

Evaluation of Fungcoal as a bioprocess technology for self-cladding of waste coal dumps

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

DOCTOR OF PHILOSOPHY

(Environmental Biotechnology)

of

Rhodes University

By

Lerato M. Sekhohola

FEBRUARY 2015

Abstract

Low-grade coal, a poor source of energy, has long been regarded as waste material by the coal mining industry. Biological degradation of this coal material by ligninolytic fungal strains presents a viable strategy towards eliminating this unusable fossil fuel. To this end, a novel and patented bioprocess termed Fungcoal was developed. Fungcoal is a biological process utilised in the *in situ* treatment of waste coal and is based on the mutualistic relationship between the fungus *Neosartorya fischeri* and the graminaceous species *Cynodon dactylon*. The process facilitates the rapid conversion of waste coal into soil-like material that stimulates establishment of vegetation for eventual coal dump rehabilitation. While a number of *in vitro* studies have identified various fungal strains as efficient coal degraders, the mechanisms involved in the Fungcoal-stimulated degradation process have not been fully elucidated. Furthermore, implementation of Fungcoal at both pilot and commercial scale has not been achieved. Thus the objective of this work was to investigate Fungcoal as a bioprocess via examining the role of coal degrading fungi (CDF) and grasses as biocatalysts in coal biodegradation and for the self-cladding of waste coal dumps.

Initially, waste coal degradation by *N. fischeri*, strain ECCN 84, was investigated, specifically focusing on the mechanisms underpinning the process. *In vitro* studies showed the addition of waste coal induced active fungal colonisation resulting in increased fungal biomass. Increased extracellular laccase (LAC) activity, occurring concomitantly with an increase in hyphal peroxisome proliferation, was also observed in the coal supplied fungal cultures. Analysis of the colonised waste coal revealed a time dependent reduction in the percentage weight of elemental carbon coupled with an increase in elemental oxygen. The results supported metabolism and degradation of waste coal by *N. fischeri* strain ECCN 84 and involvement of fungal extracellular laccase.

The contribution of *C. dactylon*, a C4 grass species to *in situ* biodegradation of waste coal in the presence of coal degrading and mycorrhizal fungi (MF) was also investigated. Enhanced degradation of the waste coal into a humic soil-like material was observed within the rhizosphere. Analysis of the resultant substrate revealed an increased concentration of highly oxidised humic-like substances (HS). Fungi remained viable in the rhizosphere up to 47 weeks post-inoculation and cultivation of *C. dactylon*, indicating the resultant humic substance-rich rhizosphere provided an environment conducive for microbial proliferation and activity. Furthermore, humic substance enrichment of waste coal substrates supported germination and seedling emergence of several agronomic species including *Zea mays* (corn), *Phaseolus vulgaris* (bean), *Pisum sativum* (pea), and *Spinacia oleracea* (spinach).

Use of various cladding materials to support coal biodegradation, by fungus-grass mutualism—and rehabilitation of waste dumps was evaluated at commercial scale. While substantial physico-chemical changes were not evident in the absence of cladding or where waste coal was used as cladding material, successful establishment of grass cover and diversity was achieved within three hydrological cycles on dumps cladded with weathered coal.

Work presented in this thesis successfully demonstrates the degradation of waste coal by *N. fischeri*. The biodegradation process included enhanced extracellular LAC activity coupled with increased

waste coal oxidation. Increased HS concentration of waste coal substrate supported germination and early seedling establishment of several agronomic species. At commercial scale a co-substrate in the form of carbon-rich weathered coal was essential to support fungus-grass mutualism and Fungcoal-induced rehabilitation. These findings support the developed Fungcoal concept and the underpinning rationale that the phyto-biodegradation of waste coal indeed depends on the mutualistic interactions between grass root exudates and the ligninolytic and mycorrhizal fungi. Taken together, these findings provide practical evidence of the contribution of fungi and grasses as mutualists in the biodegradation of waste coal and sustainable rehabilitation of waste coal dumps.

Contents

Abstract	2
Contents	4
Appendix	5
Abbreviations	6
Acknowledgements	7
1. Introduction	9
1.1 Coal degrading fungi	11
1.2 Coal biotechnology	13
1.3 Application of fungal degradation of coal: a remediation strategy	16
1.4 Intergrated phyto-bioremediation technology	17
2. Aims and objectives	20
3. Results and Discussions	21
3.1 Early studies in our laboratory	21
3.2 Fungal colonization and metabolism of waste coal	21
3.3 <i>Cynodon dactylon</i> as a biocatalyst in biodegradation of waste coal	24
3.4 A strategy towards self-cladding and remediation of waste coal dumps	28
4. Conclusions and Perspectives	31
References	34
Paper 1: Biological degradation and solubilisation of coal: a review	43
Paper 2: Fungal colonization and enzyme-mediated metabolism of waste coal	64
Paper 3: Biological degradation of waste coal and seedling establishment in the resultant humic-like material	86
Paper 4: <i>In situ</i> bioremediation and self-cladding of South African coal discard dumps	104
Supplementary information	117

Appendix

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

II - Sekhohola, L.M., Isaacs, M.L. and Cowan, A.K. (2014) Fungal colonisation and enzyme-mediated metabolism of waste coal by *Neosartorya fischeri* strain ECCN 84. *Bioscience, Biotechnology and Biochemistry* 78: 1797 - 1802.

III - Sekhohola, L.M. and Cowan, A.K. (2015) Biological degradation of waste coal and seedling establishment in the resultant humic-like material. (Manuscript in revision for *CLEAN – Soil, Air and Water*)

IV - Sekhohola, L.M., Edeki, O.G. and Cowan, A.K. (2015) *In situ* remediation and self-cladding of South African discard coal dumps. (Manuscript in revision for *The Journal of The South African Institute of Mining and Metallurgy*)

Abbreviations

ABTS	2,2' [azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt]
AMD	Acid Mine Drainage
CEC	Cation exchange capacity
CDF	Coal degrading fungi
EBRU	Institute for Environmental Biotechnology-Rhodes University
EC	Electrical conductivity
ECCN	EBRU Culture Collection Number
EDS	Energy Dispersive x-ray Spectroscopy
FT-IR	Fourier Transform Infrared
HS	Humic-like substances
LAC	Laccase
LiP	Lignin peroxidase
MF	Mycorrhizal fungi
MnP	Manganese peroxidase
PAH	Polycyclic Aromatic Hydrocarbons
SEM	Scanning Electron Microscopy
TOC	Total Organic Carbon

Acknowledgements

This thesis is dedicated to my beloved son

Mohale Christopher Dlamini

The love of God has seen me through this journey and may all the glory and honour be unto Him.

My sincere and deepest gratitude goes to Professor Keith Cowan, my supervisor, for all I have learned from him and for his continuous guidance in all stages of this thesis. I would also like to thank him for inspiring and encouraging me to believe in my work. I am grateful to Anglo American Thermal Coal for the doctoral bursary granted.

I am greatly thankful to Dr Eric Igbinigie for his supervision at the beginning of this work. To the members of staff at the Institute for Environmental Biotechnology – Rhodes University (EBRU); Gisela Eustace, David Render, Richard Laubscher, Michelle Isaacs, Andile Magaba, Nomaindia Toto, Norman Singapi, Tata Elvis (RIP) and Olwethu Baba, many thanks for all your assistance and support which went far and beyond the call of duty from time to time. My dear colleagues at EBRU who have become my brothers and sisters; Lwazikazi Madikiza, Zanele Mlambo, Prudence Mambo, Gerald Edeki, Dirk Westensee, Kubura Tijjani, Hailey Johnson, Dr Yvonne van Breugel, and Dr Bonga Zuma, may you never lose that spirit of *Ubuntu* which kept us going during challenging times – giving up is never an option!

The great mentors from the Women's Academic Solidarity Association (WASA) at Rhodes University who instilled in me great appreciation of being an African woman and a young researcher in the academic world, I salute you all and I believe you will continue to give birth to many great women leaders. To my colleagues and staff members at the Carnegie Research Commons of the Rhodes University library, thank you for your continuous support and enriching conversations during my shifts.

To my dear husband Dr Phesheya Dlamini, I will forever be grateful for your resources which shaped the foundation for my work. Your continuous efforts and endless support kept my vision alive. My greatest appreciation goes to my parents, my siblings Refiloe and Boitumelo Sekhohola,

and the entire family. Without your great patience, love and prayers I would not have been able to push harder every day. My many beloved friends around the world, each one of you is sincerely appreciated for the kind words of encouragement and daily prayers, may the Lord richly bless you all!

1. Introduction

The coal mining industry generates tonnes of low-grade waste coal which is of little or no value, and its handling and storage present an environmental problem. Thus far attempts to rehabilitate such coal material on a commercial scale have been largely unsustainable due to, amongst others, the environmental cost of the rehabilitation measures. Subsequently, the challenge faced by the mining companies is development and implementation of efficient remediation technology for the unsightly dumps of the waste coal material to minimize negative impacts on the environment. The currently employed rehabilitation strategy relies heavily on the use of top soil and this practice further compromises the environment. Coal biodegradation technology has offered a platform for a number of commercial process opportunities, particularly for the remediation and recovery of value from waste and inaccessible coal resources. Among such opportunities is the development of efficient large-scale rehabilitation processes for the waste coal dumps which eliminates the use of top soil.

Previous studies have been carried out at the Institute for Environmental Biotechnology-Rhodes University (EBRU) to explore and develop a novel approach to biological degradation of South African waste coal as a platform technology on which a range of potential large-scale applications could be built. These studies aimed to determine whether the waste hard coal could be biologically oxidised (or weathered) and whether weathered coal can serve as substrate for bioprocesses developed for coal dump rehabilitation. It was further investigated whether the process could translate into large-scale processing technology and thus provide commercially viable opportunities for adding value to waste coal. In the first study, Igbini et al. (2008) isolated and identified a strain of *N. fischeri* from the rhizosphere of *C. dactylon* on coal dumps, which was found to degrade acid-treated waste hard coal *in vitro* however the mechanisms driving the process were never investigated. A subsequent study investigating phyto-bioconversion of hard coal by a consortium of nonmycorrhizal fungi demonstrated coal degradation in the *C. dactylon*/coal rhizosphere (Igbini et al., 2010) but the authors did not investigate the possibility of upscaling the process to commercial scale. Nevertheless, results from these studies led to the development of a patented process for the rehabilitation of waste coal dumps, open cast spoil and backfill (Rose et al., 2010, Patent ZA 2010/02354). The patent sought to provide a biological process of treating coal whereby value is imparted to waste coal by recovery and processing. The biological processing entails; inoculating dumped coal material with degrading microorganism(s), establishing plant growth on coal dump surface and allowing the coal to be degraded. Successful application of this novel technology requires understanding of the degradation mechanisms involved and process scale-up development following proof-of-concept. As a result the present study aimed to address the mentioned research

gaps in this field and to add to the contribution of the earlier studies in order to improve understanding on phyto-biodegradation of South African waste coal and possible development of commercial scale rehabilitation technology. These aims have been achieved in this work by:

- 1) Investigating the colonization and degradation of waste coal by *N. fischeri*, and demonstrating production of extracellular ligninolytic enzyme laccase by the fungus during degradation process.
- 2) Exploring the contribution of *C. dactylon* as a mutualist in fungal mediated coal biodegradation and demonstrating soilification of the waste coal into humic material, and
- 3) Evaluating the effect of cladding material in the form of waste coal as support medium for the CDF/grass mutualism in facilitating rehabilitation of waste coal dumps at commercial scale.

The first section of the thesis provides an introductory discussion on coal degradation by microorganisms, particularly fungi; with more focus on the mechanisms involved, biotechnological applications of fungal bioprocessing of coal, as well as the integration of fungal degradation of coal into a phyto-bioremediation strategy, leading up to the specific aims and objectives of this work. This is followed by an integrated summary of the results and discussion. Conclusions have been drawn and future recommendations provided before presenting the four chapters/papers making up the thesis. The chapters/papers are summarized below.

Paper 1 reviews the role and contribution of ligninolytic fungi and bacteria as well as mycorrhizal fungi and plant root exudates in degradation and solubilisation of low-grade waste coal. In this paper a conceptual model has been presented to describe possible pathways involved in mutualistic interaction between the ligninolytic microorganisms and plants believed to result in coal biodegradation and solubilisation.

Paper 2 describes the colonisation and metabolism of South African low rank discard coal by a fungal strain ECCN 84, *N. fischeri*, previously isolated from a coal environment. The work presented in this paper accounts for possible mechanisms involved in coal biodegradation by this fungus.

Paper 3 focuses on proof-of-concept of *in situ* phyto-biodegradation of waste coal by CDF, paying attention to the nature of the coal bioconversion product in *C. dactylon* rhizosphere. The paper further explores possible utilisation of the resultant humic-like substrate for establishment of agronomic crops.

Paper 4 addresses process scale-up development, with more focus on the effect of cladding waste coal substrate on CDF/ grass species mutualism towards self-cladding of coal discard dumps. The results reported in this paper demonstrate possible beneficiation of waste coal material and large scale application of phyto-biodegradation of waste coal.

1.1 Coal degrading fungi

Fungi and bacteria, particularly the ligninolytic species, have been widely reported to degrade and metabolise a number of recalcitrant and complex organic substrates including coal (Quigley et al., 1989; Maka et al., 1989; Torzilli and Isbister 1994; Polman et al., 1994; Laborda et al., 1999; Fakoussa and Frost, 1999; Gotz and Fakoussa, 1999; Machnikowska et al., 2002; Igbinigie et al., 2008). The ligninolytic fungi such as white and brown rot fungi (classified under the phylum Basidiomycota) are the major players in solubilisation and degradation of coal due to an ability to produce some or all of the well known ligninolytic enzymes, lignin peroxidase (LiP, E.C. 1.11.1.13), manganese peroxidase (MnP, E.C. 1.11.1.14) and laccase (LAC, E.C. 1.10.3.2) (Fakoussa and Hofrichter, 1999; Laborda et al., 1999; Zavarzina et al., 2004; Tao et al., 2010; Sekhohola et al., 2013; Klein et al., 2014). Ascomycetes and Deuteromycetes have also been reported to degrade coal even though their enzymology underpinning coal degradation is usually not specified in literature (Sekhohola et al., 2013). Nevertheless it appears oxidative action of ligninolytic enzyme systems plays the major role in coal degradation by fungi, allowing the microorganisms to metabolise coal and extract carbon for growth and proliferation. For example, metabolism of low rank coal material by Deuteromycete *N. fischeri* was coupled with increased activity of extracellular laccase (Sekhohola et al., 2014) while solubilisation of lignite by the Basidiomycetes *Trametes hirsula* and *Trametes maxima* involved both manganese and lignin peroxidases (Klein et al., 2014). In each of these cases it was reported that addition of substrate coal increased enzyme activity and resulted in accumulation of fungal biomass (Sekhohola et al., 2014; Klein et al., 2014).

The CDF are able to breakdown coal because, despite the complex and often highly aromatic chemical structure of coal, there are some preserved moieties in coal that resemble those of the parent material lignin (Polman et al., 1994; Hofrichter et al., 1999; Brown et al., 2014). These molecular structures, which include phenolics, carboxylic acids and alkanes, render coal susceptible to degradation by lignin degraders such as ligninolytic fungi and bacteria (Orth et al., 1993; Fakoussa and Hofrichter, 1999; Grinhut et al., 2007; Huang et al., 2013). For example the white and brown rot

fungi are well known for their coal degrading ability and are ubiquitous in nature as natural degraders of lignocelluloses in wood (Lee et al., 2013; Doria et al., 2014). It is possible there are numerous fungal strains thriving within natural ecosystems capable of degrading coal that have not yet been discovered. For example, coal degradation by litter decomposing and ectomycorrhizal fungi has not been reported even though these have also been identified as laccase producers (Chaurasia et al., 2013). Furthermore, much research has investigated the mechanisms for most of the fungal strains identified as coal degraders however there are some cases where it is still not specified how the coal material is broken down. For example *Penicillium citrinum*, previously isolated as a laboratory contaminant, has shown ability to breakdown coal but the mechanisms involved have not yet been elucidated (Polman et al., 1994). Sufficient understanding of the mechanisms involved in coal breakdown is essential for upscaling of these processes at an industrial scale and development of functional technologies.

As shown in Figure 1, the chemical structure of coal is heterogeneous and complex and CDF deploy other mechanisms that, in addition to oxidative enzymatic action, cleave the various molecular structures including: alkaline substances, chelators, surfactants and esterases (Hofrichter and Fakoussa, 2001; Sekhohola et al., 2013). It appears the higher fungi are mostly responsible for enzymatic breakdown of coal while the non-enzymatic processes are associated with microfungal and bacterial action (Hofrichter and Fakoussa, 2001).

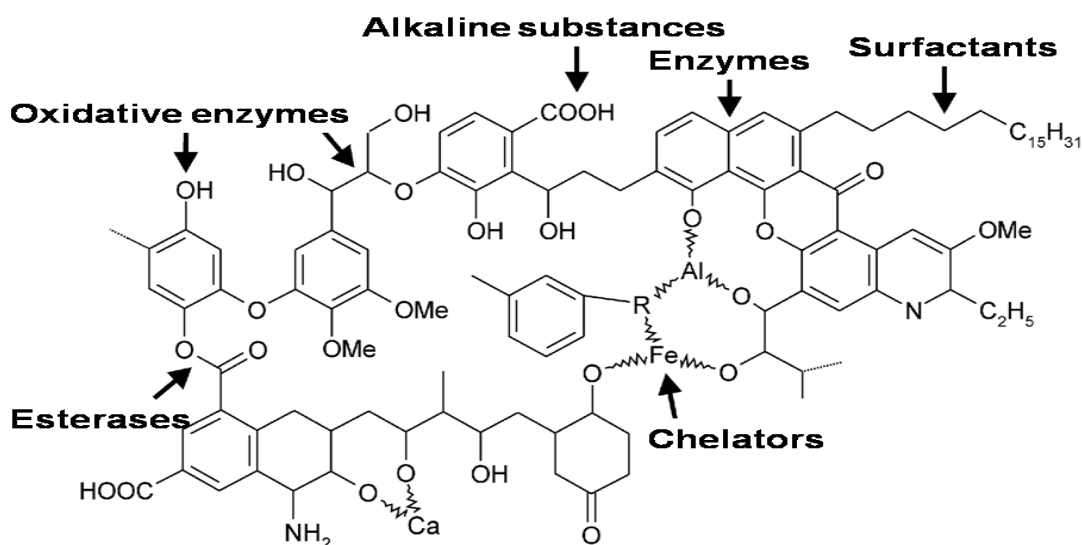


Figure 1: Purported catalysts involved in bioconversion and solubilisation of coal and the molecular structures cleaved as shown on hypothetical structural model of brown coal (adapted from Hofrichter and Fakoussa 2001)

1.2 Coal biotechnology

Bioprocessing of low-grade coal has attracted interest due to its advantages over the conventional chemical and thermal processing (Kang 2014). Re-mining and burning of waste coal to produce electricity has for a long time been regarded as an innovative and economically advantageous way to utilise the low-grade coal both locally and internationally (Niemi et al., 2009). This strategy is generally perceived as a self-financing way of solving the problems that arise from dumping of coal. Before burning, waste coal has to be processed into viable fuel and to reduce the levels of emitted toxic gases in compliance with the laws and legislations. Processing involves pulverizing and mixing coal with limestone to reduce emission of sulphur dioxide gas when burning the coal for power generation (Baldwin, 2004). Mixing with limestone also changes the acidity of the coal material resulting in alkaline ash after burning, which can be utilised on mine dumps to alleviate problems of Acid Mine Drainage (AMD) (Baldwin, 2004). Nonetheless, such processing increases the cost of utilising this material as fuel and subsequently power production from waste coal becomes more expensive than the conventional approach (Niemi et al., 2009). This poses an increased cost to consumers who have to cover the costs of complying with restrictions on emission of toxic gases into the environment. On the other hand, the ambient temperatures and pressure under which biological processes operate favour development and implementation of biological technologies, especially on large industrial scale (Fakoussa and Hofrichter 1999; Tripathi et al., 2010; Jinghua et al., 2012). A number of bioprocesses involving coal degradation by microorganisms, particularly fungi, have been investigated to explore possible development of technologies. It has been established that most fungal isolates studied either break down the organic component of the coal chemical structure or sequester some interspersed inorganics through different processes including the following:

- I. Solubilisation resulting from sequestering of inorganic cations from macromolecules and ionization of acidic functional groups (Ralph and Catcheside 1997)
- II. Depolymerisation of higher molecules to smaller fractions through enzymatic cleavage of covalent bonds within coal structure (Ralph and Catcheside 1997; Klein et al., 1999)

- III. Mineralisation of the organic component with the release of carbon dioxide (Steffen et al., 2002)
- IV. Decolourisation (bleaching) resulting from splitting of double bonds due to breakdown of humic acid to fulvic acid (Klein et al., 1999; Steffen et al., 2002)
- V. Liquefaction (physical change from solid to liquid) resulting from conversion of inorganic components (Hofrichter and Fakoussa 2001; Basaran et al., 2003)

Coal biotechnology promises to re-shape the coal mining industry as indicated by its beneficial contribution towards the utilisation of low rank coals (Fakoussa and Hofrichter 1999). The low rank coals are known to be a poor source of energy and for a long time have been regarded as waste material by the coal mining industry (Chassapis and Roulia, 2008; Baldwin, 2004). Large amounts of this waste coal are disposed of in the form of unsightly dumps which pose an environmental hazard, (Limptlaw et al., 2005; Jawarkar and Jambhulkar, 2008; Zhao et al., 2013), in which the landscape is transformed into unaesthetic swamps of acid mine drainage (AMD) and dumps of waste coal (Figure 2) (Katzur and Haubold-Rosar, 1996; Bech et al., 1997; Szczepanska and Twardowska, 1999; Li 2006). Not only is the topography transformed but the hydrology is also affected as sediments and AMD from the dumped waste are deposited into nearby water bodies through run-offs (Tiwary 2001; Hopkins et al., 2013). Recent advances in the field of microbial degradation of coal are gradually changing the negative perception of handling the so-called waste coal and certainly presenting opportunities for technology development aimed at remediation and eco-friendly use of such coal material. For example, solubilisation of low rank coal from Pakistan by the fungal isolate *Penicillium chrysogenum* served as a pre-treatment step in the production of complex organic fractions that could be converted to biogenic natural gas methane by methanogens (Haider et al., 2013). Opara et al. (2012) also examined microbial production of methane from lignite, bituminous coal and coal waste materials. Within 30 days the extrapolated amount of methane produced was equivalent to 16 000 scf of gas produced per tonne of coal in a year.



