pesticides: Recent Advances, *Critical Reviews in Environmental Science and Technology*, 39 (10), 843-907, DOI: 10.1080/10643380801910090.

Igbinigie, E. E. (2007) The rhizosphere as a bioprocess environment for the bioconversion of hard coal. PhD Thesis, Rhodes University, South Africa.

Igbinigie, E. E., Aktins, S., van Breugel, Y., van Dyke, S., Davies-Coleman, M. T. and Rose, P. D. (2008) Fungal Biodegradation of Hard Coal by a Newly Reported Isolate, *Neosartorya fischeri*, *Biotechnology Journal* **3,** 1407-1416.

Igbinigie, E. E., Mutambanengwe C. C. Z. and Rose, P. D. (2010) Phyto-bioconversion of hard coal in the *Cynodon dactylon*/coal rhizosphere. *Biotechnology Journal* **5**, 292-303 DOI 10.1002/biot.200900201.

Ingvarsson, P. T., Yang, M., Nielsen, H. M., Rantanen, J. and Foged, C. (2011) Stabilization of liposomes during drying. *Expert Opinion on Drug Delivery* **8**(3), 375-388

Innes, L., Hobbs, P. J. and Bardgett, R. D. (2004) The impacts of individual plant species on rhizosphere microbial communities in soils of different fertility. *Biology and Fertility of Soils* **40,** 7-13.

Integrated Resource Plan (2019) Department of Mineral resources and Energy, South Africa. http://www.energy.gov.za/IRP/2019/IRP-2019.pdf.

Janusz, G., Pawlik, A., Sulej, J., Swiderska-Burek, U., Jarosz-Wilkołazka, A. and Paszczynski, A. (2017) Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. *FEMS Microbiology Reviews* **41,** 94-962 doi: 10. 1093/femsre/fux049.

Janusz, G., Pawlik, A., Swiderska-Burek, U., Polak, J., Sulej, J., Jarosz-Wilkołazka A. and Paszczynski, A. (2020) Laccase properties, physiological functions, and evolution. *International Journal of Molecular Sciences* **21,** 966. doi:10.3390/ijms21030966.

Jensen, K. A., Bao, W., Kawai, S., Srebotnik, E. and Hammel, K. E. (1997) Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis subvermispora* in the absence of lignin peroxidase. *Applied and environmental microbiology* **63**(2), 815.

Jimoh, T. A. and Cowan, A. K. (2017). Extracellular polymeric substance production in high rate algal oxidation ponds. *Water Science & Technology* **76**, (10) 2647- 2654.

Jimoh, T. A., Keshinro, M. O. and Cowan, A. K. (2019). Microalgal–bacterial flocs and extracellular polymeric substances: two essential and valuable products of integrated algal pond systems. *Water, Air, & Soil Pollution* **230**, 95. https://doi.org/10.1007/s11270-019-4148-3.

Johansson, T. and Nyman, P. O. (1993) Isozymes of lignin peroxidase and manganese (II) peroxidase from the white-rot basidiomycete *Trametes versicolor* .1. Isolation of enzyme forms and characterization of physical and catalytic properties. *Archives of Biochemistry and Biophysics* **300**, 49-56.

Jones, D. D. and Moore, D. J. (1973) Golgi apparatus mediated polysaccharide secretion by outer root cap ceils of *Zea mays.* III. Control by exogenous sugars. *Physiologia Plantarum* **29,** 68-75

Jones, D. L., Hodge, A. and Kuzyakov, Y. (2004) Plant and mycorrhizal regulation of rhizodeposition. *New Phytologist* **163,** 459-480.

Jones, D., Nguyen, C. and Finlay, D. R. (2009). Carbon flow in the rhizosphere: carbon trading at the soil root interface. *Plant Soil* **321**, 5-33.

Jonker, A., Gruber, M. Y., Wang, Y., Coulman, B., McKinnon, J. J., Christensen, D. A. and Yu, P. (2012). Foam stability of leaves from anthocyanidin accumulating Lc-alfalfa and relation to molecular structures detected by Fourier-transformed infrared vibration spectroscopy, *Grass and Forage Science* **67**, 369-381.

Kumla, J., Suwannarach, N., Matsui, K. and Lumyong, S. (2020) Biosynthetic pathway of indole-3-acetic acid in ectomycorrhizal fungi collected from northern Thailand. *PLoS ONE* **15**(1), e0227478. https://doi.org/10.1371/journal.pone.0227478

Kannenberg, S. A. and Phillips, R. P. (2016). Plant responses to stress impacts: The C we do not see. *Tree Physiology* **37**, 151-153. doi:10.1093/treephys/tpw108.

Karst, J., Gaster, J., Wiley, E. and Landhäusser, S. M. (2016). Stress differentially causes roots of tree seedlings to exude carbon. *Tree Physiology* **37**, 154-164. doi:10.1093/treephys/tpw090.

Kersten, P. and Cullen, D. (2007). Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. *Fungal Genetics Biology* **44,** 77-87. doi:10.1016/j.fgb.2006.07.007.

Keshinro, M. O., Kadiri, M., Ojewumi, A. W. and Augustine, T. E. (2017). Vegetative growth analysis of *Hibiscus sabdariffa* L. (Roselle) treated with some plant growth substances. *Direct Research Journal of Agriculture and Food Science* **5** (12), 401-408.

Khan, A. G. (2005) Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. *Journal of Trace Elements in Medicine and Biology* **18,** 355-364.

Kiefer-Meyer, M. C., Gomord, V., Connell, A. O., Halpin, C. and Faye, L. (1996) Cloning and sequence analysis of laccase-encoding cDNA clones from tobacco. *Gene* **78**(1-2), 205-207. https://doi.org/10.1016/0378-1119(96)00381-2.

Klein, O. I., Kulikova, N. A., Konstantinov, A. I., Fedorova, T. V., Landesman, E. O. and Koroleva, O. V. (2014) Humic substances of highly oxidised brown coal by Basidiomycetes *Trametes hirsuta* and *Trametes maxima*. *Applied Microbiology and Biochemistry* **49**, 287-295.

Kota, S., Barlaz, M. A. Borden, R. C. (2004). Spatial heterogeneity of microbial and geochemical parameters in gasoline contaminated aquifers. *Journal of Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management* **8** (2), 105-18.

Kruse, N. A., Bowman, J. R., Mackey, M. L., McCament, B. and Johnson, K. S. (2012) The lasting impacts of offline periods in lime dosed streams: a case study in raccoon creek, Ohio, *Mine Water* and the *Environment* **31**(4), 266-272.

Kuiper, I., Lagendijk, E. L., Bloemberg, G. V. and Lugtenberg, B. J. (2004) Rhizoremediation: A beneficial plant-microbe interaction. *Molecular Plant-Microbe Interactions* **17,** 6-15.

Kumla, J., Suwannarach, N., Matsui, K. and Lumyong, S. (2020) Biosynthetic pathway of indole3-acetic acid in ectomycorrhizal fungi collected from northern Thailand. *PLoS ONE* **15**(1), e0227478.

Kupriashina, M. A., Selivanov, N. and Nikitina, V. E. (2012) Isolation and purification of Mn-peroxidase from *Azospirillum brasilense* Sp245. *Prikl Biokhim Mikrobiol* **48**, 23-6.

Kuwahara, M., Glenn, J. K., Morgan, M. A. and Gold, M. H. (1984). Separation and characterization of two extracellular H_2O_2 dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS Letters* **169,** 247-250.

Kuzyakov, Y. and Domanski, G. (2000). Carbon input by plants into the soil. *Journal of Plant Nutrition and Soil Science* **163,** 421- 431.

Kwiatos, N., Jędrzejczak-Krzepkowska, M., Strzelecki, B. and Bielecki, S. (2018) Improvement of efficiency of brown coal biosolubilization by novel recombinant *Fusarium oxysporum* laccase. *AMB Express* **8,** 133. https://doi.org/10.1186/s13568-018-0669-1.

Kwiatos, N., Jędrzejczak-Krzepkowska, M., Krzemińska, A., Delavari, A., Paneth, P. and Bielecki, S. (2020) Evolved *Fusarium oxysporum* laccase expressed in *Saccharomyces cerevisiae*. *Scientific Reports* **10,** 3244. https://doi.org/10.1038/s41598-020-60204-1.

Land Rehabilitation Guidelines for Surface Coal Mines (2018) Land Rehabilitation Society of Southern Africa, Coaltech, Minerals Council of South Africa.

Lange, M., Eisenhauer, N., Sierra, C. A., Bessler, H., Engels, C., Griffiths, R. I. and Gleixner, G. (2015) Plant diversity increases soil microbial activity and soil carbon storage. *Nature Communications* **6,** 67071-8.

Lass-Florl, C. (2012) *Aspergillus terreus*: how inoculum size and host characteristics affect its virulence. *Journal of Infectious Diseases* **205,** 1268-1277.

Learman, D. R., Wankel, S. D., Webb, S. M., Martinez, N., Madden, A. S. and Hansel, C. M. (2011) Coupled biotic–abiotic Mn (II) oxidation pathway mediates the formation and structural evolution of biogenic Mn oxides. *Geochimica et Cosmochimica Acta* **75**, 6048-6063.