Figure 2: Dumped waste coal (left) and the associated acid mine drainage (right) polluting the environment

While research is generally lacking in the field of coal biotechnology despite coal mining being one of the leading industries in South Africa, it seems China is at the forefront with research output on fungal bioprocessing of low rank coal and advancing to clean coal technology (Chen and Xu, 2010). China's invested interest in this field is due to the country's large reserves of lignite and the need to utilize this fossil fuel in "clean ways". A number of fungal strains isolated from various ecosystems such as soil, coal and decaying woods have been subject to Chinese lignite bioprocessing studies including *Penicillium sp* (Dong et al., 2006), *Trichoderma sp* (Xiu-xiang et al., 2009), *Hypocrea lixii* (Tao et al., 2010), and several unspecified white rot fungi (Su-dong et al., 2009; Jinghua et al., 2012). In the same regard, Ying et al. (2010) investigated degradation of lignite model compounds by *Chrysosporum versicolor*, Golden mushroom, *Schizophyllum* and an unspecified fungi, AH. In their experiments they discovered that the fungi were able to decompose the model compounds whereby *C. versicolor* and *Golden mushroom* grew better than *Schizophyllum* and fungi AH in the presence of these compounds.

1.3 Application of fungal degradation of coal: a remediation strategy

The application of biological methods for remediation of recalcitrant pollutants such as coal spoil is regarded as cost-effective and eco-friendly (Lee et al., 2014). As a result biological treatment processes have received much scientific attention. Though coal is well known for its complex chemical structure, sufficient understanding and application of its degradation and solubilisation forms a necessary foundation for development and implementation of biological intervention

strategies (Sekhohola et al., 2013). Furthermore coal-degrading microorganisms form part of the indigenous microbial consortia that naturally colonise coal environments though they may thrive in relatively small population sizes (Igbini et al., 2008). For instance, disposed coal with a highly aromatic chemical structure becomes highly oxidised (Figure 3) as it undergoes biotic weathering resulting from microbial solubilization and depolymerization (Cimadevilla et al., 2003; Igbini et al., 2008). Such oxidation of the organic constituents increases the oxygen content and decreases the hydrogen-to-carbon ratio, molecular weight, calorific value, and aromaticity of the coal material (Cimadevilla et al., 2003). With highly oxidised and less aromatic chemical structure the coal material becomes more susceptible to further biological conversion and degradation by less advanced microorganisms such as ligninolytic bacteria. Even though such bioprocessing of coal by fungi occurs naturally, Szulc et al. (2014) pointed out that the relatively small population sizes of the degrading microorganisms in natural environments delay the remediation process. It was further pointed out that inoculating the contaminated environment with selected degrading microorganisms, a process known as bioaugmentation, would increase efficiency of the bioremediation process (Szulc et al., 2014).

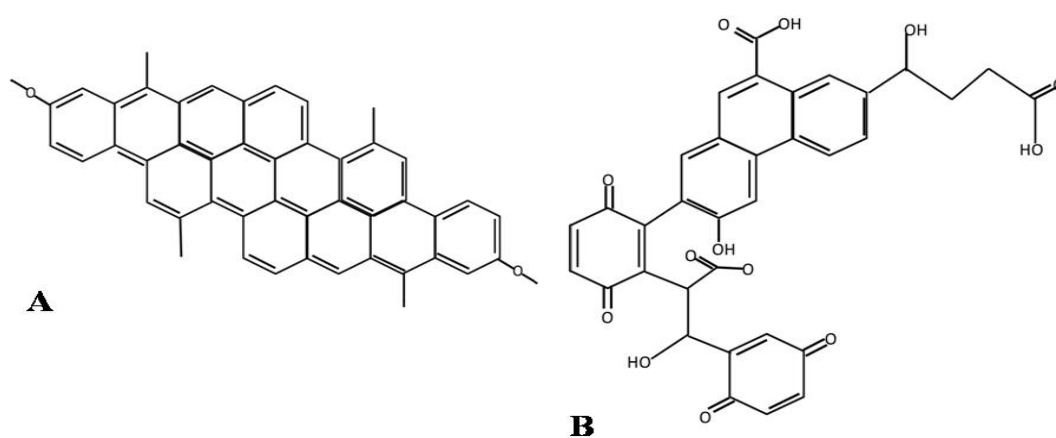


Figure 3: Hypothetical chemical structures of coal showing high aromaticity typically found in hard coal (A) and less aromatic but highly oxidised structure typical of low rank coal (B), as adapted from Fakoussa and Hofrichter (1999).

While the mechanisms involved have not been fully elucidated it has been postulated that fungal interaction with coal particles results in coal bioconversion into a mixture of heterogeneous macromolecules that are mainly humic acids (Cohen and Gabriele, 1982; Henning et al., 1997; Catcheside and Ralph, 1999; Grinhut et al., 2007). The resultant humic substances have long been reported to have beneficial attributes such as loosening compacted soils, stimulating the development of microbial populations and enhancing uptake of essential nutrients and trace elements required by

plants in soil (Piccolo et al., 1997). Fungal bioconversion of disposed coal therefore renders what is regarded as waste material into an organic humic-rich resource and allows for carbon recycling and beneficiation of this so-called waste, particularly as a substrate for plant establishment (Klein et al., 2014).

1.4 Integrated phyto-bioremediation technology

Observations from recent studies show that direct contact between metabolically active biocatalysts and coal facilitates the transformation of this recalcitrant substrate into a material rich in HS (Mukasa-Mugerwa et al., 2010; Tripathi et al., 2010; Oboirien et al., 2013). Furthermore a conceptual model (Figure 4) describing possible pathways involved in biodegradation of coal indicates that while microbial enzymatic systems play a major role, plants also participate in achieving the desired biotransformation of the coal material (Sekhohola et al., 2013). It is hypothesised that plant root exudate stimulates microbial colonization by providing simple carbon in the form of organic acids, and proliferation of microbial populations within the rhizosphere results in a higher level of coal-microorganism interaction, and subsequently more efficient coal degradation (Sekhohola et al., 2013). On the other hand, mycorrhizal hyphae are more extensive and more effective than plant roots at absorbing nutrients from the soil, and therefore increase plant uptake of nutrients and water from the soil (Barea and Azcon-Aguilar, 1982). As a result grass tends to show improved drought tolerance, rapid recovery from wilting and lowered nutrient requirement in the presence of mycorrhizal fungi. Mycorrhizae also promotes establishment of plants even under stressful environmental conditions such as those on coal waste (Leyval et al., 1997; Mukasa et al., 2010). It appears that the fundamental principles underpinning the processes involved in the integrated phyto-biodegradation of coal include: 1) fungal and bacterial degradation of coal, 2) beneficiation of plants from HS resulting from coal degradation and 3) the mutual and reciprocally beneficial relationship between plants and MF (Sekhohola et al., 2013). However, within an industrial context, there is insufficient understanding of complex microbial interactions as well as changing rhizospheric environments (Burns et al., 2013). These limitations together with limited research on different aspects of soil extracellular enzymes makes it difficult to translate the knowledge gathered thus far into development of viable technologies (Burns et al., 2013). Informed selection of the biocatalysts; that is the grass and microbial species is therefore very important.

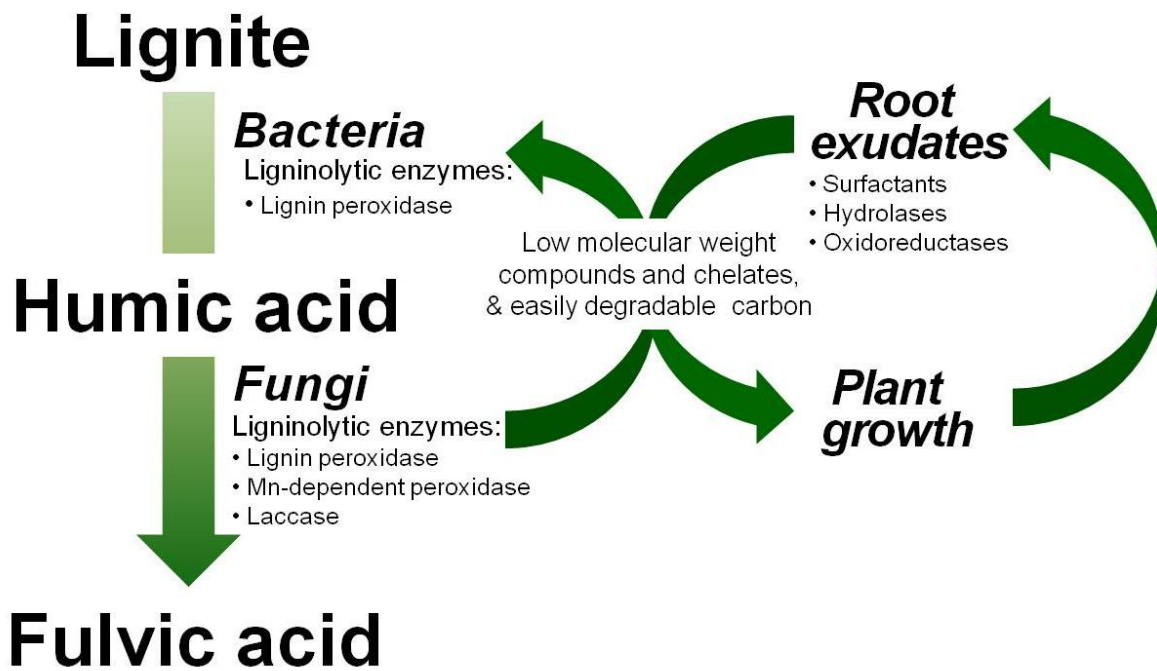


Figure 4: Model describing the phyto-biodegradation of coal to humic acids and fulvic acids and illustrating the main interactive pathways between ligninolytic microorganisms and root exudates to facilitate plant growth (Sekhohola et al., 2013).

Sporadic growth of *C. dactylon* was observed on bituminous hard coal dumps that had not undergone any form of rehabilitation. It was observed that coal material within the rhizosphere of this grass had been broken down into a loose dark soil-like material (Igbini et al., 2008; Mukasa-Mugerwa et al., 2010). Studies were carried out on the coal rhizosphere in an effort to elucidate the processes involved (Igbini et al., 2008; Igbini et al., 2010; Mukasa-Mugerwa et al., 2010). Following intensive screening of coal samples taken from the rhizosphere a fungal strain *N. fischeri* was isolated for further laboratory investigations (Igbini et al., 2008). The fungus was able to solubilise acid treated hard coal and humic acids were tentatively identified as major products (Igbini et al., 2008). Investigations also revealed a symbiotic association between plant roots and arbuscular mycorrhizal populations, which were identified as *Glomus clarum*, *Paraglomus occultum*, *Gigaspora gigantea*, and *Glomus mossea* (Mukasa-Mugerwa et al., 2010). Organic acids which were thought to aid in oxidation of coal material were detected in root exudate of mycorrhizal plants, but not in exudate from plants without mycorrhizal associations (Mukasa-Mugerwa et al., 2010). The results suggested that fungal species such as *N. fischeri* with the ability to breakdown the complex substrate coal may be actively involved in the coal degradation process resulting in production of HS that may enhance plant growth (Igbini et al., 2008; Mukasa-Mugerwa et al., 2010). It appeared the

fungi could also be benefiting from the stimulatory effect of mycorrhizal fungi (MF) in association with plant root systems. However, it still remains unclear how each process links to the other. These findings nevertheless led the authors to propose a technology for rehabilitation of waste coal dumps, which they termed Fungcoal (Rose et al., 2010, Patent ZA 2010/02354). The abovementioned observations have thus presented a platform bioprocess for further investigation and development of an *in-situ* coal rehabilitation technology that can be deployed for efficient reclamation of waste coal dumps.

The Fungcoal technology has shown great potential on small scale field trials (20 ×20 m) on waste coal dumps (Kleinkopje Roofcoal Dump, Mpumalanga, South Africa). *In-situ* bioconversion of coal into an organic rich soil-like material was observed after adding *N. fischeri*, mycorrhizal fungi, and *C. dactylon* to a cladding layer of weathered coal (Rose et al., 2007). Grass cover became well established and it was noted that transformation of the dumped coal discard to a depth of 500 mm had occurred within six years. This result indicated that the technology not only facilitates coal degradation but also promotes revegetation and thus has the potential for development into a full rehabilitation strategy. The merits of such a strategy that promotes vegetation establishment include control of pollution of ecosystems that could potentially result from dump run offs, improved visual aesthetic and most importantly, facilitated microbial activity within the rhizosphere resulting in breakdown of the waste coal (Maiti, 2007). Application of such an integrated phyto-bioremediation technology becomes particularly important in South Africa where conventional approaches towards rehabilitation on waste coal dumps rely on use of topsoil as a key resource for establishment of vegetation cover. Unfortunately, the practice requires excavation of large quantities of fertile soil to be re-deposited on dump surfaces, leaving behind a void that is susceptible to erosion. In some cases low nutrient content in the soil coupled with accelerated mineralisation of soil organic matter reduce efficiency of mineral fertilizers applied to supplement the soil nutrient content (Glaser et al., 2002). This leads to deterioration of plant cover and ultimate failure of the process.

2. Aims and objectives

The overall objective of this thesis was to investigate the role and contribution of CDF, using *N. fischeri* as the fungal biocatalyst, and grasses, using *C. dactylon* as the mutualist, in evaluating the patented Fungcoal bioprocess developed for the self-cladding and rehabilitation of waste coal dumps.

The specific aims were;

- 1) To demonstrate the colonization and degradation of waste coal by *N. fischeri*,
- 2) To explore the contribution of *C. dactylon* in fungal mediated coal biodegradation and soilification, and
- 3) To evaluate the effect of cladding material as support medium for the CDF/grass mutualism in facilitating rehabilitation of waste coal dumps at commercial scale.

3. Results and Discussions

3.1 Early studies in our laboratory

Results from previous studies at the Institute for Environmental Biotechnology, Rhodes University (EBRU) were used to underpin a patented process for the rehabilitation of waste coal dumps, open cast spoil and backfill (Rose et al., 2010, Patent ZA 2010/02354). Of particular relevance to the present study is the mutualistic interaction between CDF and the C4 grass *C. dactylon* in the self-cladding and rehabilitation of waste coal dumps. Even so, a strain of *N. fischeri* isolated from rhizosphere of *C. dactylon* solubilised hard coal *in vitro* (Igbini et al., 2008). However, no information was provided on colonisation and the metabolic mechanisms involved and the nature of products formed remains unknown. Furthermore, it was suggested that a consortium of CDF together with *C. dactylon* through mutualism facilitated coal biodegradation in the rhizosphere (Igbini et al., 2010). Again, very little evidence to support this conjecture was provided by the authors. Furthermore, at the outset of the current study no *in situ* experimental evidence (e.g. pot or field trials) existed to support the interaction of the CDF and *C. dactylon*. To improve understanding of the proposed phyto-bioremediation process involving *N. fischeri* and *C. dactylon* it seemed important to initially investigate the colonization, metabolism and utilisation of waste coal for development of a rehabilitation strategy.

3.2 Fungal colonization and metabolism of waste coal

Neosartorya fischeri was shown to actively colonise waste coal and together with MF and other putative CDF was sustained in a coal/soil environment for a period of 42 wks (Figure 5). In order for any microorganism to break down a substrate it needs first to colonise it. The colonization of waste coal by *N. fischeri* in liquid cultures occurred coincidentally with a decline in weight percentage of elemental carbon and an increase in weight percentage of elemental oxygen and, enhanced activity of an extracellular LAC (Sekhohola et al., 2014). *N. fischeri*, strain ECCN 84, also tested positive for production of LAC in the presence of a non-natural LAC mediator 2,2'-(azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt] (ABTS) (Figure 6). Production of extracellular laccases is thought to be a typical response to xenobiotics by many ligninolytic fungi. The xenobiotics bind to recognition sites present in promoter regions of genes encoding for laccase and induce enzyme production (Kunamneni et al., 2007). Availability of complex aromatic carbon substrates containing xenobiotics therefore triggers substrate mineralisation through LAC catalysis. For example, LAC production was detected in degradation of polycyclic aromatic hydrocarbons

(PAH) by *Trametes versicolor* (Hans et al., 2004) and mineralisation of petroleum asphaltenes by *N. fischeri* (Uribe-Alvarez et al., 2011). With coal, constituents of its heterogenous chemical structure comprising various aromatic carbon compounds such as phenolics and carboxylic acids induce microbial mineralisation through extracellular LAC catalysis (Huang et al., 2013; Kaushik and Takur 2013).

Laccases produced by different fungal strains are heterogenous in structure and mode of action (Zapata-Castilo et al., 2012; Chaurasia et al., 2013). The enzyme consists of four Cu atoms and it is generally accepted that oxidation of organic aromatic compounds is catalysed by removal of electrons at one Cu atom and this is coupled with reduction of molecular oxygen to water via generation of free radical intermediates at the trinuclear Cu cluster (Stoilova et al., 2010; Reiss et al., 2011; Chaurasia et al., 2013). This oxidation enhances solubilisation and/or depolymerisation of the macromolecular coal structure into smaller molecular weight entities (Chong et al., 2014). In addition to enzyme production, resolution images of *N. fischeri* cultures growing on coal revealed formation of peroxisomes in the mycelia (Sekhohola et al., 2014). The fungal synthesis of peroxisomes is typically associated with metabolism of complex carbonaceous substrates such as coal through enzyme catalysis (Bartoszewska et al., 2010; Gabaldon, 2010). Such metabolism involves oxidation of complex hydrocarbon compounds such as fatty acids through catalysis by naturally occurring peroxides such as hydrogen peroxide (Saraya et al., 2010). Together, the results of this study support oxidative metabolism of the waste coal by the fungus, *N. fischeri*. Production of manganese peroxidase (MnP) by *N. fischeri* was also investigated but the test gave a negative response.