Lee, S. C., Ni, M., Li, W., Shertz, C. and Heitman, J. (2010) The evolution of sex: a perspective from the fungal kingdom. *Microbiology and Molecular Biology Reviews* **74,** 298-340.

Leontievsky, A., Myasoedova, N., Pozdnyakova, N. and Golovleva, L. (1997) 'Yellow' laccase of *Panus tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators. *FEBS Letters* **413** (3), 446–448.

Leontovyčová, H., Trdá, L., Dobrev, P. I., Šašek, V., Gay, L., Balesdent M. H. and Burketová L. (2020) Auxin biosynthesis in the phytopathogenic fungus *Leptosphaeria maculans* is associated with enhanced transcription of indole-3-pyruvate decarboxylase LmIPDC2 and tryptophan aminotransferase LmTAM1. *Research in Microbiology* **171,** 174-184.

Lie, S. (1973). The EBC-Ninhydrin method for determination of free alpha amino nitrogen. *Journal of the Institute of Brewing* **79** (1), 37- 41.

Limpitlaw, D. and Briel, A. (2015) 'Post-mining land use opportunities in developing countries-a review', *The Journal of The Southern African Institute of Mining and Metallurgy* **114,** 1-5.

Li, Q., Liu, J. and Gadd, G, M. (2020) Fungal bioremediation of soil co-contaminated with petroleum hydrocarbons and toxic metals. *Applied Microbiology and Biotechnology* **104,** 8999–9008. https://doi.org/10.1007/s00253-020-10854-y.

Liu, L., Dean, J. F.D., Friedman, W. E. and Eriksson, K. E. L. (1994) A laccase-like phenoloxidase is correlated with lignin biosynthesis in *Zinnia elegans* stem tissues. *The Plant Journal* **6**(2), 213-224. https://doi.org/10.1046/j.1365-313X.1994.6020213.x.

Li, X. G., Liu, B., Heia, S., Liu, D. D., Han, Z. M., Zhou, K. X., Cui, J. J., Luo, J. Y. and Zheng, Y. P. (2009) The effect of root exudates from two transgenic insect-resistant cotton lines on the growth of *Fusarium oxysporum*. *Transgenic. Research* **18,** 757-767.

Li, Y., Huang, W., Jiu, B., Sun, Q. and Che, Q. (2020) Modes of occurrence and origin of minerals in Permian coals from the Huainan coalfield, Anhui, China. *Minerals* **10,** 399. doi:10.3390/min10050399.

Lu, H., Sun, J. and Zhu, L. (2017) The role of artificial root exudate components in facilitating the degradation of pyrene in soil. *Scientific Reports* **7,** 7130. DOI:10.1038/s41598-017-07413-3.

Luterbacher, J. S., Alonso, D. M. and Dumesic, J. A. (2014). "Targeted chemical upgrading of lignocellulosic biomass to platform molecules," *Green Chemistry* **16,** 4816- 4838.

Lu, Y., Lu, Y. C., Hu, H. Q., Xie, F. J., Wei, X. Y. and Fan, X (2017). Structural characterization of lignin and its degradation products with spectroscopic methods. *Journal of Spectroscopy* **2017**: 15. https://doi.org/10.1155/2017/8951658.

Maier, R. M., Pepper, I. L. and Gerba, C. P. (2000) Environmental biotechnology. Academic Press. A Harcourt Science and Technology Company, Carlifonia, USA.

Malcolm, R. E. and Vaughan, D. (1979). Effects of humic acid fractions on invertase activities in plant tissues. *Soil Biology and Biochem*istry **11**, 65-72. https://doi.org/10.1016/0038-0717(79)90120-2.

Maobe, M. and Nyarango, R. (2013). Fourier transformer infra-red spectrophotometer analysis of *Warbur giaugandensis* medicinal herb used for the treatment of diabetes, malaria and pneumonia in Kisii Region, Southwest Kenya. *Global Journal of Pharmacology*, **7,** 61-68.

Marschner, P., Crowley, D. and Yang, C. H. (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition, and soil type. *Plant Soil* **261,** 199-208.

Marshall, P. D. (2002). Use of microsensors for studying the physiological activity of plant roots. In: Waisel, Y., Eshel, A., Kafkafi, U. (eds) Plant roots the hidden half, 3rd edn. Marcel Dekker, New York, USA, **pp 333**-347.

Martin, B. C., George, S. J., Price, C. A., Ryan, M. H. and Tibbett, M. (2014) The role of root exuded low molecular weight organic anions in facilitating petroleum hydrocarbon degradation: Current knowledge and future directions. *Science of the Total Environment* **472,** 642–653.

Martin, H. (2002) Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology* **30,** 454 - 466. doi:10.1016/S0141-0229(01)00528-2.

Martinez, A. T. (2002). Molecular biology and structure-function of lignin-degrading heme

peroxidases. *Enzyme Microbial Technology* **30,** 425–444, doi:10.1016/S01410229(01) 00521-X.

Martins, L. O., Soares, C. M., Pereira, M. M., Teixeira, M., Costa, T., Jones, G. H. and Henriques, A. O. (2002) Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *Journal of Biological Chemistry* **277**, 18849–59.

Matera, I., Gullotto, A., Tilli, S., Ferraroni, M., Scozzafava, A. and Briganti, F. (2008) Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complexed with p-toluate. *Inorganica Chimica Acta* **361,** 4129-4137. doi:10.1016/j.ica.2008. 03.091.

McCutcheon, S. C. and Schnoor, J. L. (2003) Overview of phytotransformation and control of wastes. In *Phytoremediation: Transformation and Control of Contaminants*, pp. 3-58. New York: Wiley.

Melzer, B., Steinbrecher, T., Seidel, R., Kraft, O., Schwaiger, R. and Speck, T. (2010) The attachment strategy of English ivy: a complex mechanism acting on several hierarchical levels. *Journal of the Royal Society Interface* **7,** 1383-1389.

Menon, V. and Rao, M. (2012) Trends in bioconversion of lignocellulose: biofuels, platform chemicals and biorefinery concept. *Progress in Energy and Combustion Science* **38,** 522-50.

Mester, T. and Field, J. A. (1997). Optimization of manganese peroxidase production by the white rot fungus *Bjerkandera* sp. strain BOS55. *FEMS Microbiology Letter* **155,** 161–168. doi:10.1111/j.1574-6968.1997.tb13873.x.

Micallef, S. A., Channer, S., Shiaris, M. P. and Colon-Carmona, A. (2009) Plant age and genotype impact the progression of bacterial community succession in the *Arabidopsis* rhizosphere. *Plant Signal Behaviour* **4,** 777-780.

Minussi, R. C., Pastore, G. M. and Durán, N. (2007) Laccase induction in fungi and laccase/N-OH mediator systems applied in paper mill effluent. *Bioresource Technology* **98,** 158-164.

Mishra, S. K., Suh, W. I., Farooq, W., Moon, M., Shrivastav, A., Park, M. S. and Yang, J. (2014). Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. *Bioresource Technology* **155**, 330-333.

Mitchell, R. J., Hester, A. J., Campbell, C. D., Chapman, S. J., Cameron, C. M., Hewison, R. L. and Potts, J. M. (2010). Is vegetation composition or soil chemistry the best predictor of the soil microbial community? *Plant and Soil* **333,** 417-430.

Mochida, I., Okuma, O. and Yoon S. H. (2014) Chemicals from direct coal liquefaction. *Chemical Reviews* **114**, 1637-1672.

Mohan, J. (2004). Organic spectroscopy*:* Principles and applications. Harrow, UK: Alpha Science International Ltd.

Moreira, P. R., Duez, C., Dehareng, D., Antunes, A., Almeida-Vara, E., Frere, J. M., Malcata, F. X. and Duarte, J. C. (2005) Molecular characterization of a versatile peroxidase from a Bjerkandera strain. *Journal of Biotechnology* **118,** 339-352, doi:10.1016/j. jbiotec.2005.05.014.

Mot, A. C. and Silaghi-Dumitrescu, R. (2012) Laccases: complex architectures for one-electron oxidation. *Biochemistry Moscow* **77,** 1395-1407. https://doi.org/10.1134/S0006297912120085.

Mukasa-Mugerwa, T. T., Dames, J. F. and Rose, P. D. (2011) The Role of a Plant/Fungal Consortium in the Degradation of Bituminous Hard Coal, *Biodegradation* **22,** 129-141. DOI 10.1007/s10532-010-9382-8.

Narula, N., Kothe, E., and Behl, R. K. (2009). Role of root exudates in plant-microbe interactions. *Journal of Applied Botany and Food Quality* **82,** 122-130.

National Research Council (1993) *In Situ Bioremediation: When Does it Work?*. Washington, DC: The National Academies Press. https://doi.org/10.17226/2131.

Neal, A. L., Ahmad, S., Gordon-Weeks, R. and Ton, J. (2012). Benzoxazinoids in root exudates of maize attract Pseudomonas putida to the rhizosphere. PLOS ONE **7,** e35498.