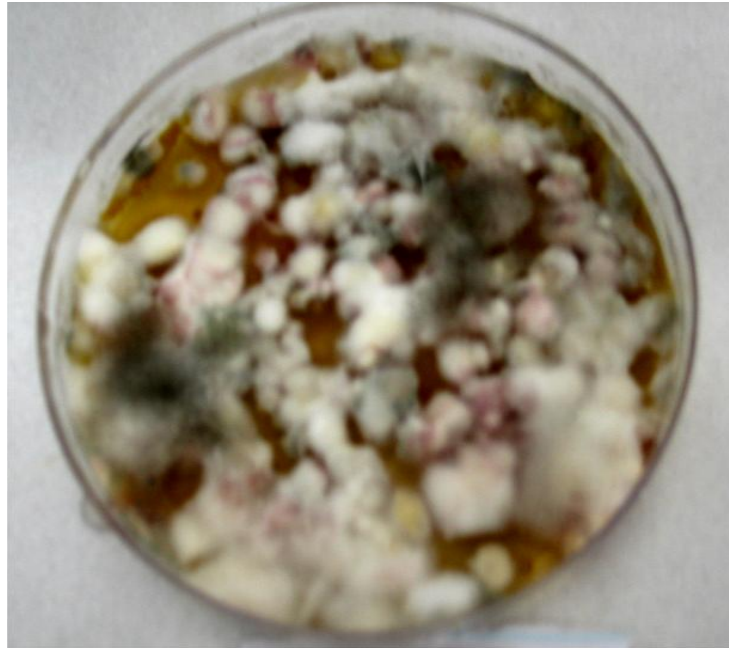


Figure 5: Consortium of fungal cultures isolated from waste coal inoculated with coal degrading and mycorrhizal fungi prior to grass cultivation is seen here growing on potato dextrose agar with added penicillin (added to eliminate bacterial growth).

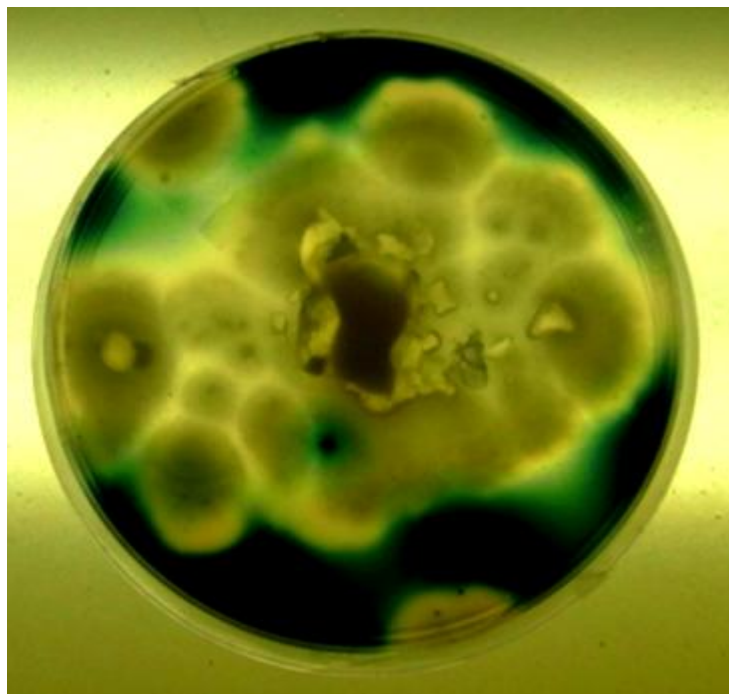


Figure 6: Plate culture of *Neosartorya fischeri* growing on potato dextrose agar containing 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt] (ABTS) showing the indigo green coloured media characteristic of ABTS oxidation by laccase.

Results from pot trials also revealed waste coal biodegradation within the rhizosphere of *C. dactylon* in the presence of CDF and *N. fischeri* which resulted in highly oxidised substrate (Sekhohola et. al. appendix III). Analysis of the HS extracted from the biodegradation product by Fourier Transform – Infrared spectroscopy (FT-IR) revealed spectra showing abundance of O-H and COOH groups coupled with the presence of C=O stretching of various carbonyl groups. In the study conducted by Traversa et al. (2014) the FT-IR analysis of naturally occurring humic acids extracted from compost, which benefited germination and growth of switchgrass, also showed similar spectra of highly oxidised compounds. Traversa et al. (2014) did not determine the biochemical processes underpinning their observations. However, Willmann and Fakoussa, (1997) have reported that humic substances are excellent chelators of nutrient ions in the soil due to their oxidised chemical structure and thus allow for easy nutrient uptake by plants, while Valdrighi et al. (1996) suggested that they are mainly involved in enhancement of cell membrane permeability to nutrients. Furthermore, humic substances at different stages of decomposition can activate the Krebs’s cycle, photosynthesis, and production of amino acids, and these processes enhance plant growth (Muscolo et al., 2012). Humic acid macromolecules extracted from earthworm compost were reported to consist of bioactive molecules such as 3-indole acetic acid, which regulates lateral root development (Canellas et al., 2002). Although the mechanisms are not well understood, it is postulated that the acid disassociates from the humic acid molecule and is released into the rhizosphere once the humics interact with grass root exudates. This induces acidification within the rhizosphere that results in root cellular expansion and mitosis, hence lateral root elongation (Rayle and Cleland, 1992). It is therefore possible that in the present work the resultant humic-rich substrate enhanced establishment of *C. dactylon*. Humic substances can also serve as source of carbon and energy for soil microorganisms (Coates et al., 2002; Anesio et al., 2004). The mechanisms by which they influence the biological activities of microorganisms are still not fully understood, however Visser (1985) demonstrated metabolism of soil humic acids by soil microorganisms resulting in increased populations.

3.3 *Cynodon dactylon* as a biocatalyst in the degradation of waste coal

Cultivation of *C. dactylon* on a waste coal substrate inoculated with CDF, including *N. fischeri*, resulted in soilification of coal material. The substrate remaining after cultivation of *C. dactylon* on waste coal was assayed for HS and the results showed an increase after 23 and 47 wks (Sekhohola and Cowan, appendix III). This contrasts with results obtained from 1 ha commercial scale trials in which *C. dactylon*, and a combination of annual and perennial grasses including *Eragrostis tef*, *Chloris gayana*, *Pennisetum clandestinum* and *Paspalum notatum* were used together with *N.*

fischeri and a suite of MF as inocula (Sekhohola et al., appendix IV). In the latter, no discernable increase in HS concentration was evident after two seasons. However, these commercial trials were designed to determine the effect of cladding material on CDF/ grass species mutualism and were not specific for *C. dactylon*. Furthermore, it is unlikely that one would observe physico-chemical changes in such a short period of time as many similar field studies have been evaluated after 3 to 18 years (Jawarkar and Jambhulkar, 2008; Jawarkar et al., 2009). Nevertheless, the merits of grass cover establishment on the dumps include carbon sequestration, both above and below ground, and addition of organic matter through grass decomposition, which is the key component that influences soil properties and bioactivities (Chen et al., 2004; Makela et al., 2014; Erfanzadeh et al., 2014). Establishment of grass cover is therefore expected to enhance microbial activity which in turn will result in soilification of the waste coal material overtime, as observed in the pot trials (Sekhohola and Cowan, appendix III). In a pot trial using spoil from an opencast mine as soil parent material, Daynes et al. (2013) indeed demonstrated that organic matter in the form of compost together with plants and arbuscular mycorrhizal fungi are key factors that influence soil structure and stability through aggregate formation. Their findings showed that organic matter enhanced development of plant biomass and root growth while adhesive bonding by root exudates and mycorrhizal fungi contributed to soil aggregate formation (Daynes et al., 2013). They concluded that the results support the hypothesis that the soil becomes a self-organizing system through interrelated feedback between soil structure and activity of biological systems.

Although pot trial studies indicated an increase in HS the increase in mass was very small (Sekhohola and Cowan, appendix III). In these experiments, the humics were 0.42 and 1.58 g/kg waste coal after 23 and 47 wks respectively. Similar results were observed on the large-scale demonstration trials carried out at Witbank (Sekhohola et al., appendix IV). Nonetheless, the results agree with previous propositions that humic substances are one of the end products of microbial degradation of coal (Cohen and Gabriele, 1982; Henning et al., 1997; Catcheside and Ralph, 1999; Holker and Hofer 2002; Grinhut et al., 2007). Several possibilities might explain the low levels of retained humics. Firstly, the humics may not be a direct product of fungal biodegradation of coal as other microorganisms such as bacteria have been isolated from coal environments and are implicated in coal biotransformation resulting in production of humics. For example, bacterial consortia, dominated by *Bacillus licheniformis*, solubilised 50% of Leonardite coal into humic acids (Gao et al., 2012). Secondly, leaching out by irrigation and precipitation and chelation by metal ions (Piccolo and Mbagwu, 1990; Willmann and Fakoussa, 1997; Erdogan et al., 2007) may have reduced the amount of extractable humics. Thirdly, the product once formed, could have been rapidly utilised by

either the fungi or plants, or both (Moliszewska and Pisarek, 1996). In addition, other microorganisms such as bacteria may have colonized the substrate over time and mineralised the humics. Some studies have demonstrated that humic substances stimulate initial growth and increase in microbial population and enhance co-metabolism of complex carbon compounds by serving as simple readily accessible co-substrate. Bacterial degradation of anthracene was achieved only when humic acid extract was supplied as co-substrate (Kalantary and Badkoubi 2006). The bacteria were unable to metabolise anthracene in the absence of humic acids, however, addition of humic acids enhanced bacterial proliferation, and upon depletion of the humic acids anthracene was subsequently metabolised.

Many studies have revealed that *C. dactylon* (Bermuda grass) exhibits physiological and chemical mechanisms that enable it to adapt in stressful environments and better serve as a biocatalyst in remediation strategies. The study conducted by Krutz et al. (2005) demonstrated enhanced remediation of pyrene-contaminated soil cultivated with *C. dactylon*. A large population of pyrene degrader microorganisms was detected within the rhizosphere in comparison to the bulk soil and the authors suggested that this might have been the result of root exudation providing simple carbon for microbial proliferation and metabolic activity (Krutz et al., 2005). Exuded nutrients, usually in the form of organic acids, sugars and amino acids add to the carbon pool in the rhizosphere, which not only benefits the microorganisms but the host plant also utilises the carbon for growth (Leyval et al., 1997). The major anions such as malate, lactate, acetate, oxalate, citrate, fumarate, succinate and isocitrate found in the exuded organic acids induce a negative charge. As a result, the organic acids complex with the cations in the soil in solution and mobilise nutrients, enhancing their uptake by plants (Leyval et al., 1997). The anions also serve as intermediates in biochemical processes (e.g. citrate and malate in Tricarboxylic acid cycle) within microbial cells. In this way the exudates serve as an energy source that stimulates microbial activity in the rhizosphere (Azaizeh et al., 1995; Kuiper et al., 2004). Other anions such as malonate and oxalate maintain the osmotic potential in microbial cells (Leyval et al., 1997). In another study *C. dactylon* was among the grass species growing on mine tailings (Leung et al., 2007). The grass roots were found to be colonised by vesicles, arbuscules and coiled hyphae of indigenous mycorrhizal fungi, which are believed to have promoted grass establishment on the contaminated tailings. The mycorrhizal network enhances plant root morphology and metabolic changes resulting in better growth of the entire plant (Barea and Azcon-Aguilar, 1982). The extra-radical fungal hyphae are more extensive and more effective than plant roots at absorbing nutrients from the soil, therefore they increase uptake of nutrients and water by the plant (Baslam et al., 2013). As a result, the grass tends to show improved drought tolerance, rapid

recovery from wilting and lowered nutrient requirement in the presence of the mycorrhizal fungi. Mycorrhizae promotes establishment of plants even under stressful environmental conditions such as those on coal waste (Leyval et al., 1997; Mukasa-Mugerwa et al., 2010; Kumar et al., 2010; Guo et al., 2012). The mycorrhizal fungi also modify interactions of the grass roots with other soil organisms that may be pathogenic. For example; the mycorrhizae can alter the plant response to pathogenic fungi invading the root system. The fungal hyphae grow into a thick protective network in the root zone that prevents invasion by the root-feeding nematodes, and produces hormones and antibiotics that enhance root growth and suppress diseases (Barea and Azcon-Aguilar, 1982). Nutrient translocation through mycorrhizae decreases exudates exposure to the surrounding environment that could result in sorption and complex formation, and thus sufficient quantities of the exudates are distributed to the microorganisms (Barto et al., 2012). Barnett and Naylon (1966) investigated the survival mechanism by *C. dactylon* when growing under extremely dry conditions that may inhibit photosynthesis and protein synthesis. Results from their studies using C-labelled clones of the grass indicated that the free amino acid proline is readily synthesized from glutamic acid and accumulates to serve as a storage compound for carbon and nitrogen in the shoot. Ability of *C. dactylon* to tolerate drought and heavy metal enables the grass to establish on coal dumps even under such stressful conditions. This grass establishment in turn promotes continued microbial activity within the rhizosphere. *C. dactylon* was also selected as a suitable candidate for *in situ* phyto-remediation of the metal contaminated Nakivubu stream in Kampala, Uganda after exhibiting high accumulation of iron, manganese, zinc, copper and lead (Sekabira et al., 2011). Perhaps it is not surprising that aluminum-tolerant genotypes of *C. dactylon* have also been screened as suitable candidate grasses for establishment of vegetation cover on gold mine tailings in South Africa (Ramgareeb et al., 1999). All these reported attributes of *C. dactylon* grass, namely 1) root exudation, 2) mycorrhizal colonization, 3) drought tolerance and 4) heavy metal tolerance are indeed essential and render *C. dactylon* an ideal biocatalyst for successful rehabilitation of waste coal dumps. Growth of *C. dactylon* observed by Igbini et al. (2008) on unrehabilitated discard coal dumps has also indicated clearly that the grass can adapt and survive on waste coal dumps, which are characterized by high temperatures, high clay content resulting in poor porosity, aeration, water infiltration and uncertain structure (Juwarkar and Jambhulkar, 2008). Such physical factors are key characteristics that usually compromise natural establishment of vegetation cover on the dumps.

3.4 A strategy towards self-cladding and remediation of waste coal dumps

The 1 ha commercial scale trials showed establishment of annual and perennial grass species including *Cynodon dactylon*, *Eragrostis tef*, *Chloris gayana*, *Pennisetum clandestinum* and *Paspalum notatum* where weathered coal was used as substrate. All cultivated species were present and diversity was sustained with 100% cover within two seasons, thus indicating the potential for successful rehabilitation. In contrast, in the absence of weathered coal grass establishment was not successful (Sekhohola et al., appendix IV). Indeed the patented bioprocess of the self-cladding of waste coal dumps indicates that a weathered coal co-substrate be used for this process. Weathered coal is a humic-containing (contains 38% humic acid, 1.6% fulvic acid and 60% humin) brown coal material (Figure 7) in abundance at the Kromdraai coal mine. Substrate analyses carried out on the cladding coal material used on the dumps indicated that weathered coal exhibits similar physico-chemical characteristics (22 – 24% moisture retention, slightly acidic pH of 5.2 – 6.0, EC of 75 mS.m⁻¹, CEC of 5.0 Cmol_c.kg⁻¹, 90% ash content and 4 wt % elemental carbon) to topsoil soil but has much higher humic substance content (Sekhohola et al., appendix IV). Small-scale field trials have been carried out on Kleinkopje Roofcoal Dump (Mpumulanga, South Africa) whereby the weathered coal inoculated with *N. fischeri* and mycorrhizal fungi was used as cladding substrate. Establishment of *C. dactylon* and *in situ* “manufacture” of an organic rich soil-like material were achieved on the dump. Furthermore, transformation of dumped coal discard to a depth of 650 mm occurred within six years. Due to the absence of a similar substrate at other South African coal mines coupled with the scarcity of top soil, the primary aim of the 1 ha trials reported in the present work was to investigate whether indeed a co-substrate was required. The 100% grass cover achieved on plots cladded with weathered coal therefore demonstrated that a co-substrate is necessary and this coal material has great potential to sustain efficient rehabilitation on the coal dumps.

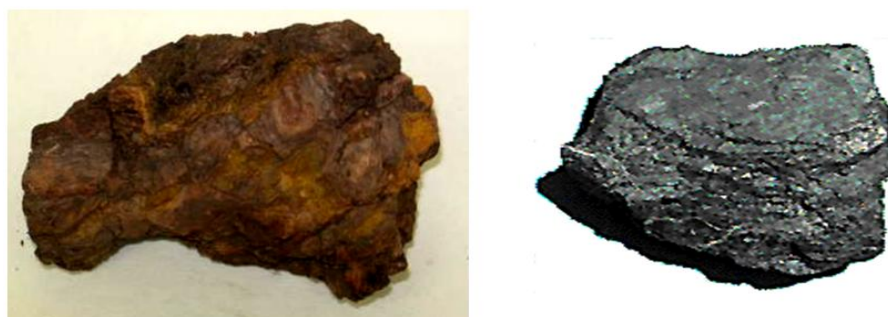


Figure 7: Brown weathered coal (left) exhibits characteristics that differ from those of black coal material (right)

The concept of utilising mutualistic interactions between plants and fungi to rehabilitate coal mine waste therefore appears to depend on the presence of a substrate containing readily accessible carbon to support early fungal growth and proliferation. Indeed the use of alternative cost effective carbon substrates to improve efficiency of phyto-remediation strategies has been explored. For example, Jawarkar and Jambhulkar (2008) used sludge obtained after waste water treatment as a carbon source and biofertilizers together with mycorrhizal fungi to facilitate growth of native tree species on coal mine spoil. In another example, Jawarkar et al. (2009) used pressmud (the residue from filtration of sugarcane juice) as an organic carbon source in combination with free living soil bacteria, nitrogen fixing bacteria and vesicular arbuscular mycorrhizal fungi to enhance plant growth on manganese mine overburden. It was found that applications of these materials, which are considered waste by the respective industries, added substantial amounts of organic matter which promoted healthy plant cover, sustained microbial activity and improved physico-chemical properties on the dump/spoil environments. Polycyclic aromatic hydrocarbons (PAH) are often introduced into the environment as byproducts of incomplete combustion and pyrolysis of coal, and Cebon et al. (2013) highlighted how readily available carbon enhances their biodegradation in contaminated soils. They demonstrated that presence of lower molecular weight PAH extracts stimulates fungal and bacterial proliferation and mineralisation activity and the overall PAH contamination is significantly decreased after 2 months.

In the present study, oxidised waste coal used as cladding material and absence of cladding substrate resulted in poor sporadic grass establishment on the dumps (Sekhohola et al., appendix IV). It is highly suspected that the initial physico-chemical characteristics on these dumps including high acidity and salinity could have compromised the vegetative cover. Nonetheless application of 100% Fungcoal was able to ameliorate the acidity on uncladded dump at Klippan. This clearly indicates that the use of this rehabilitation strategy can potentially mitigate acid mine drainage which is often characteristic of waste coal dumps. On the other hand application of Fungcoal to waste dumps either uncladded or cladded with oxidised waste coal increased electrical conductivity. Though this may not be desirable it could be a result of the required fungal-catalysed waste coal biodegradation, which induces negative charges on coal substrate and binding of cations. As the coal-metal ion complexes disassociate in solution salinity is increased (Willmann and Fakoussa, 1997; Skodras et al., 2014). Indeed successful application of Fungcoal requires the use of an appropriate carbon-rich cladding substrate.

Current mine rehabilitation protocols in South Africa involve a process to clad dumps with a minimum of 50 cm top soil. Many mines have exhausted top soil thus the soil is transported from remote locations which adds substantial cost to the rehabilitation programmes. Once applied, the top soil is fertilised and seeded with a mix of grass species. The clad layer creates a micro soil environment on the dump surface to facilitate plant establishment on the otherwise hostile coal AMD instead of rehabilitating. Excavation of the top soil also creates problems of soil erosion and deterioration of viable ecosystems as land is stripped and left bare. The study is at an early developmental stage; nonetheless the results presented in this work indicate that the proposed integrated phyto-bioremediation strategy which incorporates CDF, MF and grass could achieve a better more sustainable and long term remediation of dumped waste coal while still addressing the aesthetics of the mine environment.

4. Conclusions and Perspectives

The overall objective of this thesis was to investigate the role and contribution of CDF, using *N. fischeri* as the fungal biocatalyst, and grasses, using *C. dactylon* as the mutualist, in evaluating the patented Fungcoal bioprocess developed for the self-cladding and rehabilitation of waste coal dumps. In order to address this objective it should be appreciated that application of fungi in rehabilitation of waste coal dumps depends on their catabolic activities, optimised conditions for their growth and degradation of the coal material. The work in this thesis revealed that *N. fischeri* interacts directly with and metabolises waste coal resulting in accumulation of fungal biomass. The results demonstrated for the first time that colonisation of coal by *N. fischeri* induces production of an extracellular LAC by the fungus. This coupled with EDS analysis which showed a decline in carbon and associated increase in elemental oxygen content, supports fungal mediated oxidative metabolism of waste coal. *C. dactylon* served as a mutualist and supported fungal proliferation, possibly through exudation of simple carbon compounds that served as initial substrate, and facilitated biodegradation of waste coal *in situ*, resulting in soilification of the coal material within the rhizosphere. The lush grass establishment on the waste coal together with *in situ* fungal degradation of coal in a pot trial indicated that the waste coal material could serve as support medium for the CDF/grass mutualism. However, with large-scale field trials, weathered coal proved to be an ideal substrate to support grass establishment and potentially facilitate rehabilitation of waste coal dumps. Grass establishment on the dumps is crucial for successful rehabilitation, as it appears the coal degradation process by fungi is stimulated by and commences upon grass root exudation. Furthermore, the grass root system helps to spread the fungal inoculums applied on the surface to other parts of the dump, thus increasing coal degradation and soilification. Ultimately fungi and grasses through their mutualistic interactions facilitate waste coal dump rehabilitation, as described by the patented Fungcoal bioprocess, and the process is self-sustaining and efficient.

The roles of both plants and fungi in the biodegradation of waste coal were demonstrated in a pot trial within 23 and 47 wks but only grass establishment was demonstrated on large scale trials on the discard coal dumps within two seasons. Perhaps the notion of such seemingly contradicting results is what Kuiper et al. (2004) were alluding to when they stated that “lab results of seeding (wild type) microbes for degradation of soil and water pollutants are ambiguous”. They highlighted a number of studies, which reflected the importance of parameters such as temperature, pH, water activity, oxygen, electron acceptors, bioavailability of pollutant and availability of nutrients for success of bioremediation. These parameters have different influences on the biodegradation process when

upscaling laboratory studies to field trials, hence the different outcomes. The inoculated fungi have the genetic tools to degrade the coal material however the fungi may need to acclimatize to the extreme environmental conditions on dumps first. Moreover, biodegradation of the dumped coal chunks is naturally a slow process. This has been apparent with other large field scale rehabilitation studies similar to the one presented in this work (Jawarkar and Jambhulkar 2008; Jawarkar et al. 2009), which have demonstrated that even with addition of simple organic substrates such as sludge and pressmud biotransformation of the spoil takes many years.

A good rehabilitation strategy focuses on establishment of vegetation cover and improving physico-chemical properties to accelerate natural recovery. On waste coal dumps soil formation remains the most important aspect in restoration of functional ecosystems. Rehabilitation of discard coal dumps therefore focuses on vegetative cladding of the dump, and physico-chemical transformation of dumped coal material into less recalcitrant substrate. It is expected that successful establishment of grass cover positively influences microbial activity on the dump and speeds up the rate at which the coal material is degraded. In the present work, grass establishment achieved on the dumps is considered the first step towards rehabilitation. Thus more process evaluation and monitoring should focus on microbial persistence in coal environment and coal bioconversion activities on longer terms to demonstrate the technology at commercial scale. Biological parameters such as composition, size and degradation activity of inoculated CDF should be enumerated using techniques such as total DNA extraction, direct mRNA isolation and assaying for metabolic biomarkers such as extracellular enzyme laccase.

Indeed, the outcomes of this thesis contribute significantly to the development and implementation of a novel approach to the longstanding challenges associated with biodegradation of waste coal. The fungal processing of waste coal investigated in the present work promises sustainability and efficiency in rehabilitation of waste coal dumps. Nonetheless, regulatory authorities need to be more involved and more mines need to adopt the technology for its successful implementation. Mining authorities should acknowledge that rehabilitation is a long term process and appreciate that biological treatment of waste coal is a naturally slow process but the most efficient method for remediation and even for beneficiation of this substrate. Often the responsible ownership of a newly developed technology is not established by the mine personnel and in such cases the technology developers are expected to take responsibility for research development and implementation. This becomes difficult since the mining industry generally operates within regulations that do not allow

for uncompromised participation of outsiders. Indeed considerable cooperation between all parties in development of technology implementation protocols is crucial.

References

- Anesio, A. M., Hollas, C., Granéli, W., & Laybourn-Parry, J. (2004) Influence of humic substances on bacterial and viral dynamics in fresh waters. *Applied and Environmental Microbiology* **70**:4848-4854.
- Azaizeh, H. A., Marschner, H., & Romheld, V. (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.
- Baldwin, M. (ed). (2004) Burning Waste Coal. *MAGS, Rockhound news* **50**: 1-8.
- Barea, J. M., & Azcon-Aguilar, C. (1982) Production of plant growth-regulating substances by the Vesicular-Arbuscular mycorrhizal fungus *Glomus mosseae*. *Applied and Environmental Microbiology* **43**:810-813.
- Barnett, N.M., & Naylor, A.W. (1966) Amino acid and protein metabolism in Bermuda grass during water stress. *Plant Physiology* **41**:1222-1230.
- Barto, E.K., Weidenhamer, J.D., Cipollini, D., & Rillig, M.C. (2012). Fungal superhighways: do common mycorrhizal networks enhance below ground communication? *Trends in Plant Science* **17**:633-637.
- Bartoszewska, M., Opalinski, L., Veenhuis, M., & Klei, I. (2011). The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. *Biotechnology Letters* **33**:1921-1931.
- Basaran, Y., Denizli, A., Sakintuna, B., Taralp, A., & Yurum, Y. (2003) Bio-liquefactio/solubilisation of low rank Turkish lignites and characterisation of the products. *Energy and Fuels* **17**:1068-1074.
- Baslam, M., Garmendia, I., & Goicoechea, N. (2013). Enhanced accumulation of vitamins, nutraceuticals and minerals in lettuces associated with arbuscular mycorrhizal fungi (AMF): a question of interest for both vegetables and humans. *Agriculture* **3**:188-209.
- Bech, J., Poschenrieder, C., Llugany, M., Barcelo, J., Tume, P., & Tobias, F. J. (1997) Arsenic and heavy metal contamination of soil and vegetation around a copper mine in Northern Peru. *The Science of the Total Environment* **203**:83-91.
- Brown, N.A., Ries, L.N.A., & Goldman, G.H. (2014) How nutritional status signalling coordinates metabolic and lignocellulolytic enzyme secretion. *Fungal Genetics and Biology* **72**:48-63.
- Burns, R.G., Deforest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., & Zoppini, A. (2013) Soil enzymes in a changing environment: current knowledge and future directions. *Soil Biology and Chemistry* **58**:216-234.

- Canellas, L.P., Olivares, F.L., Okorokova-Façanha, A.L., & Façanha, A.R. (2002). Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H⁺-ATPase activity in maize roots. *Plant Physiology* **130**:1951-1957
- Catcheside, D.E.A., & Ralph, J.P. (1999) Biological processing of coal. *Applied Microbiology and Biotechnology* **52**:16-24.
- Cébron, A., Faure, P., Lorgeoux, C., Ouvrard, S., & Leyval, C. (2013). Experimental increase in availability of a PAH complex organic contamination from an aged contaminated soil: consequences on biodegradation. *Environmental Pollution* **177**:98-105
- Chassapis, K. & Roulia, M. (2008) Evaluation of low-rank coals as raw material for Fe and Ca organomineral fertilizer using a new EDXRF method. *International Journal of Coal Geology* **75**:185-188.
- Chaurasia, P.K., Bharati, S.L., & Singh, S.K. (2013). Comparative studies on the blue and yellow laccases. *Research in Plant Sciences* **1**:32-37.
- Chen, W., & Xu, R. (2010) Clean coal technology development in China. *Energy Policy* **38**: 2123-2130.
- Chen, C.R., Xu, Z.H., Mathers, N.J. (2004) Soil carbon pools in adjacent natural and plantation forests of subtropical Australia. *Soil Science Society of America Journal* **68**: 282 – 291.
- Chong, N.H.H., Marjo, C.E., Das, T., Rich, A.M., & Manefield, M. (2014) Surface analysis reveals biogenic oxidation of sub-bituminous coal by *Pseudomonas fluorescens*. *Applied Microbiology and Biotechnology* **98**:6443-6352.
- Cimadevilla, J.L. G., Álvarez, R., & Pis, J.J. (2003) Photoacoustic FT-IR study of weathered stockpiled coking coals. *Vibrational Spectroscopy* **31**:133-141.
- Coates, J.D., Cole, K.A., Chakraborty, R., O'Connor, S.M., & Achenbach, L.A. (2002) Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. *Applied and Environmental Microbiology* **68**: 2445 – 2452.
- Cohen, M. S., & Gabriele, P. D. (1982) Degradation of Coal by the Fungi *Polyporus versicolor* and *Poria monticola*. *Applied Microbiology and Biotechnology* **44**:23-27.
- Daynes, C.N., Field, D.J., Saleeba, J.A., Cole, M.A., & McGee, P.A. (2013) Development and stabilization of soil structure via interactions between organic matter, arbuscular mycorrhizal fungi and plant roots. *Soil Biology & Biochemistry* **57**:683-694.
- Dong, L.H., Yuan, Q., & Yuan, H.L. (2006) Changes of chemical properties of humic acids from crude and fungal transformed lignite. *Fuel* **85**:2402-2407.

- Doria, E., Altobelli, E., Girometta, C., Nielsen, E., Zhang, T., & Savino, E. (2014) Evaluation of lignocelulolytic activities of ten fungal species able to degrade poplar wood. *International Biodeterioration and Biodegradation* **94**:160-164.
- Erdogan, S., Baysal, A., Akba, O., & Hamamci, C. (2007) Interaction of Metals with Humic Acid Isolated from Oxidized Coal. *Polish Journal of Environmental Studies* **16**:671-675.
- Erfanzadeh, R., Bahrami, B., Motamedi, J., & Pétilion, J. (2014) Changes in soil organic matter driven by shifts in co-dominant plant species in a grassland. *Geoderma* **213**:74-78.
- Fakoussa, R. M., & Hofrichter, M. (1999) Biotechnology and Microbiology of coal degradation. *Applied Microbiology and Biotechnology* **52**:25-40.
- Fakoussa, R.M., & Frost, P.J. (1999) In vivo-decolorization of coal-derived humic acids by laccase excreting fungus *Trametes versicolor*. *Applied Microbiology and Biotechnology* **52**:60–65.
- Fakoussa, R.M. (1981) Coal as a substrate for microorganisms. Investigations of the microbial decomposition of (untreated) bituminous coals. PhD Desertation, Rhein Friedrich-Wilhelm University, Germany.
- Gabalton, T. (2010) Peroxisome diversity and evolution. *Philosophical Transactions of the Royal Society* **365**:765-773.
- Gao, T.G., Jiang, F., Yang, J.S., Li, B.Z., & Yuan, H.L. (2012) Biodegradation of Leonardite by an alkali-producing bacterial community and characterisation of degraded products. *Applied Microbial and Cell Physiology* **93**:2581-2590.
- Gotz, G.K.E, & Fakoussa, R.M. (1999) Fungal biosolubilization of Rhenish brown coal monitored by Curiepoint pyrolysis/gas chromatography/mass spectrometry using tetraethylammonium hydroxide. *Applied Microbiology and Biotechnology* **52**:25-40
- Glaser, B., Lehmann, J., & Zech, W. (2002) Ameliorating physical and chemical properties of highly weathered soils in the tropics with charcoal - a review. *Biology and Fertility of Soils* **35**:219-230.
- Grinhut, T., Hadar, Y., & Chen, Y. (2007) Degradation and transformation of humic substances by saprotrophic fungi: processes and mechanisms. *Fungal Biology Reviews* **21**:179-189.
- Guo, W., Zhao, R., Fu, R., Bi, N., Wang, L., Zhao, W., Guo, J., & Zhang, J. (2014) Contribution of arbuscular mycorrhizal fungi to the development of maize (*Zea mays* L.) grown in three types of coal mine spoil. *Environmental Science and Pollution Research* **21**:3592-3603.
- Haider, R., Ghauri, M.A., SanFilipo, J.R., Jones, E.J., Orem, H.W., Tatu, C.A., Akhtar, K., & Akhtar, N. (2013) Fungal degradation of coal as a pretreatment for methane production. *Fuel* **104**:717-725.

- Hans, M.J., Choi, H.T., & Song, H.G. (2004) Degradation of phenanthrene by *Trametes versicolor* and its laccase. *Journal of Microbiology* **42**:94 – 98.
- Henning, K., Steffes, H. & Fakoussa, R.M. (1997) Effects on the molecular weight distribution of coal-derived humic acids studied by ultrafiltration. *Fuel Processing Technology* **52**:225-237.
- Hofrichter, M. & Fakoussa, R. (2001) Microbial degradation of coal. In *Lignin, humic substances and coal*. Sleibuchel, A. & Hofrichter, M. (eds). Vol. 1, Wiley-VCH, Weinheim, Germany.
- Hofrichter, M., Ziegenhagen, D., Sorge, S., Ullrich, F., Bublitz, R., & Fritsche, W. (1999) Degradation of lignite (low rank coal) by ligninolytic basidiomycetes and their manganese peroxidase system. *Applied Microbiology and Biotechnology* **52**:78-84
- Holker, U. & Hofer, M. (2002) Solid substrate fermentation of lignite by the coal-solubilizing mould, *Trichoderma atroviride*, in a new type of bioreactor. *Biotechnology Letters* **24**:1643-1645.
- Holker, U., Ludwig, S., Scheel, T. & Hofer, M. (1999) Mechanisms of coal solubilization by the deuteromycetes *Trichoderma atroviride* and *Fusarium oxysporum*. *Applied Microbiology and Biotechnology* **52**:57-59.
- Hopkins II, R.L., Altier, B.M., Haselman, D., Merry, A.D., & White, J.J. (2013) Exploring the legacy effects of surface coal mining on stream chemistry. *Hydrobiologia* **713**:87-95.
- Huang, Z., Urynowicz, M.A., & Colberg, P.J.S. (2013) Bioassay of chemically treated subbituminous coal derivatives using *Pseudomonas putida* F1. *International Journal of Coal Geology* **115**:97-105.
- Igbinigie, E. E., Mutambanengwe, C. Z., & Rose, P. D. (2010) Phyto-bioconversion of hard coal in the *Cynodon dactylon*/coal rhizosphere. *Biotechnology Journal* **5**:292-303.
- Igbinigie, E. E., Aktins, S., van Breugel, Y., van Dyke, S., Davies-Coleman, M. T., & Rose, P. D. (2008) Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri*. *Biotechnology Journal* **3**:1407-1416.
- Jawarkar, A. A., Yadav, S.K., Thawale, P.R., Kumar, P., Singh, S.K. & Chakrabarti, T. (2009) Developmental strategies for sustainable ecosystem on mine spoil dumps: a case study. *Environmental Monitoring and Assessment* **157**:471-481.
- Jawarkar, A. A., & Jambhulkar, H. P. (2008) Phytoremediation of coal mine spoil dump through integrated biotechnology approach. *Bioresource Technology* **99**:4732-4741.
- Jinghua, Y., Lei, X., & Liqiang, W. (2012) Separation and analysis of lignite bioconversion products. *International Journal of Mining Science and Technology* **22**: 529-532.

- Kalantary, R.R., & Badkoubi, A. (2006) Effect of humic compounds on Bacterial growth bioremediation of PAHs. *Iran Journal of Environmental Health, Science and Engineering* **3**: 31 – 38.
- Kang, J.W. (2014) Removing environmental organic pollutants with bioremediation and phytoremediation. *Biotechnology Letters* **36**:1129-1139.
- Katzur, J., & Haubold-Rosar, M. (1996) Amelioration and reforestation of sulfurous mine soils in Lusatia (Eastern Germany). *Water, Air and Soil Pollution* **91**:17-32.
- Kaushik, G. & Thakur, I.S. (2013) Purification, characterization and usage of thermotolerant laccase from *Bacillus*. sp. for biodegradation of synthetic dyes. *Applied Biochemistry and Microbiology* **49**:352-359.
- Klein, J., Catcheside, D.E.A., Fakoussa, R., Gazso, L., Fritsche, W., Hofer, M., Laborda, F., Margarit, I., Rehm, H., Reich-Walber, M., Sand, W., Schacht, S., Schmiers, H., Setti, L., & Steinbuchel, A. (1999). Biological processing of fossil fuels: Resume of the bioconversion session of ICCS'97. *Applied Microbiology and Biotechnology* **52**:2-15.
- Klein, O.I., Kulikova, N.A., Konstantinov, A.I., Fedorova, T.V., Landesman, E.O., & 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.
- Krutz, L.J., Beyrouthy, C.A., Gentry, T.J., Wolf, D.C., & Reynolds C.M. (2005) Selective enrichment of a pyrene degrader population and enhanced pyrene degradation in Bermuda grass rhizosphere. *Biology and Fertility of Soils* **42**:359-364.
- Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., & Lugtenberg, J.J. (2004) Rhizoremediation: a beneficial plant-microbe interaction. *The American Phytopathological Society* **17**:6-15.
- Kumar, A., Raghuwanshi, R., Upadhyaya, R.S., (2010) Arbuscular mycorrhizal technology in reclamation and revegetation of coal mine spoils under various revegetation models. *Engineering* **2**: 683 – 689.
- Kunamneni, A., Ballesteros, A., Plou, F.J., & Alcalde, M. (2007) Fungal laccase – a versatile enzyme for biotechnological applications. In *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*. Mendez-Villas, A. (ed) Vol 1, Formex, Badajoz, Spain.
- Laborda, F., Monistrol, I.F., Luna, N., & Fernandez, M. (1999) Processes of liquefaction/solubilization of Spanish coals by microorganisms. *Applied Microbiology and Biotechnology* **52**:49-56.

- Lee, H., Jang, Y., Choi, Y.S., Kim, M.J., Lee, J., Lee, H., Hong, J.H., Lee, Y.M., Kim, G.H., & Kim, J.J. (2014) Biotechnological procedures to select white rot fungi for the degradation of PAH's. *Journal of Microbiological Methods* **97**:56-62.
- Leung, H.M., Ye, Z.H., & Wong, M.H. (2007) Survival strategies of plants associated with arbuscular mycorrhizal fungi on toxic mine tailings. *Chemosphere* **66**:905-915.
- Leyval, C., Turnau, K., & Haselwandter, K. (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza* **7**:139-153.
- Li, M. S. (2006) Ecological restoration of mineland with particular reference to the metalliferous mine wasteland in China: A review of research and practice. *Science of the Total Environment* **357**:38-53.
- Limptlaw, D., Aken, M., Lodewijks, H., & Viljoen, J. (2005) Post mining rehabilitation, land use and pollution at collieries in South Africa. *The South African Institute of Mining and Metallurgy* 1-10.
- Machnikowska, H., Pawelec, K., & Podgórska, A. (2002) Microbial degradation of low rank coals. *Fuel Processing Technology* **77-78**:17-23
- Maiti, S. K. (2007) Bioreclamation of coalmine overburden dumps - with special emphasis on micronutrients and heavy metal accumulation in tree species. *Environmental Monitoring and Assessment* **125**:111-122.
- Maka, A., Srivastava, V.J., Kilbane, J.J., & Akin, C. (1989) Biological solubilisation of untreated North Dakota lignite by a mixed bacterial and bacterial/fungal culture. *Applied Biochemistry and Biotechnology* **20/21**:715 –729
- Makela, M.R., Donofrio, N., & De Vries, R.P. (2014) Plant biomass degradation by fungi. *Fungal Genetics and Biology* **72**:2-9.
- Moliszewska, E., & Pisarek, I. (1996) Influence of humic substances on the growth of two phytopathogenic soil fungi. *Environment International* **22**: 579-584.
- Mukasa-Mugerwa, T. T., Dames, J. F., & Rose, P. D. (2010) The role of a plant/fungal consortium in the degradation of bituminous hard coal. *Biodegradation* **1**:1-13.
- Musco, A., Sidari, M., & da Silva J.A.T (2010) Biological effects of water-soluble soil phenol and soil humic extracts on plant systems. *Acta Plant Physiology* **35**: 309 – 320.
- Niemi, E., Reich, S., & Charlton, D. (2009) Economic Analysis of Alternative Programs for Managing Waste Coal in Central Appalachia. *Economic Analysis of Managing Waste Coal* **1**: 1-45.