Nealson, K. H., Tebo, B. M. and Rosson, R. A. (1988) Occurrence and mechanisms of microbial oxidation of manganese. *Advances in Applied Microbiology* **33**, 279-318.

Nealson, K. H. (2006) The manganese-oxidizing bacteria in the prokaryotes*, A Handbook on the Biology of Bacteria Third Edition* **5,** 222-231.

Neumann, G. and Römheld, V. (2007) The release of root exudates as affected by the plant physiological status. In: Pinton, R., Varanini, Z., Nannipieri, Z. (eds) The rhizosphere: biochemistry and organic substances at the soil-plant interface, 2nd edn. CRC, Boca Raton, Florida, USA, **pp 23**-72.

Neumann, G., George, T. S. and Plassard, C. (2009). Strategies and methods for studying the rhizosphere—the plant science toolbox. *Plant Soil* **321**, 431-456.

Neumann, G., Bott, S., Ohler, M. A., Mock, H. P., Lipmann, R., Grosch, R. and Smalla, K. (2014). Root exudation and root development of lettuce (Lactuna sativa L. cv. Tizian) as affected by different soils. *Frontiers in Microbiology* **5**, 2.

Newman, L. A., Strand, S. E., Choe, N., Duffy, J., Ekuan, G., Ruszaj, M., Shurtleff, B. B., Wilmoth, J., Heilman, P. and Gordon, M. P. (1997) Uptake and biotransformation of trichloroethylene by hybrid poplars. *Environmental Science & Technology* **31**(4), 1062-1067. DOI: 10.1021/es960564w.

Nguyen C. (2003). Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomoie* **23,** 375-396.

Nhuchhen, D. R., Basu, P. and Acharya, B. A. (2014) Compressive review on biomass torrefecation. *International Journal of Renewable Energy & Biofuels* **1**-57. DOI: 10.5171/2014.506376.

Niladevi, K. N. and Prema, P. (2005) Mangrove *Actinomycetes* as the source of ligninolytic enzymes. *Actinomycetologica* **19**, 40-7.

Nordestgaard, B. G. and Rostgaard, J. (1985) Critical-point drying versus freeze drying for

scanning electron microscopy: a quantitative and qualitative study on isolated hepatocytes. *Journal of Microscopy* **137**(2),189-207. doi: 10.1111/j.1365-2818.1985 .tb02577.x.

Numponsak, T., Kumla, J., Suwannarach, N., Matsui, K. and Lumyong, S. (2018) Biosynthetic pathway and optimal conditions for the production of indole-3-acetic acid by an endophytic fungus, *Colletotrichum fructicola* CMU-A109. *PLoS ONE* 13(10), e0205070. https://doi.org/10.1371/journal.pone.0205070.

Oboirien, B. O., Burton, S. G., Cowan, D. and Harrison, S. T. L. (2008) The effect of the particulate phase on coal biosolubilisation mediated by *Trichoderma atroviride* in a slurry bioreactor. *Fuel Processing Technology* **89**(2),123-30. doi:10.1016/j.fuproc. 2007.06.019.

Oboirien, B. O., Ojumu, T. V. and Obayopo, O. S. (2013) Fungi solubilisation of low rank coal: Performances of stirred packed bed reactors. *Fuel Processing Technology* **106**, 295-302.

O'Keefe, J. M. K., Bechtel, A., Christanis, K., Dai, S., DiMichele, W. A., Eble, C. F., Esterle, J. S., Mastalerz, M., Raymond, A. L., Valentim, B. V., Wagner, N. J., Ward, C. R. and Hower, J. C. (2013) On the fundamental difference between coal rank and coal type. *International Journal of Coal Geology* **118,** 58-87.

Okuda, T., Klich, M. A., Seifert, K. A. and Ando, K. (2000). Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification (Samson RA, Pitt JI, eds). Harwood Academic Publishers, The Netherlands. **83**-99.

Olawale, J. T., Edeki, O. G. and Cowan A. K. (2020) Bacterial degradation of coal discard and geologically weathered coal. *International Journal of Coal Science and Technology* **7**(2), 405-416 https://doi.org/10.1007/s40789-020-00306-3.

Pande, V., Pandey, S. C., Sati, D., Pande, V. and Samant, M. (2020) Bioremediation: an emerging effective approach towards environment restoration. *Environmental Sustainability* **3,** 91-103.

Paszczynski, A., Huynh, V. B. and Crawford, R. (1986) Comparison of ligninase-I and

peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* **244**,750-65.

Patten, C. L., and Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied Environmental and Microbiology* **68,** 3795–3801. doi: 10.1128/AEM.68.8.3795-3801.2002.

Pawlik, A., Wójcik, M., Rułka, K., Motyl-Gorzel, K., Osińska-Jaroszuk, M., Wielbo, J., Marek-Kozaczuk, M., Skorupska, A., Rogalski, J. and Janusz, G. (2016) Purification and characterization of laccase from *Sinorhizobium meliloti* and analysis of the lacc gene. *International journal of biological macromolecules* **92,** 138-147.

Perez-Boada, M., Ruiz-Duenas, F. J., Pogni, R., Basosi, R., Choinowski, T., Martinez, M. J., Piontek, K. and Martinez, A. T. (2005) Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways. *Journal of Molecular Biology* **354**,85-402.

Perret, X., Staehelin, C. and Broughton, W. J. (2000) Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews* **64**, 180-201.

Personeni, E., Nguyen, C., Marchal, P. and Pagès, L. (2007). Experimental evaluation of an efflux-influx model of C exudation by individual apical root segments. *Journal of Experimental Botany* **58**, 2091-2099.

Phillips, L. A., Greer, C. W., Farrell, R. E. and Germida, J. J. (2012) Plant root exudates impact the hydrocarbon degradation potential of a weathered-hydrocarbon contaminated soil. *Applied Soil Ecology* **52,** 56-64.

Plácido, J. and Capareda, S. (2015) Ligninolytic enzymes: a biotechnological alternative for bioethanol production. *Bioresources and Bioprocessing* **2**, 23.

Pilon-Smits, E. (2005) Phytoremediation. *Annual Review of Plant Biology* **56,** 15-39. doi:10.1146/annurev.arplant.56.032604.144214.

Piontek, K., Glumoff, T. and Winterhalter, K. (1993) Low pH crystal structure of glycosylated

lignin peroxidase from *Phanerochaete chrysosporium* at 2.5 Å resolution. *FEBS Letters* **315,**119-124. doi:10.1016/0014-5793(93)81146-Q.

Pino, N. J., Muñera, L. M. and Peñuela, G. A. (2016). Root exudates and plant secondary metabolites of different plants enhance polychlorinated biphenyl degradation by rhizobacteria, *Bioremediation Journal* **20** (2), 108-116.

Polman, J. K., Stoner, D. L. and Delezene-Briggs, K. M. (1994) Bioconversion of coal, lignin and dimethoxybenzyl alcohol by *Penicillium citrinum*. *Journal of Industrial Microbiology* **13,** 292-299.

Prevost, X. M. (2003) SA coal resources and reserves, a present-day outlook. *Application of Computers and Operations Research in the Minerals Industries*, South African Institute of Mining and Metallurgy. https://www.saimm.co.za/Conferences/Apcom2003/099-Prevost.pdf.

Pritchard, S. L., Charlton, W. L., Baker, A. and Graham, I. A. (2002). Germination and storage reserve mobilization are regulated independently in Arabidopsis. *The Plant Journal* **31**(5), 639- 647.

Prusty, R., Grisafi, P. and Fink, G. R. (2004) The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences of the United State of America **101,** 4153-7. PMID:15010530; http://dx.doi.org/10.1073/pnas.0400659101.

Quaratino, D., Federici, F., Petruccioli, M., Fenice, M. and D'Aannibale, A. (2007) Production, purification and partial characterisation of a novel laccase from the white-rot fungus *Panus tigrinus* CBS 577.79. *Antonie Leeuwenhoek* **91**, 57-69.

Raeder, U. and Broda, P. (1984) Comparison of the lignin-degrading white-rot fungi *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum* at the DNA level. *Current Genetics* **8,** 499-506.

Rajashekhar, D., Srilatha, M., Rao, P. C., Sharma, S. H. K. and Rekha, K. B. (2017) Functional

and spectral characterization of humic fractions obtained from organic manures. *International Journal of Pure and Applied Bioscience* **5,** 1254-1259. http://dx. doi.org/10.18782/2320-7051.5454.

Ralph, J. P. and Catcheside, D.E.A. (1994) Decolourisation and depolymerisation of solubilised low-rank coal by the white-rot basidiomycete *Phanerochaete chrysosporium. Applied Microbiology and Biotechnology* **42,** 536 -542.

Rashid, G. M., Taylor, C. R., Liu, Y., Zhang, X., Rea, D., Fulop, V. and Bugg, T. D. H. (2015) Identification of manganese superoxide dismutase from *Sphingobacterium* sp. T2 as a novel bacterial enzyme for lignin oxidation. *ACS Chemical Biology* **10**, 2286-94.