- Oboirien, B.O., Ojumu, T.V., & Obayopo, O.S. (2013) Fungi solubilisation of low rank coal: Performances of stirred packed bed reactors. *Fuel Processing Technology* **106**:295-302.
- Opara, A., Adams, D.J., Free, M.L., McLennan, J., & Hamilton, J. (2012) Microbial production of methane and carbon dioxide from lignite, bituminous coal, and coal waste materials. *International Journal of Coal Geology* **96 – 97**: 1 – 2.
- Orth, B. A., Royse, D., & Tien, M. (1993) Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. *Applied and Environmental Microbiology* **59**: 4017-4023.
- Piccolo, A., Pietramellara, G., & Mbagwu, J. S. C. (1997) Reduction in soil loss from erosion-susceptible soils amended with humic substances from oxidized coal. *Soil Technology* **10**:235-245.
- Piccolo, A., & Mbagwu, J. S. C. (1990) Effects of different organic waste amendments on soil microaggregates stability and molecular sizes of humic substances. *Plant and Soil* **123**:27-37.
- Polman, J. K., Stoner, D. L., & Delezene-Briggs, K. M. (1994) Bioconversion of coal, lignin and dimethoxybenzyl alcohol by *Penicillium citrinum*. *Journal of Industrial Microbiology* **13**:292-299.
- Quigley, D.R., Ward, B., Crawford, D.L., Hatcher, H.J., & Dugan, P.R. (1989) Evidence that microbially produced alkaline materials are involved in coal biosolubilization. *Applied Biochemistry Biotechnology* **20/21**:753–763
- Ralph, J.P. & Catcheside, D.E.A., (1997) Transformations of low rank coal by *Phanerochaete chrysosporium* and other wood rot fungi. *Fuel Processing Technology* **52**: 79-93.
- Ramgareeb, S., Watt, M.P., Marsh, C., & Cooke, J.A. (1999) Assessment of Al³⁺ availability in callus culture media for screening tolerant genotypes of *Cynodon dactylon*. *Plant Cell Tissue and Organ Culture* **56**:65-68.
- Rayle, DL., & Cleland, R.E. (1992) The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiology* **99**: 1271 – 1274.
- Reiss, R., Ihssen, J., & Thony-Meyer, L. (2011) *Bacillus pumilus*: a heat stable enzyme with a wide substrate spectrum. *BMC Biotechnology* **9**:1-11.
- Rose, P.D., Egbinigie, E.E., Horan, M., Atkins, S., van Dyk, S., & van Breugel, Y. (2007b) Biotechnology of coal biosolubilisation and applications in waste coal beneficiation. **Anglo Coal Report, Phase II.**: 1-349.
- Rose, P.D., Egbinigie, E.E., Horan, M.P., Dames, J.F., & Mukasa-Mugerwa, T.T. (2010) Beneficiation of coal. *South African Patent Office*. ZA 2010/02354:

- Saraya, R., Veenhuis, M., & Van der Klei, I.J. (2010) Peroxisomes as dynamic organelles: peroxisome abundance in yeast. *The FEBS Journal* **277**: 3279 – 3288.
- Sekabira, K., Oryem-Origa, H., Mutumba, G., Kakudidi, E., & Basamba, T.A. (2011) Heavy metal phytoremediation by *Commelina benghalensis* (L) and *Cynodon dactylon* (L) growing in urban stream sediments. *International Journal of Plant Physiology and Biochemistry* **3**:133-142.
- Sekhohola, L., Igbini, E., & Cowan, A. (2013) Biological degradation and solubilisation of coal. *Biodegradation* **24**:305-318.
- Sekhohola, L.M., Issacs, M.L., & Cowan, A.K. (2014) Fungal colonisation and enzyme-mediated metabolism of waste coal by *Neosartorya fischeri* strain ECCN 84. *Bioscience, Biotechnology and Biochemistry* **78**:1797-1802.
- Skodras, G., Kokorotsikos, P., & Serafidou, M. (2014) Cation exchange capability and reactivity of low-rank coals and chars. *Central European Journal of Chemistry* **12**: 33-43.
- Steffen, K.T., A. Hatakka, & M. Hofrichter. (2002) Degradation of humic acids by the litter-decomposing basidiomycetes *Collybia dryophila*. *Applied and Environmental Microbiology* **68**:3442-3448
- Stoilova, I., Krastanov, A., & Stanchev, V. (2010) Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. *Advances in Bioscience and Biotechnology* **1**:208-215.
- Su-dong, Y., Xiu-xiang, T., & Kai-yi, S. (2009) Bio-solubilization of Chinese lignite II: protein adsorption onto the lignite surface. *Mining Science and Technology* **19**:0363 – 0368.
- Szczepanska, J., & Twardowska, I. (1999) Distribution and Environmental impact of coal-mining wastes in Upper Silesia, Poland. *Environmental Geology* **38**:249-258.
- Szulc, A., Ambrozewicz, D., Sydow, M., Lawniczak, L., Piotrowska-Cyplic, A., Marecik, R., & Chrzanowski, L. (2014) The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: feasibility during field studies. *Journal of Environmental Management* **132**:121-128.
- Tao, X.X., Chen, H., Shi, K.Y. & Lv, Z.P. (2010) Identification and biological characteristics of a newly isolate fungus *Hypocrea lixii* and its role in lignite bioconversion. *African Journal of Microbial Research* **4**:1842-1847.
- Tiwary, R.K. (2001) Environmental impact of coal mining on water regime and its management. *Water, Air, and Soil Pollution* **132**:185-199.
- Torzilli, A.P., & Isbister, J.D. (1994) Comparison of coal solubilisation by bacteria and fungi. *Biodegradation* **5**:55 – 62

- Traversa, A., Loffredo, E., Gattullo, C.E., Palazzo, A.J., Bashore, T.L., & Senesi, N. (2014) Comparative evaluation of compost humic acids and their effects on the germination of switchgrass (*Panicum virgatum* L.). *Journal of Soils Sediments* **14**:432-440.
- Tripathi, R.C., Jain, V.K., & Tripathi, P.S. M. (2010) Fungal solubilisation of Neyveli lignite into humic acid. *Energy Source Part A* **32**:72-82.
- Uribe-Alvarez, C., Ayala, M., Perezgasga, L., Naranjo, L., Urbina, H., & Vazquez-Duhalt, R. (2011) First evidence of mineralisation of petroleum asphaltene by a strain of *Neosartorya fischeri*. *Microbial Biotechnology* **4**:663-672.
- Valdrighi, M. M., Pera, A., Agnolucci, M., Frassinetti, S., Lunardi, D., & Vallini, G. (1996) Effects of compost-derived humic acids on vegetable biomass production and microbial growth within a plant (*Cichorium intybus*) – soil system: comparative study. *Agriculture, Ecosystems and Environment* **58**:133-144.
- Visser, S. A. (1985) Physiological action of humic substances on microbial cells. *Soil biology and biochemistry* **17**:457-462.
- Willmann, G., & Fakoussa, R. (1997) Extracellular oxidative enzymes of coal-attacking fungi. *Fuel Processing Technology* **52**:27-41.
- Xiu-xiang, T., Lan-ying, P., Kay-yi, S., Hui, C., Su-dong, Y., & Zhen-fu, L. (2009) Bio-solubilisation of Chinese lignite I: extra-cellular protein analysis. *Mining Science and Technology* **19**: 0358-0362.
- Ying, D., Xiu-xiang, T., Kaiyi, S., & Yang, L. (2010) Degradation of lignin model compounds by the action of white rot fungi. *Mining Science and Technology* **20**: 0076-0081.
- Zapata-Castillo, P., Vilallonga-Santana, M.L., Tamayo-Cortes, J., Rivera-Munoz, G., & Solis-Pereira, S. (2012) Purification and characterisation of laccase from *Trametes hirsuta* Bm-2 and its contribution to dye and effluent decolorization. *African Journal of Biotechnology* **11**:3603-3611.
- Zavarzina, A. G., Leontievsky, A. A., Golovleva, L. A., & Trofimov, S. Y. (2004) Biotransformation of soil humic acids by blue laccase of *Panus tigrinus* 8/18: an in vitro study. *Soil Biology and Biochemistry* **36**:359-369.
- Zhao, Z., Shahrour, I., Bai, Z., Fan, W., Feng, L., & Li, H. (2013) Soils development in opencast coal mine spoils reclaimed for 1-13 years in the West-Northern Loess Plateau of China. *European Journal of Soil Biology* **55**:40-46.

Paper 1

Biological degradation and solubilisation of coal: a review

Lerato M. Sekhohola, Eric E. Igbini and A. Keith Cowan

Published in Biodegradation

Biological degradation and solubilisation of coal: a review

Lerato M. Sekhohola • Eric E. Igbini • A. Keith Cowan

Received: 20 November 2011 / Accepted: 13 September 2012 / Published online: 22 September 2012

Abstract This review focuses on ligninolytic fungi, soil bacteria, plants and root exudates in the degradation and solubilisation of low grade and waste coal and the interaction between these mutualistic biocatalysts. Coal represents a considerable portion of the total global fossil fuel reserve and continued demand for, and supply of this resource generates vast quantities of spoil and low grade waste. Large scale bioremediation technologies for the beneficiation of waste coal have unfortunately not yet been realised despite the many discoveries of microorganisms capable of lignite, lignin, and humic acid breakdown. Even so, solubilisation and depolymerization of low grade coal appears to involve either ligninolytic enzyme action or the production of alkaline substances or both. While the precise mechanism of coal biosolubilisation is unclear, a model for the phyto-biodegradation of low rank coal by mutualistic interaction between ligninolytic microorganisms and higher plants is proposed. Based on accumulated evidence this model suggests that solubilisation and degradation of lignite and waste coals commences upon plant root exudate and ligninolytic microorganism interaction, which is mutualistic, and includes soil bacteria and both mycorrhizal and non-mycorrhizal fungi. It is envisaged that this model and its further elaboration will aid in the development of functional technologies for commercial bioremediation of coal mine spoils, contribute to soil formation, and the overall biogeochemistry of organic carbon in the global ecosystem.

Keywords Waste coal • Coal discard dumps • Rehabilitation • Phyto-biodegradation • Fungi • Arbuscular mycorrhizal fungi • Soil bacteria

Introduction

The coal mining sector is a major player in the global economy and by its very nature and activity impacts the biophysical, social and economic environment. Coals are formed by the accumulation of large quantities of plant remains and their subsequent decomposition and consolidation. Over extended geological periods, these processes result in the formation of peat, lignite or brown coals, bituminous coals, and finally anthracites. Coal is one of the world's most abundant fossil energy resources and it is estimated that world coal reserves are currently at 1.53×10^{20} Btu or 71.4% of the total world fossil fuel resource. Coal bio-solubilisation technology has the potential to elevate low rank and waste coal to either a clean, cost-effective energy feedstock or source of complex aromatic compounds for biocatalytic conversion to value-added products. Included here are the spoils of coal mining such as roof coal, coal fines generated during mining and coal washing operations, and waste coal i.e. discard left in the void following extraction of the main coal seam. These forms of coal are typically low calorific and have little or no apparent use and pose handling and storage problems in particular for land rehabilitation after mine closure.

A number of terms are used to describe different stages in coal biodegradation and include solubilisation, liquefaction, depolymerization, and decolourisation (Ralph and Catcheside 1996; Hofrichter et al. 1997; Laborda et al. 1999). Solubilisation and depolymerization are used frequently

and refer respectively to the dissolution of all or part of the coal molecule and the subsequent catabolic reduction of higher molecular-mass coal to smaller fractions with or without a loss of chromophores (Klein et al. 1999; Hofrichter and Fakoussa 2001). A number of microorganisms have been reported in the literature to be actively involved in one or another form of coal degradation and for some the extracellular enzymes involved are known. However, the underlying mechanism(s) employed in the process of coal biodegradation are not yet fully established which is in part due to the highly complex structure of coals (Ralph and Catcheside 1996; Kulikova et al. 2005). It has been postulated though, that chelators, alkaline substances, oxidative and hydrolytic enzymes produced by ligninolytic microorganisms play a role in coal solubilisation and depolymerization (Ralph and Catcheside 1996; Hölker et al. 1999; Laborda et al. 1999). Due to their diversity and complex enzymatic function, the manner and rate at which these microorganisms solubilise and depolymerise coals differs and is influenced by prevailing environmental conditions (Grinhut et al. 2007). Also, it seems that consortia of microorganisms are more efficient as biocatalysts than individual strains (Hofrichter et al. 1999; Grinhut et al. 2007). Thus, while the concept of population dynamics and environmental interactions amongst populations of different microbes involved in the degradation of complex substrates is not new, focus on coal solubilisation appears to have been overlooked.

In this review ligninolytic fungi and soil bacteria, and the contribution of plants and root exudates, and their interaction in waste coal biosolubilisation and degradation is highlighted. The principal importance of ligninolytic fungi and bacteria to the field of coal bioremediation appears to lie in the ability of these microorganisms to degrade aromatic compounds. Perhaps a major difficulty in developing practical bioremediation processes for treatment and valorization of low grade and waste coals has been the inability to bring metabolically active biocatalysts into direct contact with the coal substrate. Recently, studies in our laboratory utilizing the mutualistic interaction between plant roots and ligninolytic fungi to aid plant growth on coal discard dumps strongly suggest that a phyto-bioconversion process is facilitated by just such an interaction (Igbini et al. 2008; 2010).

Coal diagenesis and structure

A detailed account of the diagenesis and structure of coal is beyond the scope of this review. It is however necessary to provide some information on coal formation, classification and structure for the non-specialist and researchers in allied fields. Briefly, coal deposits are believed to have been formed through massive accumulation of plant debris that was deposited in sedimentary basins over geological time by the process known as coalification (Hatcher et al. 1982). The formation of coal begins with peatification, a process in which buried woody material is transformed into peat and this is in turn, gradually transformed into coal (Fakoussa and Hofrichter 1999). The process follows a biochemical phase and a geochemical phase that lead to transformation of both the chemical structure and physical properties of the parent material. During peatification (biochemical phase) hemicelluloses in the buried wood material are removed by microbial hydrolysis; cellulose and hexose sugars are initially enriched and thereafter gradually broken down, while resistant lignin is selectively preserved (Stout et al. 1988). This is followed by coalification (geochemical phase) in which a gradual transformation of peat into coal occurs by a moderate rise in temperature and pressure (resulting in the loss of water) from the increasing weight of the overlying sediments (DEL Rio et al. 1994). Such processes increase the structural complexity of the materials which comprise coal (Catcheside and Ralph 1999; Fakoussa and Hofrichter 1999). As lignin residues are converted to

coalified material the oxygen content of intermediates remains constant while the hydrogen and carbon contents decrease and increase respectively due to loss of methoxyl groups, water, and C3 side chains (Hatcher et al. 1982). In the later stages of coalification, increasing compaction and dewatering of the coalified material causes loss of soluble, oxygen-rich humic acids to decrease the overall oxygen content but increase carbon content without any significant change in hydrogen (Hatcher et al. 1982).

Different forms of coal arise due to differences in parent material, duration of formation, and the many different factors influencing its formation which makes it almost impossible to present a definitive structure. As a consequence, only hypothetical models of the structure of coals are possible although, aromaticity declines from hard to low rank coal (Fakoussa and Hofrichter 1999). Thus, coal is generally classified into four ranks, namely anthracite (hard coal), bituminous, sub-bituminous and lignite (low rank coal) based on fixed-carbon content and heating value (Table 1).

Degradation of coal

Coal degradation can be classified into thermal, chemical and biological (Hodek 1994). It is the latter process that is elaborated in this review with attention focused on phyto-biodegradation. At the outset, it is important to acknowledge that biodegradation studies are underpinned by studies and observations from the thermal and chemical denaturation of coal. Product analysis from these studies of coal have provided insight into the strength of the chemical bonds in coal and whether or not these are amenable to microbial attack. In addition, the chemistry of both thermal and chemical degradation reactions has provided information on the molecular structure and arrangements in coal (Kögel-Knabner 2000). As a consequence, coal has for the most part been viewed as a recalcitrant substrate for biological conversion. Thus, coal processing (consumptive degradation) has been restricted largely to thermal and chemical technologies.

Besides heat and electricity generation, destructive distillation (carbonization) of coal was developed and is used to produce hydrocarbon gases and coal tar from which various drugs, dyes, plastics, solvents, and numerous other organic chemicals were and continue to be synthesized. High pressure coal hydrogenation or liquefaction and the indirect liquefaction of coal using Fischer–Tropsch syntheses are today also used to produce clean-burning liquid fuels and lubricants (Hodek 1994; Hofrichter et al. 1997; Laborda et al. 1999). As might be expected however, there are many disadvantages to using coal as a fuel or raw material due to its pollutant potential during both its mining and consumption. Of particular concern is the accumulating stock pile of low rank coal (e.g. discard; and, coal fines generated during washing and storage) and the difficulty associated with remediation and land rehabilitation once mining ceases. Also, thermal power plants produce large quantities of fly ash and this poses handling, storage, and disposal problems, apart from the possible contamination of soils, crops, and surface and ground water with toxic trace and heavy metals (Carlson and Adriano 1993; Juwarkar and Jambhulkar 2008a; 2008b) and radionuclides (Ramachandran et al. 1990). A possible solution to such problems is the development of appropriate remedial technologies using a biological intervention strategy including saprotrophic fungi and in particular ligninolytic microorganisms as the biocatalysts.

Biological degradation and solubilisation of coal

Three groups of aromatics pose potential risk as pollutants: polyaromatic hydrocarbons (PAHs), benzene/toluene/ethyl benzene/xylene (BTEX) and synthetic substituted aromatics typified by chlorophenols (Harvey and Thurston 2001), and each group is adequately represented in various coals. Ligninolytic fungi are well known biodegraders of PAHs and do so through the production and secretion of one or more of the three principal ligninolytic enzymes (Hatakka 1994), i.e. lignin peroxidase (LiP, E.C. 1.11.1.14), Mn-dependent peroxidase (MnP, E.C. 1.11.1.13) and phenol oxidase (laccase) (LAC, E.C. 1.10.3.2) (Thurston 1994; Orth and Tien 1995). Since the first demonstration by Fakoussa in 1981 of the microbial degradation of coal, a large number of bacteria (Yuan et al. 1995), Ascomycota (Quigley 1989) and Basidiomycota (Hatakka 1994) capable of degrading coal have been identified (Table 2). In addition, several microalgae capable of utilizing aromatic pollutants have been identified and these organisms might also play a role by using coal breakdown products as a source of carbon (Semple et al. 1999). In the latter, it was shown that eukaryotic algae are capable of biotransforming and biodegrading aromatic pollutants and enhancing the degradation potential of the microbiota present. For example, degradation of benzo[*a*]pyrene by *Mycobacterium* sp. in concert with the green algae *Selanastrum capricornutum* is enhanced (Warshawsky et al. 2007).

Fungal degradation and solubilisation of lignite, lignin and humic acid

Biosolubilisation of coal has been widely studied and three general processes are accepted. These include; enzymatic (Fakoussa and Hofrichter 1999), alkaline chemicals (Quigley et al. 1988), and surfactants (Fakoussa 1988). Since low-rank coal is structurally similar to lignin (Hayatsu et al. 1979), lignin-degrading enzymes (such as LiP, MnP and LAC) might be expected to solubilise and/or degrade coal (Fakoussa and Hofrichter 1999). Alkaline chemicals also cause coal solubilisation because humic components are readily soluble in alkali (Quigley et al. 1988). Surfactants act by decreasing surface tension which tends to increase the solubility of coal particles (Fakoussa 1988). However and as recently noted, coal biosolubilisation by surfactants seems to arise as a consequence of the interaction between coal, surfactant, and microorganisms/enzymes rather than between coal and surfactant alone (Yin et al. 2011). Thus, whereas non-enzymatic mechanisms are responsible for coal degradation per se (Cohen et al. 1990) oxidases appear to act secondarily (Holker et al. 2002). In short, bacteria seem to use alkalinity and chelation to solubilise and depolymerise lignite whereas for higher fungi enzymatic oxidation plays an important role (Table 2).

The Kingdom Fungi comprises three phyla based on reproductive structure and these are; Phylum Zycomycota (conjugation fungi), Phylum Ascomycota (sac fungi), and Phylum Basidiomycota (club fungi). *Hypocrea lixii*, an ascomycete, was recently shown to solubilise oxidized lignite by degradation of carboxyl and hydroxyl groups (Tao et al. 2010) and the biochemistry and enzymology of lignite degradation in Basidiomycota, such as *Trametes versicolor* (Fakoussa and Frost 1999) and *Phanerochaete chrysosporium* (Ralph and Catcheside 1997) is well understood. In addition, there are reports indicating that species of Deuteromycota (fungi imperfecti) such as *Penicillium* sp. (Laborda et al. 1999; Yuan et al. 2006) and *Neosartorya fischeri* (Igbini et al. 2010) also degrade lignite.

Early work by Fakoussa (1981) on microbial degradation and utilization of coal was followed by demonstration of the solubilisation of low-rank coal by the fungi *Polyporus versicolor* and *Poria monticola* (Cohen and Gabriele 1982). Both organisms are wood-decaying and decompose lignified material by different mechanisms. *Polyporus versicolor* produces and utilizes polyphenol oxidase

and peroxidase enzymes to digest phenylpropanoid polymers and associated aromatics that make up the polymeric structure of lignin. *Poria monticola* by comparison, digests polysaccharides using β -glucosidases which attack mainly celluloses. Unfortunately, Cohen and Gabriele (1982) did not unequivocally establish whether coal degradation occurred as a consequence of enzyme action. The authors did however establish that some activity resulting from fungal growth was necessary for coal degradation to occur. More recently, Hölker and Hofer (2002) reported on the *in vitro* solubilisation of lignite by solid substrate fermentation. The process was catalysed by the deuteromycete *Trichoderma atroviride* CBS 349 in a semi-continuous fed-batch culture. Over a period of 40 days, the catalyst secreted esterase and LAC which purportedly solubilised approximately 10% of the lignite substrate. In the course of the experiment, digestate was harvested daily and physicochemical analysis of the soluble products revealed compounds that resembled humic and fulvic acids in a 7:3 ratio.

Lignin is a heterogeneous aromatic containing polymer and a substrate for fungal extracellular MnP and LiP (Hammel and Moen 1991; Wariishi et al. 1991; Hofrichter 2002). Higher filamentous fungi are the primary degraders of lignin in nature (Hammel 1996) and studies have shown that a significant structural component of coal is macromolecular and resembles that of lignin (Polman et al. 1994; Ralph and Catcheside 1997; Hofrichter et al. 1999; Steffen et al. 2002). Additionally, most extracts of coal contain resins and waxes and these are characterized by simple aromatic and aliphatic compounds (Hodek 1994). Such lignin-derived molecules suggest that coal might be a substrate for degradation by ligninolytic fungi (e.g. white and brown rot fungi) and ligninolytic bacteria (Orth et al. 1993; Catcheside and Ralph 1999; Fakoussa and Hofrichter 1999; Laborda et al. 1999; Mester and Tien 2000; Grinhut et al. 2007).

Although LiP was thought to be the only enzyme involved in lignin degradation by fungi MnP has been included following the demonstration that some white rot fungi do not produce LiP but still degrade lignin (Hofrichter 2002). In addition, Orth et al. (1993) reported the presence of MnP in twelve species of fungi known to degrade lignin including; *Cyathus stercoreus*, *Dichomitus squalens*, *Ganoderma lucidum*, *Grifola frondosa*, *Lentinula edodes*, *Perenniporia medulla-panis*, *Phanerochaete chrysosporium*, *Pleurotus sapidus*, *Pleurotus eryngii*, *Pleurotus pulmonaris*, *Polyporus versicolor* and *Trametes cingulata*. Interestingly, of the twelve species investigated *Phanerochaete chrysosporium*, *Pleurotus* sp. and *Trametes* sp. have also been reported to actively degrade coal (Table 2). Some fungal strains are able to produce all three of the major enzymes implicated in lignin degradation while others produce one or two (Perez et al., 2002). Laccase, MnP and LiP require the presence of low molecular weight co-factors and mediators to destabilize lignin and facilitate further depolymerization (Perez et al., 2002; Conesa et al. 2002). Reductive enzymes such as cellobiose oxidizing enzyme, aryl alcohol oxidase and aryl alcohol dehydrogenase also appear to participate in lignin degradation (Perez et al., 2002).

Humic acid is ubiquitous in nature (Demirbas et al. 2006; Grinhut et al. 2007) and as a component in low rank coal it is present at concentrations greater than in most other sources (Table 3). Similar to peat, compost, and soil, humic acid is bound to the coal substructure by hydrogen bonds, van der Waal's forces and other weak bonds (Hodek 1994; Marzec 2002). Its highly condensed three-dimensional molecular structure is typically characterized by aromatic compounds, which are usually resistant to degradation by microorganisms (Almendros and Dorado 1999; Campitelli et al. 2006; Brunetti et al. 2007). Linkages between the aromatic macromolecules are attributed to amino acids, amino sugars, peptides and aliphatic compounds (Demirbas et al. 2006). The structure of extracted

humic acids however differs and depends on origin. For example, Brunetti et al. (2007) compared the structure of humic acid extracted from native and organic amended soils which revealed an abundance of aliphatic and amide structures with fewer carbonyl and carboxylic groups in organic amendment-derived humic acids than in native soil-derived humic acids.

Despite the aromaticity of humic acids, microbial degradation has been the subject of a number of studies and results show that these complex structures can promote microbial growth (Visser 1985a; Visser 1985b; Valdrighi et al. 1996; Fakoussa and Frost 1999). For example, in our laboratory it has been demonstrated that weathered coal-derived humic acid can be used as the sole carbon source to support fungal growth on basal medium (Sekhohola et al. unpublished). In these studies, fungal biomass measured over a period of 42 days increased significantly before stationery phase was reached suggesting that humic acid is biodegradable and does serve as carbon source for microbial proliferation and activity. Furthermore, Temp et al. (1999) found that *Pycnoporus cinnabarinus* (which secretes LAC as the only extracellular phenol oxidase) can degrade coal humic acid. Also, fungi of the Ascomycota and Basidiomycota are known to actively decompose humic substances using a nonspecific ligninolytic enzyme system comprising peroxidases (e.g. LiP and MnP), phenol oxidases (e.g. LAC), supporting enzymes (e.g. hydrogen peroxide-generating oxidases) and low-molecular-weight organic acids such as oxalate, malate, and malonate (Fakoussa and Hofrichter 1999; Zavarzina et al. 2004). In addition, humic acid-degrading microorganisms appear to release chelators, which sequester inorganic cations, and alkaline substances to ionize acidic functional groups that together destabilize the humic acid structure (Ralph and Catheside 1999; Laborda et al. 1999). The resultant unstable structure is further solubilised into aromatic molecules of high molecular weight (Ralph and Catheside 1999).

Fungal peroxidases oxidize both phenolic and non-phenolic aromatic compounds using different reducing substrates (Conesa et al. 2002). Mn-dependent peroxidases generate Mn^{3+} through oxidation of Mn^{2+} , with H_2O_2 as the oxidant (Hofrichter 2002; Kersten and Cullen 2007). Mn^{3+} chelates organic acids to produce phenoxyl radical intermediates that in turn oxidize monomeric and dimeric phenols (Conesa et al. 2002; Hofrichter 2002). Chelators thus produced diffuse from the enzyme surface and oxidize the organic substrates (Conesa et al. 2002). Laccase has also been proposed to cleave non-phenolic lignin structures through a mechanism similar to that of chelated Mn^{3+} in the absence of any additional co-factors. Lignin peroxidase on the other hand catalyzes cleavage of the alkyl side chains, benzylic alcohol oxidation, and ring opening reactions in a number of lignin model compounds through aryl cation radical intermediates (Hammel and Moen 1991; Wariishi et al. 1991; Conesa et al. 2002; Kersten and Cullen 2007). Lastly, Steffen et al (2002) investigated the degradation of humic acids by the litter-decomposing basidiomycete, *Collybia dryophila* isolated from both soil litter and humus-rich soils. The humic acids used included a sample of soil litter derived humic acid (LHA) obtained by alkaline extraction and a synthetic ^{14}C -labelled humic acid synthesized by spontaneous oxidative polymerization of catechol. Liquid and solid-state cultures of *Collybia dryophila* were supplemented with or without $MnCl_2$ and/or LHA, and incubated. Results revealed that recovery of humic acids following the addition of Mn^{2+} was 75% and 60% respectively. Cultures containing Mn^{2+} also showed twice the concentration of the low molecular mass species fulvic acid compared to those without Mn^{2+} . Enzyme assays revealed that Mn^{2+} stimulated the production of MnP but had little or no effect on activity of LAC by *Collybia dryophila*. The two enzymes were thus most active in cultures containing Mn^{2+} and LHA and

resulted in prominent decolourisation of LHA lending support to the idea that similar microorganisms catalyse coal solubilisation.

Bacterial degradation and solubilisation of lignite, lignin and humic acid

Reports on bacterial solubilisation of coal date as far back as 1987 and soil bacteria have been implicated in the metabolism of lignin (Vicuna 1988; Zimmermann 1990). For example, *Streptomyces viridosporus* appears to produce extracellular peroxidases (Crawford et al. 1983) while *Streptomyces viridosporus* T7A and *S. setonii* 75Vi2 both solubilised lignite and subbituminous coals when added either on solid media or in submerged cultures (Strandberg and Lewis 1987). It was also observed that in submerged cultures these bacteria released coal solubilizing agents suspected to be basic polypeptides or polyamines (Strandberg and Lewis 1987). In other studies Quigley et al. (1989) reported the liquefaction of lignite when added to mixed bacterial culture lawns growing on solid media while mixed cultures of *Bacillus cereus*, *Bacillus pumilus* and *Bacillus subtilis* were observed to solubilise oxidized lignite comparable to that of the coal solubilizing fungus *Cunning hamella* (Maka et al. 1989). Even so, all attempts to unequivocally establish biosolubilisation by enzyme action were unsuccessful and by 1999 no known coal degrading or ligninolytic enzymes had been identified from bacteria (Fakoussa and Hofrichter 1999).

Several reports indicate that soil bacteria are capable of metabolizing lignin (Vicuna 1988; Zimmermann 1990). *Streptomyces viridosporus* is reported to produce extracellular peroxidases (Crawford et al. 1983) and metabolize lignin model compounds (Ramachandra et al. 1988). Using a radiochemical assay, lignin degradation activity was reported for strains of *Nocardia autotrophica* and *Rhodococcus* sp. (Zimmermann 1990). The discovery of a naphthalene dioxygenase gene in a strain of β proteobacterium closely related to *Polaromonas vacuolata* (Jeon et al. 2003), the isolation of three novel ligninolytic bacterial strains by 16S rRNA gene sequencing and phenotypic characterization, using a model industrial lignin residue from the Kraft process (Bandounas et al. 2011) and the recent identification of DypB gene from *Rhodococcus jostii* RHA1 as a LiP, which appears to have both Mn²⁺ and lignin oxidation sites (Ahmad et al. 2011; Roberts et al. 2011), indicate the potential of bacteria to oxidize polymeric lignin.

Efficient degradation of humic acids from coal by the soil bacterium *Pseudomonas putida* has been demonstrated (Machnikowska et al. 2002). In addition, the commonality of humic acid-oxidizing soil bacteria (Coates et al. 2002), the ability of nitrate-reducing microorganisms to use reduced humic acids as electron donors (Van Trump et al. 2011), and the increased degradation of humic acid-sorbed polynuclear aromatic hydrocarbons by PAH-degrading microbes (Vacca et al. 2005) is sufficient evidence for utilization and bioconversion of humic acids by soil bacteria.

Plant root exudates and ligninolysis

Root exudates play a primary role in plant mineral nutrition and contain molecular signals that regulate microbial growth and development and chemical compounds that mediate rhizosphere processes to enhance uptake and assimilation of nutrients (Dakora and Phillips 2002). Root exudation is usually by excretion, which involves gradient dependent release of waste, or secretion, involving exudation of compounds with known functions (Bais et al. 2004; 2006). As a result, root exudates are complex, and can comprise mucilage, root border cells, extracellular enzymes,

surfactants, simple and complex sugars, phenolics, amino acids, vitamins, organic acids, nitrogenous purines and nucleosides and inorganic or gaseous molecules such as HCO_3^- , OH^- , H^+ , CO_2 and H_2 (Marschner 1995; Bais et al. 2006).

Remediation within the rhizosphere usually arises from root exudates and exudate mediated stimulation of bacterial growth resulting in more efficient pollutant degradation. For example, root-exuded enzymes of selected crop plants include those for hydroxylation and aromatic-ring cleavage (Gramss and Rudeschko 1998). Although ligninolytic activities (as indicated by the formation of Mn^{3+} chelates and the presence of decolorizing activity against dye macromolecules) are more frequent in tissue extracts, the oxidoreductases believed to degrade lignin, phenolics, and xenobiotic organo-pollutants seem to be present in aseptically collected root exudates. Furthermore, *Pseudomonas putida* associated with roots of the monocots *Zea mays* (corn) and *Triticum aestivum* (wheat) effectively removes 3-methyl benzoate and 2,4-D, respectively from polluted soils (Ronchel and Ramos 2001; Kuiper et al. 2004, Novotný et al. 2004).

The rhizosphere is both active and dynamic and newly generated carbon, derived from root exudates, and ancient carbon in organic matter (usually soil organic matter; SOM) is used by soil microbes for energy and biomass production. DNA extracted from rhizosphere soil has been used to study the impact of plant species, producing different nutrients and signalling molecules, on microbial populations assimilating root exudates and on how different plant species contribute to use of SOM. Interestingly, bacteria related to Sphingobacteriales and *Myxococcus* assimilated root exudates only whereas bacteria related to the Sphingomonadales assimilated root exudates and used SOM carbon similar to the generalist bacteria related to *Enterobacter* and Rhizobiales, but were specific for monocotyledonous species (el Zahar Haichar et al. 2008). Some members of the *Sphingomonadaceae* use a wide range of carbon sources and many are well known degraders of recalcitrant (xenobiotic) molecules (Leys et al. 2004; Keck et al. 2006). For example, Alphaproteobacteria are catalase and oxidase positive and are rich in the fatty acid *cis*-octadecanoic acid and their ability to degrade phenanthrene, naphthalene, fluorene, biphenyl, and dibenzothiophene has been confirmed (Kim and Kwon 2010). Furthermore, *Sphingomonas* sp. recovered from the deep subsurface appear to be involved in lignite degradation (Fredrickson et al. 1999) and to actively metabolize the biphenyl structures in lignin (Katayama et al. 1988). More recent studies have revealed a *Sphingomonas* species capable of causing mineral weathering in the hyphosphere of *Scleroderma citrinum* (Uroz et al. 2007) while analysis of the mycospheres of *Laccaria proxima* and *Russula exalbicans* revealed divergent community structures indicating that different fungi select for different members of the *Sphingomonadaceae* (Boersma et al. 2009). Methods have been developed to link function with phylogeny and these have improved our understanding of microbial ecology to provide a platform for establishing unequivocal 'function-identity' (Gutierrez-Zamora and Manefield 2010). Application of such methods to coal biotechnology will undoubtedly assist in the elucidation of the microbial interrelationships required to achieve successful biosolubilisation of this recalcitrant substance.

It has been argued that the greater microbial activity around plant roots compared with the bulk soil, the faster the turnover of organic material and mineralization of nitrogen. Analysis of plant and soil microbial community interactions has revealed that differences in microbial communities caused by plants are greater and clearer for arbuscular mycorrhizal fungi (AMF) than for bacteria (Millard and Singh 2010). Thus, interactions exist between AMF and plant species but not between bacterial and plant diversity and a concept model has been proposed to explain these interactions. Root

symbionts use simple organic carbon in root exudates whereas free living bacterial and fungal decomposers use predominantly complex carbon but are nevertheless influenced by root exudates, litter returns and carbon input from symbionts. The latter is assumed to explain the observation that AMF and bacterial assemblages are related *in situ* and that AMF are the major factor determining the bacterial assemblage on grass roots (Singh et al. 2008). Decomposers are also influenced by the quality of organic material. Paterson et al. (2007) demonstrated that in the rhizosphere of *Lolium perenne*, plant-derived carbon was recovered in all microbial phospholipids, but after four weeks of growth more than 80% of the bacterial biomass had been replaced by carbon from SOM. This indicates that SOM is an important source of carbon for the soil microbial community, even in the presence of more readily available plant-derived carbon. Decomposition of plant and root symbiont litter is slow and as indicated above, can be by either specialists or generalists, individually or synergistically. Because many microbes around the root are generalists utilizing carbon available from both plants and SOM, these organisms are responsive to plant mediated processes, but their overall diversity appears to be influenced more by SOM. As a consequence, bacterial community structure is determined largely by the quality and composition of the SOM, while the activity of the microbial biomass is driven in part by vegetation growth and carbon transfer below-ground (Millard and Singh 2010).

***Cynodon dactylon* as phytobiocatalyst in coal solubilisation**

Igbinigie et al. (2008) reported the effective breakdown of bituminous hard coal on discard coal dumps colonized by *Cynodon dactylon* L (bermudagrass). Root zone investigation and extensive screening led to the isolation and identification of the deuteromycete, *Neosartorya fischeri* as a major biocatalyst in coal solubilisation. Confirmation of coal solubilisation was obtained by a combination of *in vitro* experiments and use of a perfusion fixed-bed bioreactor, to simulate the coal dump environment, and physicochemical characterization of the oxidation and nitration products. This is perhaps not an unexpected result given that “the use of plants and their associated microbes for environmental cleanup” (Pilon-Smits 2005), has emerged as a viable bioprocess technology for the remediation and restoration of polluted soils, specifically the warm-climate grass *C. dactylon* and other members of the Poaceae (Penã-Castro et al. 2006). Furthermore, the percentage of mycorrhiza associated with *C. dactylon* colonizing 3 and 6 year old coal mine dumps in Orissa, India was shown to be 95-96% and comprise hyphae, arbuscules and vesicles, of which >80% were hyphal (Ekka and Behera 2010). *C. dactylon* also grows in a wide range of soils with metal contamination and the effects of metal accumulation in the roots appears to be mitigated by phytochelatin (Schmöger et al. 2000) while mycorrhizal fungi, particularly ericoid and ectomycorrhizal fungi alter the rhizosphere chemistry of different metals by excreting organic acid chelators (Meharg 2003). It is thus not surprising that *C. dactylon* has been proposed as a bio-indicator to assess the degree of land rehabilitation (Shukla et al. 2011). For example, a survey of the AMF of plants growing on mined sites has revealed that members of the Gramineae and Compositae dominate with *Chrysanthemum moritolum*, *C.dactylon*, *Miscanthus florodulus*, and *Pteris vittata* occurring most commonly. All appear to be colonized by AMF and again (see above) arbuscules, vesicles, and coiled hyphae were identified in the roots of *C. dactylon* (Leung et al. 2007).