Ray, S., Mishra, S., Bisen, K., Singh, S., Sarma, B. K. and Singh, H. B. (2018) Modulation in phenolic root exudate profile of *Abelmoschus esculentus* expressing activation of defense pathway. *Microbiological Research* **207,** 100-107.

Read, D. B., Bengough, A. G., Gregory, P. J., Crawford, J. W., Robinson, D., Scrimgeour, C. M., Young, I. M., Zhang, K. and Zhang, X., (2003). Plant roots release phospholipid surfactants that modify the physical and chemical properties of soil. *New Phytologist* **157**, 315- 321.

Redmile-Gordon, M. A., Armenise, E., White, R. P., Hirsch, P. R. and Goulding, K. W. T. (2013). A comparison of two colorimetric assays, based upon Lowry and Bradford techniques, to estimate total protein in soil extracts. *Soil Biology and Biochemistry*, **67,** 166-173.

Redmile-Gordon, M. A., Brookes, P. C., Evershed, R. P., Goulding, K. W. T. and Hirsch, P. R. (2014) Measuring the soil-microbial interface: Extraction of extracellular polymeric substances (EPS) from soil biofilms. *Soil Biology & Biochemistry* **72,** (2014) 163-171.

Rezácová, V. and Gryndler, M. (2006) Fluorescence spectroscopy: A tool to characterize humic substances in soil colonized by microorganisms? *Folia Microbiological* **51,** 215-221.

Robards, A. W. (1991) Rapid-freezing methods and their applications. In: Hall, J. L. and Hawes, C. (eds) Electron microscopy of plant cells. *Academic Press Limited*,

London. **pp 258**-301. ISBN 0-12-318880-6.

Rohrbacher, F, and St-Arnaud M. (2016). Root Exudation: The ecological driver of hydrocarbon rhizoremediation. *Agronomy* **6,** 19. doi:10.3390/agronomy6010019.

Rose, P. D., Igbinigie, E. E., Horan, M. P., Dames, J. F. and Mukasa-Mugerwa, T. T. (2010) Beneficiation of coal *South African Patent Office*, ZA 2010/02354.

Rovira, A. D., Foster, R. C. and Martin, J. K. (1979) Note on terminology: origin, nature and nomenclature of the organic materials in the rhizosphere. In J. L. Hartley, R. Scott-Russell, eds, The Soil-Root Interface. Academic Press, New York, **pp 1**-4.

Rudrappa, T., Czymmek, K. J., Pare, P. W. and Bais, H. P. (2008) Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiology* **148,** 1547–1556.

Ruiz-Duenas, F. J., Morales, M., Perez-Boada, M., Choinowski, T., Martinez, M. J., Piontek, K. and Martinez, A. T. (2007). Manganese oxidation site in *Pleurotus eryngii* versatile peroxidase: A site-directed mutagenesis, kinetic, and crystallographic study. *Biochemistry* **46,** 66-77. doi:10.1021/bi061542h.

Rutter, B. D. and Innes R. W. (2017) Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. *Plant Physiology* **173,** 728-741

Ryan, P., Delhaize, E. and Jones, D. (2001). Function and mechanism of organic anion exudation from plant roots. *Annual Review of Plant Biology* **52,** 527-560.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.

Salt, D. E., Smith, R. D. and Raskin, I. (1998) 'Phytoremediation', *Annual Review of Plant Physiology and Plant Molecular Biology* **49,** 643-668.

Samson, R. A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J. C. and Varga, J. (2007) Diagnostic tools to identify black Aspergilli. *Studies in Mycology* **59,** 129-145.

Sariaslani, F. S. (1989) Microbial enzymes for oxidation of organic molecules, *Critical Review in Biotechnology* **9,**171-257.

Sarker, A. and Al-Rashid, J. (2013) Analytical Protocol for determination of Indole 3 acetic acid (IAA) production by Plant Growth Promoting Bacteria (PGPB). *Technical Report*.

Sasse, J., Martinoia, E. and Northen, T. (2018) Feed your friends: do plant exudates shape the root microbiome? *Trends in Plant Science* **23,** 25-41.

Schilmiller, A. L., Last, R. L., and Pichersky, E. (2008). Harnessing plant trichome biochemistry for the production of useful compounds. *The Plant Journal* **54**, 702-711.

Schnoor, J. L., Licht, L. A., McCutcheon, S. C., Wolfe, N. L. and Carreira, L. H. (1995) Phytoremediation of organic and nutrient contaminants. *Environmental Science & Technology* **29,** 318A–23A. DOI: 10.1021/es00007a002.

Schweinfurth, S. P. (2009) An introduction to coal quality, *in* Pierce, B. S., and Dennen, K. O., eds., The National Coal Resource Assessment Overview: U.S. Geological Survey Professional Paper 1625–F, Chapter C, 16 p.

Sechet, J., Marion-Poll, A. and North, H. M. (2018) Emerging functions for cell wall polysaccharides accumulated during eudicot seed development. *Plants* **7,** 81.

Sekhohola, L. M., Igbinigie, E. E. and Cowan, A. K. (2013) Biological degradation and solubilisation of coal. *Biodegradation* **24**, 305-318.

Sekhohola, L. M., Isaacs, M. L.and Cowan, A. K. (2014) Fungal colonization and enzyme-mediated metabolism of waste coal by *Neosartorya fischeri* strain ECCN 84. *Bioscience, Biotechnology, and Biochemistry* **78,** (10)1797-1802. DOI:10.1080/09 168451.2014.930325.

Sekhohola, L. M. and Cowan, A. K. (2017) Biological conversion of low-grade coal discard to a humic substance-enriched soil-like material. *International Journal of Coal Science and Technology* **4,** 183–190 (2017). https://doi.org/10.1007/s40789-017-0167-0.

Shahzad, A., Siddiqui, S., Bano, A., Sattar, S., Hashmi, M. Z., Qin, M. and Shakoor, A. (2020) Hydrocarbon degradation in oily sludge by bacterial consortium assisted with alfalfa (*Medicago sativa* L.) and maize (*Zea mays* L.). *Arabian Journal of Geosciences* **13**, 879. https://doi.org/10.1007/s12517-020-05902-w.

Shen, X., Yang, F., Xiao, C. and Zhou, Y. (2020) Increased contribution of root exudates to soil carbon input during grassland degradation. *Soil Biology and Biochemistry* **146,** (2020): 107817. https://doi.org/10.1016/j.soilbio.2020.107817.

Shukla, K, Sharma, S, Sing, N, Sing, V, Tiwari, K, and Singh, S. (2011). Nature and role of root exudates: Efficacy in bioremediation. *African Journal of Biotechnology* **10**(98), 9717-9724.

Sigoillot, J. C., Berrin, J. G., Bey, M., Lesage-Meessen L., Levasseur, A., Lomascolo, A., Record, E. and Uzan-Boukhris, E. (2012) Fungal Strategies for Lignin Degradation. *Advances in Botanical Research* **61,** 263-308. DOI: 10.1016/b978-0-12-416023-1.00008-2.

Silva-Stenico, M. E., Vengadajellum, C. J., Janjua, H. A., Harrison, S. T. L., Burton, S. G. and Cowan, D. A. (2007) Degradation of low rank coal by *Trichoderma atroviride* ES11. *Journal of Industrial Microbiology & Biotechnology* **34,** 625-31. doi:10.1007/s10295-007-0223-7.

Singh, A. D. and Kumar, S. R. (2010) Ligninolytic fungal laccases and their biotechnological applications. *Applied Biochemistry and Biotechnology* **160,** 1760-88.

Singh, A., Gupta, R. and Pandey, R. (2016) Rice seed priming with picomolar rutin enhances rhizospheric Bacillus subtilis CIM colonization and plant growth. *PLoS One* **11,** e0146013.

Singh, H., Dixit, S., Singh, P. and Verma, P. C. (2013). Differential peroxidase activities in three different crops upon insect feeding. *Plant signaling & behavior* **8**(9), e25615. https://doi.org/10.4161/psb.25615.

Smit, A. and Huijgen, W. (2017) Effective fractionation of lignocellulose in herbaceous biomass and hardwood using a mild acetone organosolv process. *Green Chemistry* **19**, 5505.

Smoleňová, E., Pokorný, R., Kaliňák, M., Liptaj, T., Šimkovič, M. and Varečka, L. (2020) Degradation of low-rank coal excavated from coal-mine Záhorie by filamentous fungi. *Acta Chimica Slovaca* **13**(2), 1-9. DOI: 10.2478/acs-2020-0019.

Socrates, G. (2004) Infrared and raman characteristic group frequencies: Tables and Charts, 3rd Edition. Wiley: New York, NY. ISBN: 978-0-470-09307-8.

Somers, E., Vanderleyden, J. and Srinivasan, M. (2004) Rhizosphere bacterial signalling: A love parade beneath our feet. *Critical Reviews in Microbiology* **30,** 205-240.

Sondreal, E. A. and Wiltsee, G. A. (1984) Low-Rank Coal: Its Present and Future Role in the United States. *Annual Review of Energy* **9,** 473-99.

Spaepen, S. and Vanderleyden, J. (2011) Auxin and plant-microbe interactions. *Cold Spring Harbor Perspectives* in *Biology* **3,** a001438.

Steinauer, K., Chatzinotas, A. and Eisenhauer, N. (2016) Root exudate cocktails: the link between plant diversity and soil microorganisms? *Ecology and Evolution* **6,** 7387-7396. DOI: 10.1002/ece3.2454.

Sterjiades, R., Dean, J. F. and Eriksson, K. E. (1992) Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. *Plant Physiology* **99**(3):1162-1168. doi:10.1104/pp.99.3.1162.

Strandberg, G. W. and Lewis, S. N. (1987) A method to enhance the microbial liquefaction of lignite coals. *Biotechnology and Bioengineering symposium* **17,** 153.

Stremersch, S., Vandenbroucke, R. E., Wonterghem, E. V., Hendrix, A., De Smedt, S. C. and Raemdonck, K. (2016) Comparing exosome-like vesicles with liposomes for the functional cellular delivery of small RNAs. *Journal of Controlled Release* **232,** 51-61.

St. Thomas (2019) "Spectroscopic Tools". URL: http://www.science-and-fun.de/tools/

Sun, S., Wang, J., Zhu, L., Liao, D., Gu, M., Ren, L., Kapulnik, Y. and Xu, G. (2012). An active factor from tomato root exudates plays an important role in efficient establishment of mycorrhizal symbiosis. PLOS ONE **7,** e43385.

Sundaramoorthy, M., Kishi, K., Gold, M. H. and Poulos, T. L. (1994) The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-A resolution. *Journal of Biological Chemistry* **269,** 32759-32767.

Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K. and Inamori, Y. (2003) A thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. *Bioscience, Biotechnology and Biochemistry* **67**, 2167-75.

Tablin, F., Wolkers, W. F., Walker, N. J., Oliver, A. E., Tsvetkova, N. M., Gousset, K., Crowe, L. M., and Crowe, J. H., (2001). Membrane Reorganization during Chilling: Implications for Long-Term Stabilization of Platelets, *Cryobiology* **43**, 114-123.

Tahir, M. M., Khurshid, M., Khan, M. Z., Abbasi, M. K. and Kazmi, M. H. (2011) Lignite-derived humic acid effect on growth of wheat plants in different soils. *Pedosphere* **21**(1),124-131.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30,** 2725-2729.

Tarasov, D., Leitch, M. and Fatehi, P. (2018). Lignin–carbohydrate complexes: properties, applications, analyses, and methods of extraction: a review. *Biotechnology for Biofuels* **11**, 269. https://doi.org/10.1186/s13068-018-1262-1.

Taylor, J. W. (1995) "Making the Deuteromycota redundant: a practical integration of mitosporic and meiosporic fungi". *Canadian Journal of Botany* **73** (1), 754-759. doi:10.1139/b95-319.

Tebo, B. M., Bargar, J. R., Clement, B. G., Dick, G. J., Murray, K. J., Parker, D., Verity, R. and Webb, S. M. (2004) Biogenic manganese oxides: properties and mechanisms of formation. *Annual Review of Earth and Planetary Sciences* **32**, 287-328. https://doi.org/10.1146/annurev.earth.32.101802.120213.

Tebo, B. M., Johnson, H. A., McCarthy, J. K. and Templeton, A. S. (2005) Geomicrobiology of manganese (II) oxidation. *Trends in Microbiology* **13**, 421-428.

Thomas, C., Butler, A., Larson, S., Medina, V. and Begonia, M. (2014) Complexation of lead by Bermuda grass root exudates in aqueous media, *International Journal of Phytoremediation*, **16**(6), 634-640. DOI: 10.1080/15226514.2013.803023.

Thurston, C. F. (1994) The structure and function of fungal laccases. *Microbiology* **140,** 19-26.

Tian, J. H., Pourcher, A. M., Bouchez, T., Gelhaye, E. and Peu, P. (2014) Occurrence of lignin degradation genotypes and phenotypes among prokaryotes. *Applied Microbiology and Biotechnology* **98**, 9527-44.

Tien, M. and Kirt, T. K. (1983) Lignin-degrading enzyme from the hymenomycetes *Phanerochaete chrysosporium* burds. *Science*, **221,** 661-663, doi:10.1126/science.221.4611.661.

Titto, R. J. (1985). Phenolic constituents on leaves of northern willows: Methods for the analysis of certain phenolics. *Journal of Agricultural and Food Chemistry* **33**, 213-217.

Todorovic, C., Nguyen, C., Robin, C. and Guckert, A. (2001) Root and microbial involvement in the kinetics of 14C-partitioning to rhizosphere respiration after a pulse labelling of assimilates. *Plant and Soil* **228,** 179-189.

Toyama, T., Furukawa, T., Maeda, N., Inoue, D., Sei, K., Mori, K., Kikuchi, S. and Ike, M. (2011) Accelerated biodegradation of pyrene and benzo[a]pyrene in the *Phragmites australis* rhizosphere by bacteria-root exudate interactions. *Water Research* **45,** 1629-1638.

Truter, W. F., Rethman, N. F. G., Potgieter, C. E. and Kruger, R. A. (2009) Re-vegetation of cover soils and coal discard material ameliorated with class F fly ash. World of coal ash (WOCA) conference, May 4-7, 2009 in Lexington, KY, USA.

Turbat, A., Rakk, D., Vigneshwari, A., Kocsubé, S., Thu, H., Szepesi, A., Bakacsy, L., Škrbic, B. D., Jigjiddorj, E., Vágvölgyi, C. and Szekeres, A. (2020) Characterization of the plant growth-promoting activities of endophytic fungi isolated from *Sophora flavescens*. *Microorganisms* **8,** 683. doi:10.3390/microorganisms805068.

Upton, B. M. and Kasko, A. M. (2016) "Strategies for the conversion of lignin to high-value polymeric materials: review and perspective," *Chemical Reviews* **116,** (4) 2275-2306.

Uren, N. C. (2007) Types, amounts, and possible functions of compounds released into the

rhizosphere by soil-grown plants. *In* The Rhizosphere; CRC Press: New York, NY, USA, pp. 1-21.

Uribe-Alvarez, C., Ayala, M., Perezgasga, L., Naranjo, L., Urbina, H. and Vazquez-Duhalt, R. (2011) First evidence of mineralization of petroleum asphaltenes by a strain of *Neosartorya fischeri. Microbial Biotechnology* **4**(5), 663-672.

Vanhercke, T., El Tahchy, A., Liu, Q., Zhou, X. R., Shrestha, P., Jean-Philippe, R. U., Mansour, M. P., Nichols, P. D., James, C. N., Horn, P. J., Chapman, K. D., Beaudoin, F.,Ruiz-López, N., Larkin, P. J., de Feyter, R. C., Singh, S. P., Petrie, J. R. (2014). Metabolic engineering of biomass for high energy density: oilseed-like triacylglycerol yields from plant leaves. *Plant Biotechnology Journal* **12**, 231-239.

Varga, J., Szigeti, G., Baranyi, N., Kocsube, S., O'Gorman, C. M., and Dyer, P. S. (2014) *Aspergillus*: sex and recombination. *Mycopathologia* **178,** 349-362.

Vierheilig, H., Lerat, S. and Piché, Y. (2003) Systematic inhibition of arbuscular mycorrhiza development by root exudates of cucumber plants colonized by *Glomus mosseae*. *Mycorrhiza* **13,** 167-170.

Veitch, N. C. (2004) Structural determinants of plant peroxidase function. *Phytochemistry Reviews* **3,** 3-18. https://doi.org/10.1023/B:PHYT.0000047799.17604.94.

Venosa, A. D. and Zhu, X. (2003) Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands. *Spill Science & Technology Bulletin* **8,** 163-178.

Vidali, M. (2001). Bioremediation: An overview. *Pure and Applied Chemistry* **73,** 1163-1172.

Videla, H. A Herrera, L. K. (2005) Microbiologically influenced corrosion; looking to the future. *Journal of International Microbiology* **8,** 169-180.

Voiniciuc, C., Pauly, M. and Usadel, B. (2018) Monitoring polysaccharide dynamics in the plant cell wall. *Plant Physiology* **174,** 2590-2600.

Vranova, V., Rejsek, K. and Formanek, P. (2013) Aliphatic, cyclic, and aromatic organic acids, vitamins, and carbohydrates in soil: A review. *The Scientific World Journal* **2013,**15. http://dx.doi.org/10.1155/2013/524239.

Wagner, G. J., Wang, E. and Shepherd, R. W. (2004). New approaches for studying and exploiting an old protuberance, the plant trichome. *Annals of Botany* **93**, 3-11.

Wang, J., Feng, J., Jia, W., Chang, S., Li, S. and Li, Y. (2015) Lignin engineering through laccase modification: A promising field for energy plant improvement. *Biotechnology for Biofuels* **8,** 145.