Studies on the molecular mechanisms underlying adaptation of *C. dactylon* to pollutant stress have indicated the consistent over-expression in roots of gene products that function in general

metabolism (e.g. fatty acids ligase, alcohol dehydrogenase, profilin, glycosyl transferases), signal transduction (e.g. G-protein, serine/threonine phosphatase, histidine kinases), nuclear transport (Ladomain protein and nucleoporin 98), and protein synthesis and degradation (e.g. ribosomal proteins, ubiquitin). Based on the outcome of this functional genomics study the authors proposed a model to describe the metabolic response of Bermuda grass roots to petroleum stress and suggested that effector enzymes are transcribed and act to alleviate harmful effects induced by the pollutant (Leung et al. 2007). It is tempting to suggest that the stress-induced up-regulation of these effector enzymes in roots of *C. dactylon* acts to facilitate colonization of the rhizosphere by microbes including AMF. Some support for this comes from the restoration of lignite fly ash dumps using biofertilizers (i.e. spores of vesicular arbuscular mycorrhizal species, *Rhizobium*, and *Azotobacter*) which caused profuse root development and plant growth in the selected species (Ram et al. 2007; Juwarkar and Jambhulkar 2008a). In particular, the monocotyledonous species *Dendrocalamus strictus* (bamboo) displayed a growth rate 15 times that of the control (Juwarkar and Jambhulkar 2008b). Together, this accumulated information strongly supports the existence of a mutualistic interaction between the roots of members of the Poaceae (e.g. *C. dactylon*) and their associated microflora in the colonization and remediation of polluted soils.

Further study on the biosolubilisation of coals in our laboratory has indicated that phyto-bioconversion of hard coal involves microbes occurring in the rhizosphere which together with the host plant promote the growth of *C. dactylon* (Igbini et al. 2010). In addition, the *in situ* degradation of hard coal appears to occur predominantly due to the in concert interactions between *C. dactylon*, AMF, *Neosartorya fischeri* and other rhizosphere fungi (Mukasa-Mugerwa et al. 2011). Of course, these relationships and their contribution to colonization of coal dumps by *C. dactylon* and coal biosolubilisation by soil bacteria and in particular the sphingomonads still needs investigation.

Phyto-biodegradation of lignite and waste coal: an integrated model

Research on coal bioconversion is rapidly advancing from laboratory scale to industrial application with increased appreciation of the role of microbial activity and the contribution of associated higher plants. Based on the accumulated data from extensive studies on different modes of degradation and transformation of coal by microorganisms and information reviewed in the present work, a model for the phyto-biodegradation of low rank coal by mutualistic interaction between ligninolytic microorganisms and higher plants is proposed (Figure 1). This model suggests that solubilisation and degradation of lignite and waste coals commences upon plant root exudate and ligninolytic microorganism interaction, which is mutualistic, and includes soil bacteria and both mycorrhizal and non-mycorrhizal fungi. It is now also apparent that vesicular arbuscular mycorrhiza is crucial for the establishment of viable, diverse and self-sustaining plant communities on mine spoil (Mukhopadhyay and Maiti 2009). The solubilisation of lignite into humic acids too is effected by fungi at ambient temperature and pressure (Tripathi et al. 2010) and *Penicillium* sp. (Achi 1994; Laborda et al. 1999; Yuan et al. 2006), *Hypocrea lixii* (Tao et al. 2010), and *Neosartorya fischeri* (Igbini et al. 2010) all degrade and solubilise lignite. Indications are that solubilisation is influenced by surfactants which facilitate adsorption of ligninolytic enzymes to the coal surface (Yin et al. 2011). In addition to surfactant-like properties, root exudates also contain small bioactive

molecules involved in chemotaxis and which can act to promote intermolecular binding e.g. in host-microbe recognition.

A major purpose of the model in Figure 1 is to provide a platform on which to develop functional technologies for the bioremediation of coal mine spoils with a view to contributing to soil formation and the overall biogeochemistry of organic carbon in the global ecosystem. Thus, coal particles in close proximity to fungal mycelia are converted to a mixture of heterogeneous humic acids in a form of black liquid droplets (Henning et al. 1997; Catcheside and Ralph 1999). Humic acid which is composed of high molecular-mass organic substances must be depolymerized to yield fulvic acid. The large size humic acid macromolecules require initial degradation into unstable compounds by extracellular enzymes to allow uptake by plants and/or microorganisms.

Conclusion and perspectives

Humic and fulvic acids are regarded as the main carbon reservoir in the biosphere and are estimated to be equivalent to 1600×10^{15} g C. Due to the crucial role of these acids in reductive and oxidative processes, (bio)sorption, (bio) complexing, and transport of pollutants, minerals and trace elements, soil structure and formation, sustaining plant growth, and control of the biogeochemistry of organic carbon in the global ecosystem humics are viewed as being extremely important (Grinhut et al, 2007). Indeed, and as illustrated in Figure 1, humic and fulvic acids are the major products of degradation and biosolubilisation of coal and arise as a consequence of metabolic events catalysed by the mutualistic interaction between plants, fungi and soil bacteria.

Coal biodegradation is a naturally complex process and appears to be driven by an arsenal of extracellular enzymes in the presence of various chelators and supporting enzymes released by different microorganisms that co-inhabit the coal environment. Fundamental research on coal biodegradation has focused mainly on laboratory scale screening methodologies developed to investigate both the microorganisms and mechanisms involved and the products generated at each stage. However, there are few if any examples of the optimization of these processes for industrial scale use. Despite the slow conversion rates in the biological break down of coal, optimization of the process on a large scale could precipitate the development of efficient technologies for remediation and even for beneficiation of low rank coal. Such technologies are essential not least of all to the mining industry where waste coal (mainly low rank coal) has for many years been considered not only a recalcitrant pollutant but the *raison d'être* preventing mine closure. Efforts to mitigate environmental problems associated with the dumping of waste coal material have cost mining companies dearly and implementation of successful biodegradation protocols is urgently needed for rehabilitating waste coal dumps. Work in our laboratory is currently underway to elucidate the mechanisms and biocatalysts involved in coal bioconversion observed in the *Cynodon dactylon*/coal system to derive a novel approach to the longstanding challenges associated with the development of an industrial coal bioconversion process.

Acknowledgements The authors are grateful for financial support provided by Anglo Thermal Coal.

References

- Achi OK (1994) Growth and coal-solubilizing activity of *Penicillium simplicissimum* on coal-related aromatic compounds. *Bioresource Technol* 48:53-57
- Ahmad M, Roberts JN, Hardiman EM, Singh R, Eltis LD, Bugg TDH (2011) Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase. *Biochemistry* 50:5096-5107
- Almendros G, Dorado J (1999) Molecular characteristics related to the biodegradability of humic acid preparations. *Eur J Soil Sci* 50:227-236
- Bais HP, Park SW, Weir TL, Callaway RM, Vivanco JM (2004) How plants communicate using the underground information superhighway. *Trends Plant Sci* 9:26-32
- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* 57:233-266
- Bandounas L, Wierckx NJP, de Winde JH, Ruijsenaars HJ (2011) Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *BMC Biotech* 11:94-105
- Boersma FGH, Warmink JA, Andreote FA, van Elsas JD (2009) Selection of *Sphingomonadaceae* at the base of *Laccaria proxima* and *Russula exalbicans* fruiting bodies. *Appl Environ Microbiol* 75:1979-1989
- Brunetti G, Plaza C, Clapp CE, Senesi N (2007) Compositional and functional features of humic acids from organic amendments and amended soils in Minnesota, USA. *Soil Biol Biochem* 39:1355-1365
- Campitelli PA, Velasco MI, Ceppi SB (2006) Chemical and physicochemical characteristics of humic acids extracted from compost, soil and amended soil. *Talanta* 69:1234-1239
- Carlson CC, Adriano DC (1993) Environmental impacts of coal combustion residues. *J Environ Qual* 22:227-247
- Catcheside DEA, Ralph JP (1999) Biological processing of coal. *Appl Microbiol Biotech* 52:16-24
- Coates JD, Cole KA, Chakraborty R, O'Connor SM, Achenbach LA (2002) Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. *Appl Environ Microbiol* 68:2445-2452.
- Cohen MS, Gabriele PD (1982) Degradation of coal by the fungi *Polyporus versicolor* and *Poria monticola*. *Appl Environ Microbiol* 44:23-27
- Cohen MS, Feldmann KA, Brown CS, Grey ET (1990) Isolation and identification of the coal solubilizing agent produced by *Trametes versicolor*. *Appl Environ Microbiol* 56:3285-3290
- Conesa A, Punt PJ, van den Hondel CAMJJ (2002) Fungal peroxidases: molecular aspects and applications. *J Biotechnol* 93:143-158
- Crawford D, Pometto A III, Crawford R (1983) Lignin degradation by *Streptomyces viridosporus*: Isolation and characterization of a new polymeric lignin degradation intermediate. *Appl Environ Microbiol* 45:898-904
- Dakora FD, Phillips DA (2002) Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* 245:35-47
- DEL Rio JC, González-Villa FM, Verdejo T (1994) Characterization of humic acids from low rank coals by ¹³C-NMR and pyrolysis-methylation. Formation of benzenecarboxylic acid moieties during the coalification process. *Org Geochem* 22:885-891
- Demirbas A, Kar Y, Deveci H (2006) Humic substances and nitrogen-containing compounds from low rank brown coals. *Energy Sources Part A* 28:341-351.
- Ekka NJ, Behera N (2010) A study of the mycorrhizal association with vegetation on coal mines spoil. *The Bioscan* 5:369-372
- Fakoussa RM (1981) Coal as a substrate for microorganisms. Investigations of the microbial decomposition of (untreated) bituminous coals. PhD Dissertation, Rhein Friederich-Wilhelm University, Germany.
- Fakoussa RM (1988) Production of water-soluble coal-substances by partial microbial liquefaction of untreated hard coal, *Resour Conser Recycl* 1:251-260
- Fakoussa RM, Frost PJ (1999) In vivo-decolorization of coal-derived humic acids by laccase-excreting fungus *Trametes versicolor*. *Appl Microbiol Biotechnol* 52:60-65
- Fakoussa RM, Hofrichter M (1999) Biotechnology and microbiology of coal degradation. *Appl Microbiol Biotechnol* 52:25-40
- Fong SS, Seng L, Chong WN, Asing J, Faizal M, Nor M, Satirawaty M, Pauzan A (2006) Characterization of the coal derived humic acids from Mukah, Sarawak as soil conditioner. *J Braz Chem Soc* 17:582-587

- Fredrickson JK, Balkwill DL, Romine MF, Shi T (1999) Ecology, physiology, and phylogeny of deep subsurface *Sphingomonas* sp. *J Ind Microbiol Biotechnol* 23: 273-283
- Gotz GKE, Fakoussa RM (1999) Fungal biosolubilization of Rhenish brown coal monitored by Curie point pyrolysis/gas chromatography/mass spectrometry using tetraethylammonium hydroxide. *Appl Microbiol Biotechnol* 52:25-40
- Gramss G, Rudeschko O (1998) Activities of oxidoreductase enzymes in tissue extracts and sterile root exudates of three crop plants, and some properties of the peroxidase component. *New Phytol* 138:401-409
- Grinhut T, Hadar Y, Chen Y (2007) Degradation and transformation of humic substances by saprotrophic fungi: processes and mechanisms. *Fungal Biol Rev* 21:179-189
- Gutierrez-Zamora M-L, Manefield M (2010) An appraisal of methods for linking environmental processes to specific microbial taxa. *Rev Environ Sci Biotechnol* 9:153-185
- Hammel KE (1996) Extracellular free radical biochemistry of ligninolytic fungi. *New J Chem* 20:195-198
- Hammel KE, Moen MA (1991) Depolymerization of a synthetic lignin in vitro by lignin peroxidase. *Enzyme Microb Technol* 13:15-18
- Harvey PJ, Thurston CF (2001) The biochemistry of ligninolytic fungi In: Gadd GM (ed) *Fungi in bioremediation*. British Mycological Society Symposia, pp 27-51
- Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol Rev* 13:125-135
- Hatcher PG, Breger IA, Szeverenyi N, Maciel GE (1982) Nuclear magnetic resonance studies of ancient buried wood-II. Observations on the origin of coal from lignite to bituminous coal. *Org Geochem* 4:9-18
- Hayatsu R, Winans RE, McBeth RL, Scott RG, Moore LP, Studier MH (1979) Lignin like polymers in coals. *Nature* 278:41-43
- Henning K, Steffes H, Fakoussa RM (1997) Effects on the molecular weight distribution of coal derived humic acids studied by ultrafiltration. *Fuel Process Technol* 52:225-237
- Hodek W (1994) The structure of coal in regard of microbiological degradation. *Fuel Process Technol* 40:369-378
- Hofrichter M (2002) Review: lignin conversion by manganese peroxidase (MnP). *Enzyme Microbiol Technol* 30:454-466
- Hofrichter M, Bublitz F, Fritsche W (1997) Fungal attack on coal II. Solubilization of low-rank coal by filamentous fungi. *Fuel Process Technol* 52:55-64
- Hofrichter M, Fakoussa R (2001) Microbial degradation and modification of coal. In: Steinbuchel A and Hofrichter M (eds) *Lignin, humic substances and coal*, Vol. 1. Wiley-VCH, Weinheim, pp 393-427
- Hofrichter M, Fritsche W (1997) Depolymerization of low-rank coal by extracellular fungal enzyme systems. 2. The ligninolytic enzymes of the coal-humic acid depolymerizing fungus *Nematoloma forwardii* b19. *Fuel Energy Abstr* 38:296
- Hofrichter M, Ziegenhagen D, Sorge S, Ullrich R, Bublitz F, Fritsche W (1999) Degradation of lignite (low-rank coal) by ligninolytic basidiomycetes and their manganese peroxidase system. *Appl Microbiol Biotechnol* 52:78-84
- Hölker U, Höfer M (2002) Solid substrate fermentation of lignite by the coal-solubilizing mould, *Trichoderma atroviride*, in a new type of bioreactor. *Biotechnol Lett* 24:1643-1645
- Hölker U, Ludwig S, Scheel T, Höfer M (1999) Mechanisms of coal solubilization by the deuteromycetes *Trichoderma atroviride* and *Fusarium oxysporum*. *Appl Microbiol Biotechnol* 52:57-59
- Hölker U, Schmiere H, Grobe S, Winkelhofer M, Polsakiewicz M, Ludwig S, Dohse J, Höfer M (2002) Solubilization of low-rank coal by *Trichoderma atroviride*: evidence for the involvement of hydrolytic and oxidative enzymes by using ¹⁴C-labelled lignite. *J Ind Microbiol Biotechnol* 28:207-212
- Igbinigie EE, Aktins S, van Breugel Y, van Dyke S, Davies-Coleman MT, Rose PD (2008) Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri*. *Biotechnol J* 3:1407-1416
- Igbinigie EE, Mutambanengwe CZ, Rose PD (2010) Phyto-bioconversion of hard coal in the *Cynodon dactylon*/coal rhizosphere. *Biotechnol J* 5:292-303
- Jeon CO, Park W, Padmanabhan P, DeRito C, Snape JR, Madsen EL (2003) Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. *Proc Natl Acad Sci USA* 100:13591-13596
- Juwarkar AA, Jambhulkar HP (2008a) Phytoremediation of coal mine spoil dump through integrated biotechnological approach. *Bioresource Technol* 99:4732-4741

- Juwarkar AA, Jambhulkar HP (2008b) Restoration of fly ash dump through biological interventions. *Environ Monit Assess* 139:355-365
- Katayama Y, Nishikawa S, Murayama A, Yamasaki M, Morohoshi N, Haraguchi T (1988) The metabolism of biphenyl structures in lignin by the soil bacterium *Pseudomonas paucimobilis* Syk-6. *FEBS Lett* 233:129-133
- Keck A, Conradt D, Mahler A, Stolz A, Mattes R, Klein J (2006) Identification and functional analysis of the genes for naphthalenesulfonate catabolism by *Sphingomonas xenophaga* BN6. *Microbiol* 152:1929-1940
- Kersten P, Cullen D (2007) Extracellular oxidative systems of the lignin degrading Basidiomycete *Phanerochaete chrysosporium*. *Fungal Genet Biol* 44:77-87
- Kim S-J, Kwon KK (2010) Marine, hydrocarbon degrading alphaproteobacteria. In: Timmis KN (ed) *Handbook of hydrocarbon and lipid microbiology*. Springer-Verlag, Berlin, pp 1707-1714
- Klein J, Catcheside DEA, Fakoussa R, Gazso L, Fritsche W, Höfer M, Laborda F, Margarit I, Rehm H-J, Reich-Walber M, Sand W, Schacht S, Schmiers H, Setti L, Steinbüchel A (1999) Biological processing of fossil fuels - résumé of the bioconversion session of ICCS 97. *Appl Microbiol Biotechnol* 52:2-15
- Kögel-Knabner I (2000) Analytical approaches for characterizing soil organic matter. *Organic Geochem* 31:609-625
- Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ (2004) Rhizoremediation: A beneficial plant-microbe interaction. *MPMI* 17:6-15
- Kulikova NA, Stepanova EV, Koroleva OV (2005) Mitigating Activity of humic substances: direct influence on biota. In: Perminova IV, Hatfield K and Hertkorn N (eds) *Use of humic substances to remediate polluted environments: from theory to practice*. NATO Science Series: IV: Earth Environ Sci 52:285-309
- Laborda F, Monistrol IF, Luna N, Fernández M (1999) Processes of liquefaction/solubilization of Spanish coals by microorganisms. *Appl Microbiol Biotechnol* 52:49-56
- Leung HM, Ye ZH, Wong MH (2007) Survival strategies of plants associated with arbuscularmycorrhizal fungi on toxic mine tailings. *Chemosphere* 66:905-915
- Leys NM, Ryngaert A, Bastiaens L, Verstraete W, Top EM, Springael D (2004) Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. *Appl Environ Microbiol* 70:1944-1955
- Machnikowska H, Pawelec K, Podgórska A (2002) Microbial degradation of low rank coals. *Fuel Process Technol* 77/78:17-23
- Maka A, Srivastava VJ, Kilbane II JJ, Akin C (1989) Biological solubilisation of untreated North Dakota lignite by a mixed bacterial and bacterial/fungal culture. *Appl Biochem Biotechnol* 20/21:715-729
- Marschner H (1995) *Mineral nutrition of higher plants*. 2nd Edn. Academic Press, London
- Marzec A (2002) Towards an understanding of the coal structure: a review. *Fuel Process Technol* 77/78:25-32
- Meharg AA (2003) The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycol Res* 107:1253-1265
- Mester T, Tien M (2000) Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. *Inter Biodeter Biodeg* 46:51-59
- Millard P, Singh BK (2010) Does grassland vegetation drive soil microbial diversity? *Nutr Cycl Agroecosyst* 88:147-158
- Mukasa-Mugerwa TT, Dames JF, Rose PD (2011) The role of a plant/fungal consortium in the degradation of bituminous hard coal. *Biodegradation* 22:129-141
- Mukhopadhyay S, Maiti SK (2009) Reclamation of mine spoils with vesicular arbuscular mycorrhiza (VAM) fungi - a review. *Environ Ecol* 27:642-646
- Novotný C, Svobodová K, Erbanová P, Cajthaml T, Kasinath A, Lang E, Šašek V (2004) Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biol Biochem* 36:1545-1551
- Orth BA, Royse D, Tien M (1993) Ubiquity of lignin-degrading peroxidases among various wood degrading fungi. *Appl Environ Microbiol* 59:4017-4023
- Orth AB, Tien M (1995) Biotechnology of lignin degradation. In: Esser K and Lemke PA (eds) *The mycota. II. Genetics and biotechnology*. Springer-Verlag, Berlin, pp 287-302
- Paterson E, Gebbing T, Abel C, Sim A, Telfer G (2007) Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytol* 173:600-610
- Penã-Castro JM, Barrera-Figueroa BE, Fernández-Linares L, Ruiz-Medrano R, Xoconostle-Cázares B (2006) Isolation and identification of up-regulated genes in bermudagrass roots (*Cynodon dactylon* L.) grown under petroleum hydrocarbon stress. *Plant Science* 170:724-731