Wang, T., Su, D., Wang, X. and He, Z. (2020) Adsorption-degradation of polycyclic aromatic hydrocarbons in soil by immobilized mixed bacteria and its effect on microbial communities. *Journal of Agricultural and Food Chemistry* **68,** 14907-14916.

Wei, X., Wei, H., Viadero, R. C. (2011) Post-reclamation water quality trend in a Mid-Appalachian watershed of abandoned mine lands, *Science of Total Environment* **409**(5), 941–948.

Weston, L. A., Ryan, P. R. and Watt, M. (2012) Mechanisms for cellular transport and release of allelochemicals from plant roots into the rhizosphere. *Journal of Experimental Botany* **63,** 3445-3454.

Wheeler, B. D. and Proctor, M. C. F. (2000) Ecological gradients, subdivisions and terminology of north-west European mires. *Journal of Ecology* **88,** 187-203.

White, T. J., Bruns, T. D., Lee, S. S. and Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp 315-322 In: PCR protocols: a guide to methods and applications, eds. Innis, M. A., Gelf, D. H., Sninsky, J. J., White, T. J. Academic Press, Inc., NewYork.

Wieland, G., Neumann, R. and Backhaus, H. (2001) Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. *Applied Environmental Microbiology* **67,** 5849-5854.

Williams, A. and de Vries, F. T. (2019). Plant root exudation under drought: implications for ecosystem functioning. *New phytologist* doi: 10.1111/nph.16223

Woith, E. and Melzig, M. F. (2019) Extracellular vesicles from fresh and dried plants-

simultaneous purification and visualization using gel electrophoresis International *Journal of Molecular Sciences* 20, 357. doi:10.3390/ijms20020357.

Wolkers, W. F., Oliver, A. E., Tablin, F. and Crowe, J. H. (2004). A Fourier-transform infrared spectroscopy study of sugar glasses. *Carbohydrate Research* **339** (6),1077-85. doi: 10.1016/j.carres.2004.01.016

Wong, D. W. S. (2009) Structure and action mechanism of ligninolytic enzymes. *Applied Biochemistry and Biotechnology* **157,**174-209. DOI 10.1007/s12010-008-8279-z.

Xia, Y., Amna, A. and Opiyo, S. O. (2018). The culturable endophytic fungal communities of switchgrass grown on a coal-mining site and their effects on plant growth. *PLoS ONE* **13**(6), e0198994. https://doi.org/10.1371/journal. pone.0198994

Xiao, L., Li, Y., Liao, Y., Huirong Ma, H., Wu, J., Zhang, Y. and Yao, J. (2018) Bioconversion of lignite humic acid by white-rot fungi and characterization of products. *3 Biotech* **8,** 258. https://doi.org/10.1007/s13205-018-1281-4.

Yang, Y., Yang, J., Li, B., Wang, E. and Yuan, H. (2018) An esterase from *Penicillium decumbens* P6 involved in lignite depolymerization. *Fuel* **214**(2018), 416–422. https://doi.org/10.1016/j.fuel.2017.11.035.

Yokota, D., Moraes, M., and Pinho, S. C. (2012) Characterization of lyophilized liposomes produced with non-purified soy lecithin: A case study of casein hydrolysate microencapsulation. *Brazilian Journal of Chemical Engineering* **29**, 325–335.

Yoshida, H. (1883). Chemistry of lacquer (urushi). *Journal of the Chemical Society* **43,** 472-486.

Yoshitomi K. J. and Shann, J. R. (2001) Corn (*Zea mays* L.) root exudates and their impact on ^{14}C-Pyrene mineralization, *Soil Biology & Biochemistry* **33,** 1769-1776.

Youn, H. D., Hah, Y. C. and Kang, S. O. (1995). Role of laccase in lignin degradation by white-rot fungi. *FEMS Microbiology Letter* **132,** 183–188 doi:10.1111/j.15746968.1995.tb07831.x.

Yuan, H. L., Yang, J. S., Wang, F. Q. and Chen, W. X. (2006) Degradation and solubilization

of Chinese lignite by *Penicillium* sp. P6. *Applied Biochemistry and Microbiology* **42,** 52-55.

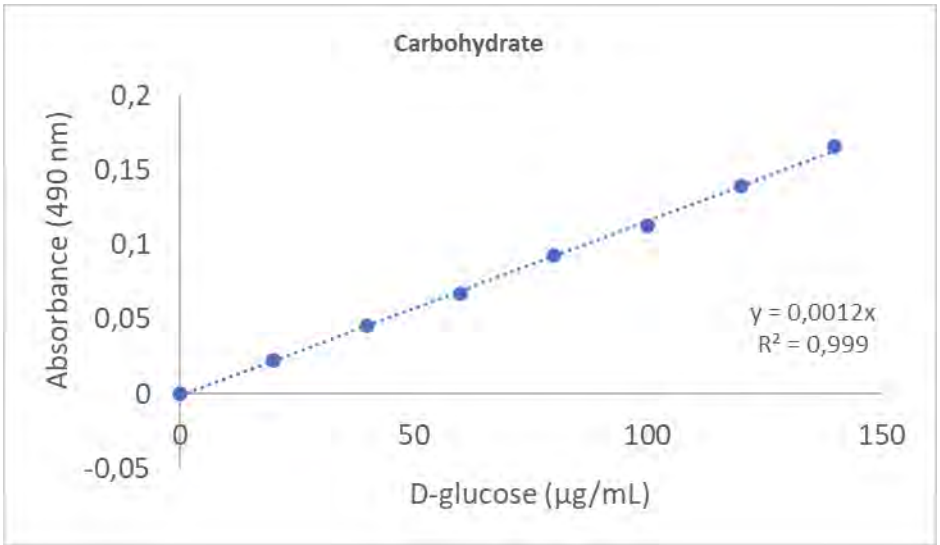
Zhao, Q., Nakashima, J., Chen, F., Yin, Y., Fu, C. and Yun, J. (2013) Laccase is necessary and nonredundant with Peroxidase for lignin polymerization during vascular development in Arabidopsis. *Plant Cell* **25,** 3976-3987. doi: 10.1105/tpc. 113.117770.

Zhou, L., Yuan, L., Zhao, B., Li, Y. and Lin, Z. (2019). Structural characteristics of humic acids derived from Chinese weathered coal under different oxidizing conditions. *PLoS ONE* **14**(5), e0217469.https://doi.org/10.1371/journal.pone.0217469.

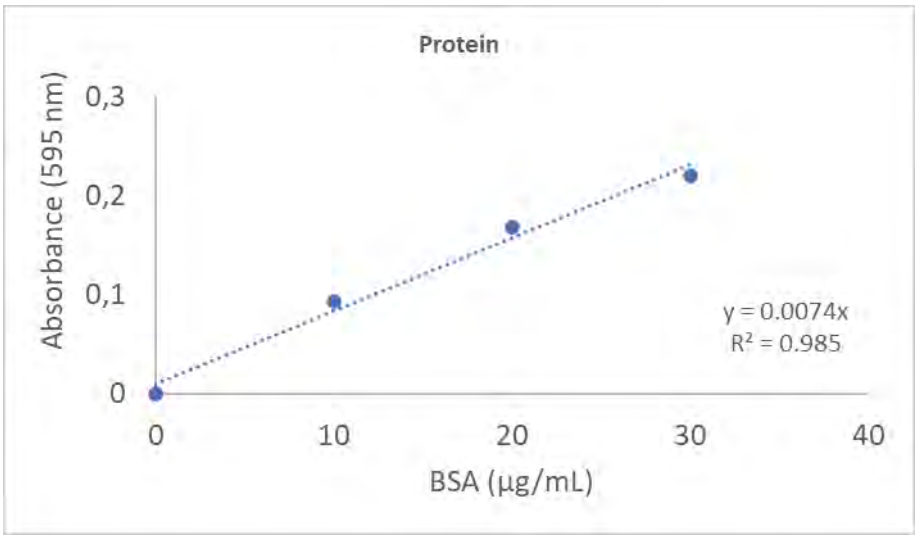
Zouari-Mechichi, H., Mechichi, T., Dhouib, A., Sayadi, S., Martínez, A. T. and Martínez, M. J. (2006) Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: Decolorization of textile dyes by the purified enzyme. *Enyzme and Microbial Technology* **39,** 141–148. https://doi.org/10.1016/j.enzmictec.2005.11.027

Appendices

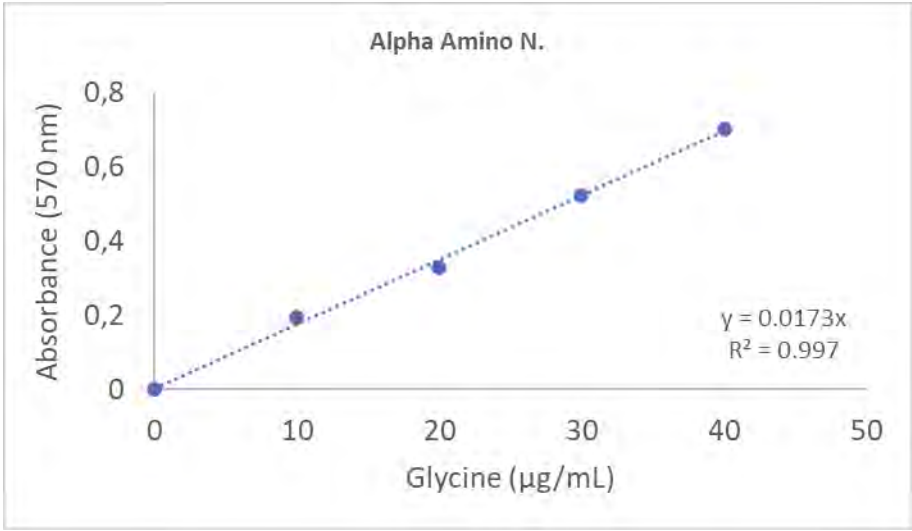
Appendix I: Carbohydrate standard calibration curve



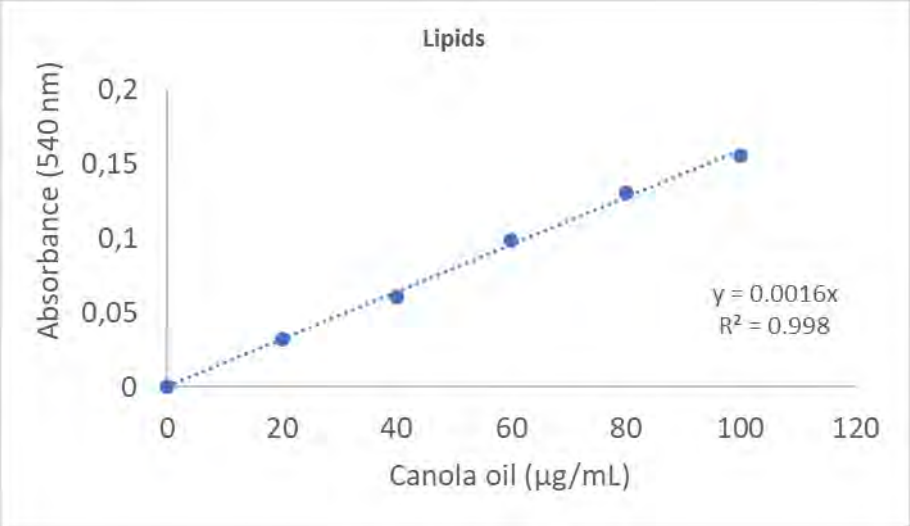
Appendix II: Protein standard calibration curve



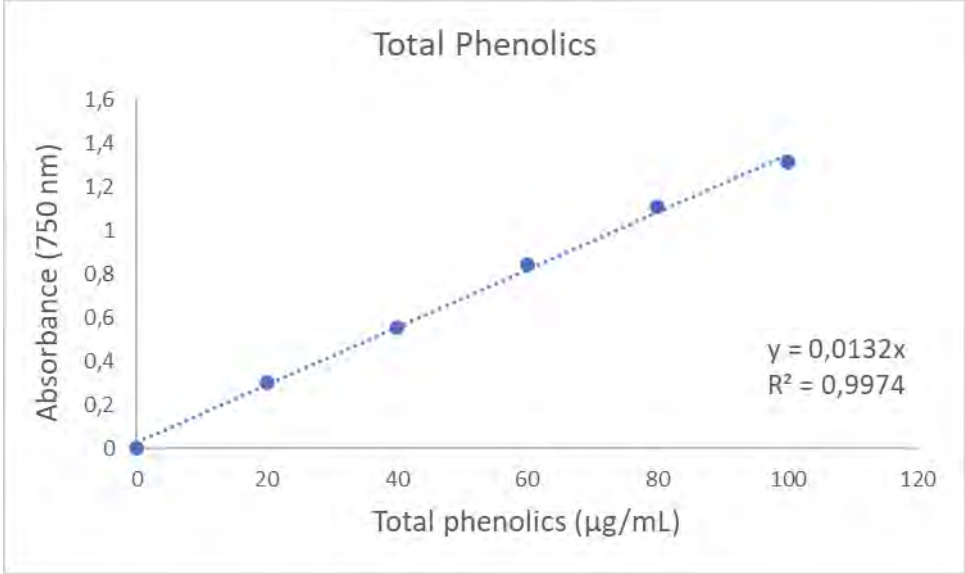
Appendix III: Alpha amino nitrogen standard calibration curve



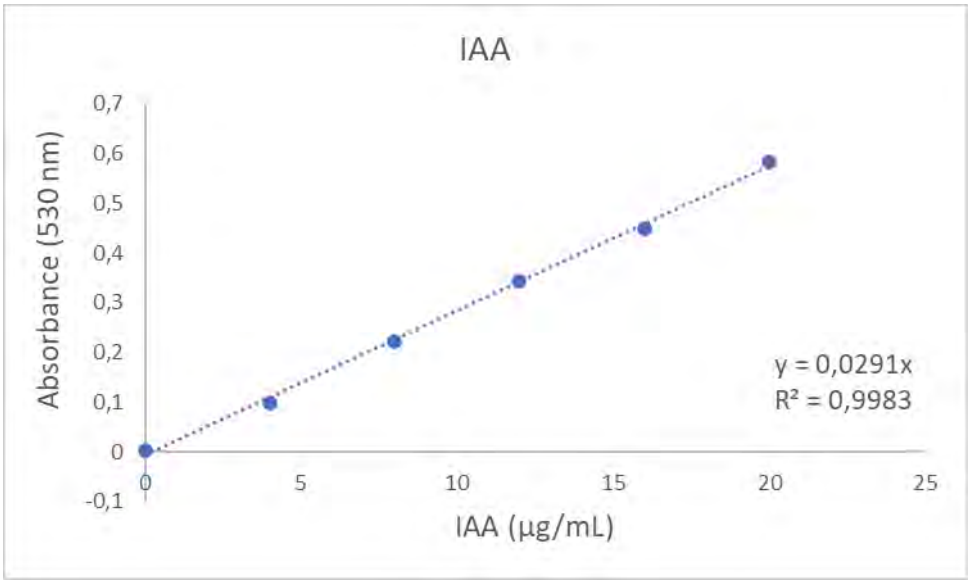
Appendix IV: Lipids standard calibration curve



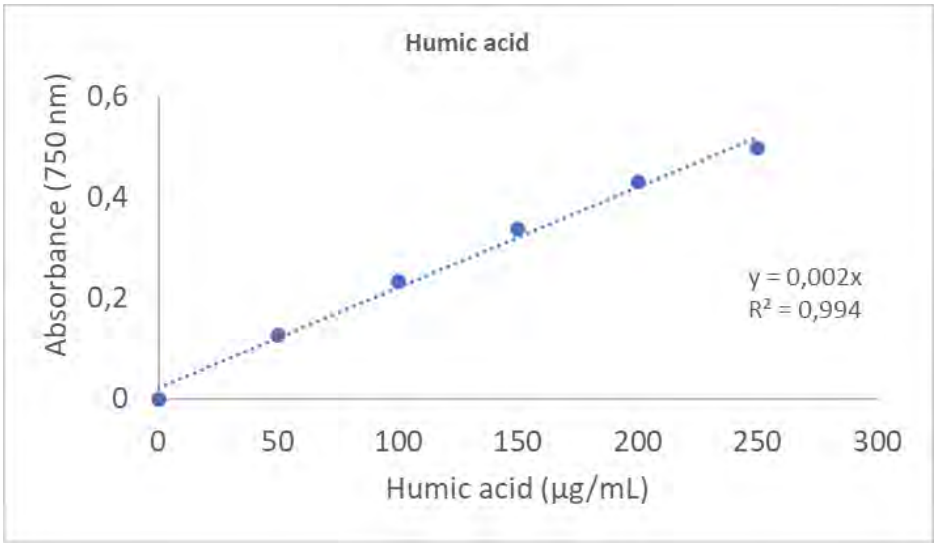
Appendix V: Total phenolics standard calibration curve



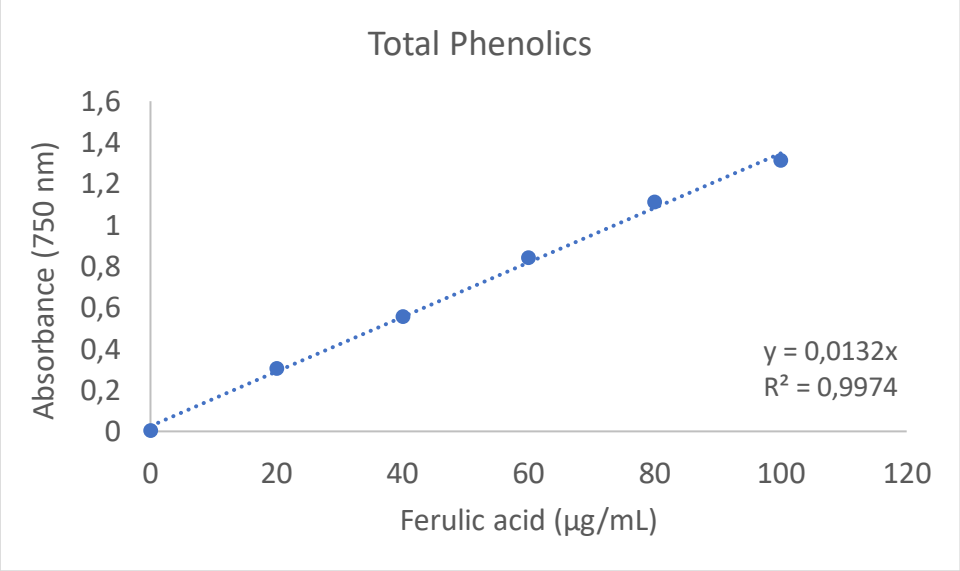
Appendix VI: IAA standard calibration curve



Appendix VII: Humic acid standard calibration curve



Appendix VIII: Total phenolics standard calibration curve



Appendix IX. Gene sequences for the fungal strains

- ***Aspergillus* sp. strain ECCN 84 (Accession number- MT239561)**

CTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCT
CCCACCCGTGTCTATTGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGG
GGAGGCCTCGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCTG
AAAGTATGCAGTCTGAGTTGATTATCATAATCAGTTAAAACTTTCAACAACGGATCTCTT
GGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAATAATGTGAATTGCAGAATT

CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC
TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCGCCGTCCCCGGTT
TCCCCCGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATG
GGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCCAGCCGACACCCAACTTTATTTCT
AAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA
GGAA

- ***Aspergillus* sp. strain ECCN 225 (Accession number- MT239564)**

CTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCT
CCCACCCGTGTCTATCGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGG
GGAGGCCTCGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCTG
AAAGTATGCAGTCTGAGTTGATTATCATAATCAGTTAAAACTTTCAACAACGGATCTCTT
GGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC
TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGTCCCCTCCCCC
GGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTT
TGTCACCCGCTCTGTAGGCCCGGCCGGCGCCAGCCGACACCCAACTTTATTTTCTAAGGT
TGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

- ***Penicillium* sp. strain ECCN 243 (Accession number- MT239576)**

CTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCT
CCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGG
GCTTACGCCCCCGGGCCCGCGCCCGCCGAAGACACCCTCGAACTCTGTCTGAAGATTGAA
GTCTGAGTGAAAATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCAT
CGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAAATTCAGTGAATCATC
GAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTC
ATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCCCGATCTCCGGGGGACGG
GCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCC
GCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAATTTTTATCCAGGTTGACCTCG
GATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

Appendix X. ABTS regression equation (The slope of the equation was used to derive the LAC activity).

Treatments	Regression Equation
ECCN 84 (Control)	y = -0,0001x + 0,0072
ECCN 84 + EPS	y = 0,0263x + 0,328
ECCN 84 + P-coal	y = -0,0001x + 0,4703
ECCN 84 + EPS + P-coal	y = 0,0149x + 0,8201
ECCN 225 (Control)	y = -9E-05x + 0,0269
ECCN 225 + EPS	y = 0,0084x + 0,1106
ECCN 225 + P-coal	y = 0,0005x + 0,7964
ECCN 225 + EPS + P-coal	y = 0,0138x + 0,8231
ECCN 243 (Control)	y = -0,0008x + 0,02
ECCN 243 + EPS	y = 0,0224x + 0,2393
ECCN 243 + P-coal	y = 0,0002x + 0,0054
ECCN 243 + EPS + P-coal	y = 0,0115x + 0,4838

Appendix XI: Physicochemical analysis: (pH, Electrical conductivity (EC) and Bulk density)

Keywords: Control = C; Spores only = Strain 84 only; Maize + Spores = MS84; Maize + Okra + Spores = MOS84

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
pH	Control	3	3.8400	.13077	.07550	3.5152	4.1648	3.75	3.99
	Spore only	3	3.3433	.05508	.03180	3.2065	3.4801	3.28	3.38
	Maize only	3	5.3200	.56630	.32696	3.9132	6.7268	4.70	5.81
	Maize and spore	3	5.7500	.26907	.15535	5.0816	6.4184	5.45	5.97
	Maize, Okra and spore	3	6.0233	.16862	.09735	5.6045	6.4422	5.83	6.14
	Total	15	4.8553	1.13314	.29258	4.2278	5.4828	3.28	6.14
EC	Control	3	120.00	17.321	10.000	76.97	163.03	110	140
	Spore only	3	136.67	37.859	21.858	42.62	230.71	110	180
	Maize only	3	246.67	83.267	48.074	39.82	453.51	180	340
	Maize and spore	3	436.67	90.738	52.387	211.26	662.07	340	520
	Maize, Okra and spore	3	623.33	170.098	98.206	200.79	1045.88	450	790
	Total	15	312.67	214.592	55.407	193.83	431.50	110	790
Bulk density	Control	3	.7300	.05292	.03055	.5986	.8614	.67	.77
	Spore only	3	.7233	.04509	.02603	.6113	.8353	.68	.77
	Maize only	3	.8000	.04000	.02309	.7006	.8994	.76	.84
	Maize and spore	3	.8133	.07506	.04333	.6269	.9998	.74	.89
	Maize, Okra and spore	3	.8400	.03000	.01732	.7655	.9145	.81	.87
	Total	15	.7813	.06457	.01667	.7456	.8171	.67	.89

Homogeneous Subsets

pH

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Spore only	3	3.3433		
	Control	3	3.8400		
	Maize only	3		5.3200	
	Maize and spore	3		5.7500	5.7500
	Maize, Okra and spore	3			6.0233
	Sig.		.068	.107	.286

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

EC

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Control	3	120.00		
	Spore only	3	136.67		
	Maize only	3	246.67		
	Maize and spore	3		436.67	
	Maize, Okra and spore	3			623.33
	Sig.		.153	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Bulk density

	Treatment	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Spore only	3	.7233	
	Control	3	.7300	
	Maize only	3	.8000	.8000
	Maize and spore	3	.8133	.8133
	Maize, Okra and spore	3		.8400
	Sig.		.071	.380

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix XII: Statistical data for Humic/humic-like substances

Keywords: Control = C; Strain 84 only = S; Maize only = M; MS84 = MS; MOS84 = MSO

Descriptives Conc(µg/mL)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
C	3	160.867	29.3655	16.9542	87.919	233.815	127.2	181.2
M	3	209.700	40.8534	23.5867	108.215	311.185	182.7	256.7
S	3	148.700	28.1469	16.2506	78.779	218.621	132.2	181.2
MS	3	166.700	14.6544	8.4607	130.297	203.103	150.2	178.2
MSO	3	139.033	9.7511	5.6298	114.810	163.256	132.2	150.2
Total	15	165.000	33.9547	8.7671	146.197	183.803	127.2	256.7

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Conc(µg/mL)

	Treatment	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	MSO	3	139.033	
	S	3	148.700	
	C	3	160.867	160.867
	MS	3	166.700	166.700
	M	3		209.700
	Sig.		.268	.060

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix XIII: Statistical data for Total phenolics

Keywords: Control = C; Strain 84 only = S; Maize only = M; MS84 = MS; MOS84 = MSO

Descriptives

Conc(µg/mL)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
C	3	12.55033	1.731190	.999503	8.24982	16.85085	11.439	14.545
M	3	18.00500	5.536280	3.196373	4.25212	31.75788	14.621	24.394
S	3	13.25733	1.513168	.873628	9.49842	17.01625	11.515	14.242
MS	3	14.87367	1.017376	.587382	12.34636	17.40097	13.712	15.606
MSO	3	14.04067	1.578868	.911560	10.11854	17.96279	12.652	15.758
Total	15	14.54540	3.080378	.795350	12.83954	16.25126	11.439	24.394

Homogeneous Subsets

Conc(µg/mL)

	Treatment	N	Subset for alpha = 0.05
			1
Duncan[a]	C	3	12.55033
	S	3	13.25733
	MSO	3	14.04067
	MS	3	14.87367
	M	3	18.00500
	Sig.		.054

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix XIV: Statistical data for indole-indole-like compounds

Keywords: Control = C; Strain 84 only = S; Maize only = M; MS84 = MS; MOS84 = MSO

Descriptives

Conc(µg/mL)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
C	3	1.66100	.248266	.143336	1.04427	2.27773	1.375	1.821
M	3	1.76400	.310140	.179060	.99357	2.53443	1.443	2.062
S	3	1.60367	.441534	.254920	.50684	2.70050	1.100	1.924
MS	3	1.79867	.277705	.160333	1.10881	2.48853	1.478	1.959
MSO	3	1.40867	.124114	.071657	1.10035	1.71698	1.271	1.512
Total	15	1.64720	.289988	.074875	1.48661	1.80779	1.100	2.062

Homogeneous Subsets

Conc(µg/mL)

	Treatment	N	Subset for alpha = 0.05
			1
Duncan[a]	MSO	3	1.40867
	S	3	1.60367
	C	3	1.66100
	M	3	1.76400
	MS	3	1.79867
	Sig.		.171

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.