- Perez J, Munoz-Dorado J, de la Rubia T, Martinez T (2002) Biodegradation and biological treatments of cellulose, hemicelluloses and lignin: an overview. *Int Microbiol* 5:53-63
- Pilon-Smits E (2005) Phytoremediation. *Annu Rev Plant Biol* 56:15-39
- Polman JK, Stoner DL, Delezene-Briggs KM (1994) Bioconversion of coal, lignin and dimethoxybenzyl alcohol by *Penicillium citrinum*. *J Indust Microbiol* 13:292-299
- Quigley DR, Ward B, Crawford DL, Hatcher HJ, Dugan PR (1989) Evidence that microbially produced alkaline materials are involved in coal biosolubilization. *Appl Biochem Biotechnol* 20/21:753-763
- Quigley DR, Wey JE, Breckenridge CR, Stoner DL (1988) The influence of pH on biological solubilisation of oxidized, low-rank coal. *Resour Conserv Recycl* 1:163-174
- Ralph JP, Catcheside DEA (1996) Size-exclusion chromatography of solubilised low-rank coal. *J Chromatog A* 724:97-105
- Ralph JP, Catcheside DEA (1997) Transformations of low rank coal by *Phanerochaete chrysosporium* and other wood-rot fungi. *Fuel Process Technol* 52:79-93
- Ralph JP, Catcheside DEA (1999) Transformation of macromolecules from a brown-coal by lignin peroxidase. *Appl Microbial Biotechnol* 52:70-77
- Ram LC, Srivastava NK, Jha SK, Sinha AK, Mastro RE, Selvi VA (2007) Management of lignite fly ash for improving soil fertility and crop productivity. *Environ Management* 40:438-452
- Ramachandra M, Crawford D, Hertel G (1988) Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete *Streptomyces viridosporus*. *Appl Environ Microbiol* 54:3057-3063
- Ramachandran TV, Lalit BY, Mishra UC (1990) Modifications in natural radioactivity content of soil samples around thermal power stations in India. *Indian J Environ Health* 32:13-19
- Roberts JN, Singh R, Grigg JC, Murphy MEP, Bugg TDH, Eltis LD (2011) Characterization of dye-decolorizing peroxidases from *Rhodococcus jostii* RHA1. *Biochemistry* 50:5108-5119
- Ronchel M, Ramos JL (2001) Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. *Appl Environ Microbiol* 67:2649-2656
- Semple KT, Cain RB, Schmidt S (1999) Biodegradation of aromatic compounds by microalgae. *FEMS Microbiol Letts* 170:291-300
- Singh BK, Nunan N, Ridgway KP, McNicol J, Young JPW, Daniell TJ, Prosser JI, Millard P (2008) Relationship between assemblages of mycorrhizal fungi and bacteria on grass roots. *Environ Microbiol* 10:534-541
- Schmöger MEV, Oven M, Grill E (2000) Detoxification of arsenic by phytochelatin in plants. *Plant Physiol* 122:793-801
- Strandberg GW, Lewis SN (1987) The Solubilization of coal by an extracellular product from *Streptomyces setonii* 75Vi2. *J Industr Microbiol* 1:371-375.
- Steffen KT, Hatakka A, Hofrichter M (2002) Degradation of humic acids by the litter-decomposing Basidiomycete *Collybia dryophila*. *Appl Environ Microbiol* 68:3442-3448
- Stout SA, Boon JJ, Spackman W (1988) Molecular aspects of the peatification and early coalification of angiosperm and gymnosperm woods. *Geochim Cosmochim Acta* 52:405-414
- Shukla SK, Singh K, Singh B, Gautam NN (2011) Biomass productivity and nutrient availability of *Cyanodon dactylon* (L.) Pers. growing on soils of different sodicity stress. *Biomass Bioenergy* 35:3440-3447
- Tao XX, Chen H, Shi KY, Lv ZP (2010) Identification and biological characteristics of a newly isolated fungus *Hypocrea lixii* and its role in lignite bioconversion. *Afri J Microbiol Res* 4:1842-1847
- Temp U, Meyrahn H, Eggert C (1999) Extracellular phenol oxidase patterns during depolymerization of low-rank coal by three basidiomycetes. *Biotechnol Lett* 21:281-287
- Thurston CF (1994) The structure and function of fungal laccases. *Microbiol* 140:19-26
- Torzilli AP, Isbister JD (1994) Comparison of coal solubilisation by bacteria and fungi. *Biodegradation* 5:55-62
- Tripathi RC, Jain VK, Tripathi PSM (2010) Fungal biosolubilization of Neyveli lignite into humic acid. *Energy Sources A* 32:72-82
- Uroz S, Calvaruso C, Turpault MP, Pierrat JC, Mustin C, Frey-Klett P (2007) Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil. *Appl Environ Microbiol* 73:3019-3027
- Vacca DJ, Bleam WF, Hickey WJ (2005) Isolation of soil bacteria adapted to degrade humic acid-sorbed phenanthrene. *Appl Environ Microbiol* 71:3797-3805

- Valdrighi MM, Pera A, Agnolucci M, Frassinetti S, Lunardi D, Vallini G (1996) Effects of compost derived humic acids on vegetable biomass production and microbial growth within a plant (*Cichorium intybus*) – soil system: comparative study. *Agric Ecosys Environ* 58:133-144
- Van Trump JI, Wrighton KC, Thrash JC, Weber KA, Andersen GL, Coates JD (2011) Humic acid-oxidizing, nitrate-reducing bacteria in agricultural soils. *mBio* 2(4):e00044-11. doi:10.1128/mBio.00044-11
- Vicuna R (1988) Bacterial degradation of lignin. *Enzyme Microb Technol* 10:646-655
- Visser SA (1985a). Physiological action of humic substances on microbial cells. *Soil Biol. Biochem.* 17:457-462
- Visser SA (1985b) Effects of humic acids on numbers and activities of micro-organisms within physiological groups. *Organic Geochem* 8:81-85
- Wariishi H, Valli K, Gold MH (1991) Depolymerization of lignin by manganese peroxidase. *Biochem Biophys Res Commun* 176:269-275
- Warshawsky D, LaDow K, Schneider J (2007) Enhanced degradation of benzo[*a*]pyrene by *Mycobacterium* sp. in conjunction with green alga. *Chemosphere* 69:500-506
- Yin S, Tao X, Shi K (2011) The role of surfactants in coal bio-solubilisation. *Fuel Process Technol* 92:1554-1559
- Yuan H-L, Toyota K, Kimura M (1995) Observation with scanning electron microscope of microbial succession on lignite along with weathering. *Bull Jap Soc Microbiol Ecol* 10:53-58
- Yuan H-L, Yang JS, Wang FQ, Chen WX (2006) Degradation and solubilization of Chinese lignite by *Penicillium* sp. P6. *Appl Biochem Microbiol* 42:52-55
- el Zahar Haichar F, Marol C, Berge OI, Rangel-Castro JI, Prosser JI, Balesdent J, Heulin T, Achouak W (2008) Plant host habitat and root exudates shape soil bacterial community structure. *The ISME Journal* 2:1221-1230
- Zavarzina AG, Leontievsky AA, Golovleva LA, Trofimov SY (2004) Biotransformation of soil humic acids by blue laccase of *Panus tigrinus* 8/18: an in vitro study. *Soil Biol Biochem* 36:359-369
- Zimmermann W (1990) Degradation of lignin by bacteria. *J Biotechnol* 13:119-130

Figure headings

Fig. 1 Model describing the phyto-bioconversion of lignite to humic and fulvic acids and, illustrating the main interactive pathways between ligninolytic microorganisms and root exudates to facilitate plant growth.

Table 1 Some distinguishing features of the four major coal ranks

Coal rank	Fixed C content (%)	Heating value (Btu/lb)*
Anthracite	86–98	13 500–15 600
Bituminous	46–86	11 000–15 000
Sub-bituminous	46–60	8 300–13 000
Lignite	46–60	5 500–8 300

*1 MJ=947.82 Btu

Table 2 Microorganisms and the purported catalysts used in coal biodegradation and biosolubilisation

Microorganism	Catalyst	References
Fungi		
<i>Aspergillus sp</i>	Lignin peroxidase	(Laborda et al. 1999)
<i>Auricularia sp</i>	Lignin peroxidase	(Hofrichter and Fritsche, 1997)
<i>Clitocybula dusenii</i>	Mn-peroxidase	(Hofrichter and Fritsche, 1997)
<i>Doratomyces sp</i>	Mn-peroxidase	(Laborda et al. 1999)
<i>Heterobasidion annosum</i>	Lignin peroxidase	(Fakoussa and Frost 1999)
<i>Lentinula edodes</i>	Mn-peroxidase	(Gotz and Fakoussa, 1999)
	Laccase	
<i>Nematoloma frowardii</i>	Laccase	(Hofrichter and Fritsche 1997)
	Lignin peroxidase	
	Mn-peroxidase	
<i>Neosartorya fischeri</i>	NS	(Igbini et al. 2008)
<i>Penicillium sp</i>	Esterase	(Laborda et al. 1999)
<i>Penicillium citrinum</i>	NS	(Polman et al. 1994)
<i>Phanerochaete chrysosporium</i>	Lignin peroxidase	(Ralph and Catchside 1999)
	Coal solubilising agent	(Torzilli and Isbister 1994)
<i>Pleurotus ostreatus</i>	Mn-peroxidase	(Fakoussa and Frost 1999)
<i>Pycnoporus cinnabarinus</i>	Lignin peroxidase	(Fakoussa and Frost 1999)
<i>Trametes versicola</i>	Lignin peroxidase	(Gotz and Fakoussa 1999)
	Laccase	(Fakoussa and Frost 1999)
	Coal solubilising agent	(Torzilli and Isbister 1994)
<i>Trichoderma sp</i>	Phenoloxidase	(Laborda et al. 1999)
Bacteria		
<i>Arthrobacter sp</i>	Coal solubilizing agent	(Torzilli and Isbister 1994)
<i>Bacillus cereus</i>	Alkaline solubilizing substances	(Maka et al. 1989)
<i>Bacillus pumilus</i>	Alkaline solubilizing substances	
<i>Bacillus subtilis</i>	Alkaline solubilizing substances	
<i>Pseudomonas putida</i>	Alkaline solubilizing substances	(Machnikowska et al. 2002)
<i>Streptomyces badius</i>	Alkaline solubilizing substances	(Quigley et al. 1989)
<i>Streptomyces setonii</i>	NS (non-enzymatic)	(Strandberg and Lewis 1987)
	Alkaline solubilizing substances	(Quigley et al. 1989)
<i>Streptomyces viridosporous</i>	NS (non-enzymatic)	(Strandberg and Lewis 1987)
	Alkaline solubilizing substances	(Quigley et al. 1989)

NS – Not specified

Table 3 Percentage humic acid in different resource samples(Fong et al. 2006)

Sample	Humic acid content (%)
Lignite	40 – 85
Black peat	10 – 40
Compost	2 – 5
Soil and sludge	1 – 5

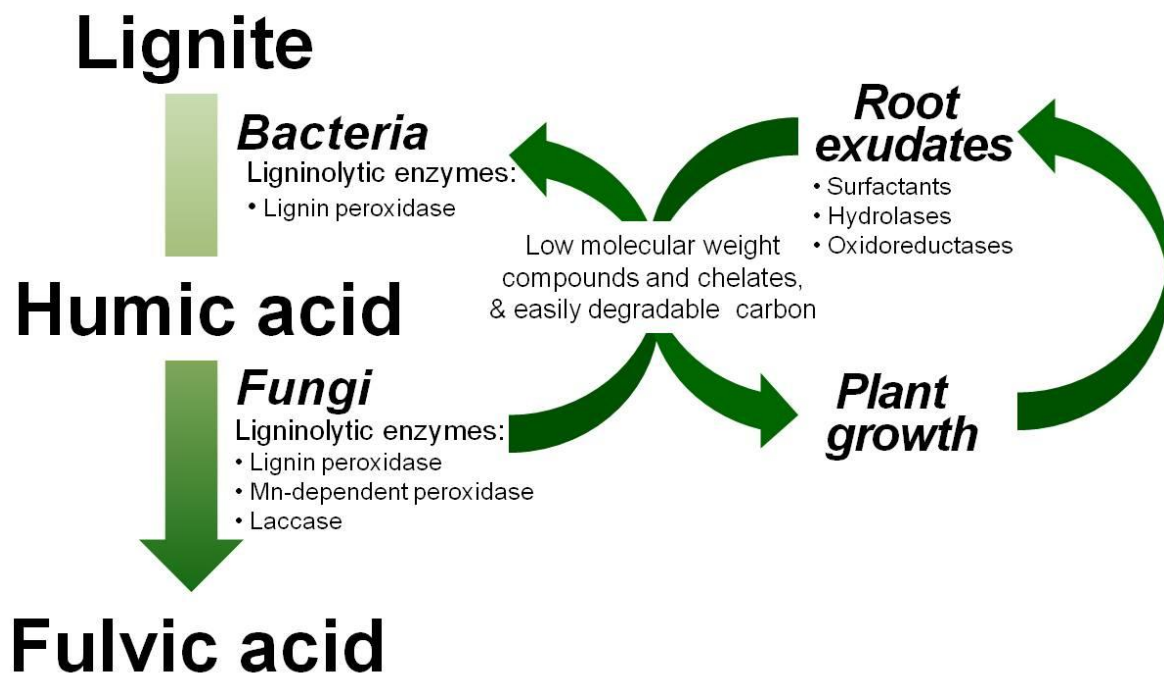


FIGURE 1. Sekhohola et al.

Paper 2

Fungal colonization and enzyme-mediated metabolism of waste coal

Lerato Mary Sekhohola, Michelle Louise Isaacs and Ashton Keith Cowan

Published in Bioscience, Biotechnology and Biochemistry

Running Head: Fungal colonisation and metabolism of waste coal

Title: Fungal Colonisation and Enzyme-Mediated Metabolism of Waste Coal by *Neosartorya fischeri* Strain ECCN 84

Authors: Lerato Mary Sekhohola, Michelle Louise Isaacs and Ashton Keith Cowan¹

Address: Institute for Environmental Biotechnology (EBRU), Rhodes University,
Grahamstown 6140, South Africa

Received: February 10, 2014; Accepted April 23, 2014

¹ To whom correspondence should be addressed. Fax: +27-46-6222-656; E-mail: a.cowan@ru.ac.za
Abbreviations: ABTS, 2,6-dimethoxyphenol, 2,2' [azino-bis-(3-ethylbonzthiazoline-6-sulphonic acid) diammonium salt]; EDS, energy dispersive x-ray spectroscopy; LAC, laccase; LiP, lignin peroxidase; MnP, Mn-dependent peroxidase; PBS, phosphate buffered saline; PDA, potato dextrose agar; SEM, scanning electron microscopy

Abstract

Colonisation and oxidative metabolism of South African low rank discard coal by the fungal strain ECCN 84 previously isolated from a coal environment and identified as *Neosartorya fischeri* was investigated. Results show that waste coal supported fungal growth. Colonisation of waste coal particles by *Neosartorya fischeri* ECCN 84 was associated with the formation of compact spherical pellets or sclerotia-like structures. Dissection of the pellets from liquid cultures revealed a nucleus of “engulfed” coal which when analysed by energy dispersive x-ray spectroscopy showed a time dependent decline in weight percentage of elemental carbon and an increase in elemental oxygen. Proliferation of peroxisomes in hyphae attached to coal particles and increased extracellular laccase activity occurred after addition of waste coal to cultures of *Neosartorya fischeri* ECCN 84. These results support a role for oxidative enzyme action in the biodegradation of coal and suggest that extracellular laccase is a key component in this process.

Key words: *Neosartorya fischeri*; coal; laccase; biodegradation

Introduction

Neosartorya fischeri, a species of Deuteromycota (fungi imperfecti) belonging to the family Trichocomaceae, a teleomorph of the *Aspergillus fischeri* series,¹⁾ is saprotrophic and colonises environments rich in decaying organic material including coal.²⁻⁵⁾ Thus, this fungus is capable of colonising and using a wide range of complex organic substrates which emphasises its biodegradation potential for use in rehabilitation of recalcitrant materials. Furthermore, the production of heat resistant ascospores⁶⁾ indicates that *Neosartorya fischeri* is a robust microbial catalyst and proliferates relatively easily irrespective of prevailing conditions. It is therefore perhaps not surprising that *Neosartorya fischeri* has been identified as a candidate species in the development of sustainable technologies for coal mine rehabilitation.

Igbinigie *et al.*³⁾ first reported on the degradation of hard coal by *Neosartorya fischeri* and pointed toward use of this organism for sustainable rehabilitation of solid coal mine waste. More recently, Taewoo *et al.*⁷⁾ reported the breakdown of petroleum hydrocarbons by this fungus. The two studies focused on the by-products of biodegradation emphasizing the potential of *Neosartorya fischeri* for exploitation as a catalyst in the development of remedial bioprocess technologies. In a further study, Uribe-Alvarez *et al.*⁵⁾ investigated the mineralization of petroleum asphaltenes by *Neosartorya fischeri* and detected activity of an extracellular oxidative laccase (LAC, E.C. 1.10.3.2). Ligninolytic enzymes like LAC, produced mostly by the Ascomycetes, Basidiomycetes and Deuteromycetes, have been widely reported to play a role in modifying and degrading the complex structure of substrates such as coal and polyaromatic hydrocarbons.⁸⁾ In fact, since the initial discovery that fungi possess the ability to breakdown and metabolise coal and polycyclic aromatic hydrocarbons researchers have focused on elucidating the underlying mechanisms. Thus, lignite degradation by Basidiomycota, such as *Trametes versicolor*⁹⁾ and *Phanerochaete chrysosporium*¹⁰⁾ appears to involve increased activity of LAC and the peroxidases, lignin peroxidase (LiP, E.C. 1.11.1.14) and Mn-dependent peroxidase (MnP, E.C. 1.11.1.13).

Industrial application of microbial catalytic mechanisms remains competent and economic due to the ability of microbial systems to perform under ambient temperatures and pressure. However, elucidation and understanding of microbial processes is crucial for their efficient exploitation and for further development of rehabilitation technologies. Fakoussa and Frost⁹⁾ argue that of the three ligninolytic enzymes involved in coal degradation biological LAC production is more economic and comparable to application of chemically produced agents. This is because LAC requires only molecular oxygen as a cofactor to oxidise a wide range of complex substrates while peroxidases require hydrogen peroxide as a co-substrate. Following its purification from a number of fungal strains,¹¹⁾ application of extracellular LAC was explored in different industrial sectors including the food, textile, pulp and paper industries as well as in medical, pharmaceutical and bioremediation fields.^{12,13)} Furthermore, LAC production by fungi is not confined to degradation of complex molecules but is also involved in the synthesis of

pigments associated with fungal development such as dihydroxynaphthalene melanins produced in response to environmental stress, formation of fruiting bodies, morphogenesis, detoxification, spore formation and pathogenesis.¹³⁾ This intimate association between LAC and fungal physiology highlights the potential of *Neosartorya fischeri* to survive and rejuvenate under harsh environmental conditions characteristic of waste coal dumps.

In the present work the colonisation and oxidative metabolism of South African low rank coal by *Neosartorya fischeri* strain ECCN84 was investigated by quantifying fungal growth in response to substrate, determining the interaction between this fungal biocatalyst and coal particles, and by screening and monitoring activity of oxidative enzymes and in particular, laccase.

Materials and Methods

Materials. All chemicals and reagents, unless stated otherwise, were purchased from Merck (Pty) Ltd., Modderfontein, South Africa. *Neosartorya fischeri* strain ECCN 84 was previously isolated from waste coal dumps³⁾ 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. Waste coal comprising a mix of low grade roof coal and discards, from the void following extraction of the high grade coal seam and with the following characteristics: total organic carbon = $10.3 \pm 2.0 \text{ mg.kg}^{-1}$; ash content = $55.5 \pm 0.3 \text{ wt\%}$, and calorific value = $8\text{-}10 \text{ MJ.kg}^{-1}$, was obtained from coal mines in eMalahleni (Witbank), Mpumalanga Province, South Africa. Aliquots were powdered using a HP-M 100 Pulverizer (HERZOG Maschinenfabrik GmbH Co., Osnabrück, Germany) to yield particles of approximately 0.2-0.5 mm in diameter. Powdered coal was sterilised by freeze thawing using liquid nitrogen (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 which was incubated at 30°C for 48 h.

Fungus cultivation, spore preparation, inoculation, and incubation. *N. fischeri* strain ECCN 84 was cultured from stock mycelial plugs on PDA at 30°C which was determined as the optimum temperature for spore formation, established by culturing ECCN 84 on PDA at 15, 30, 35 and 45°C, and production of spores confirmed by light microscopy (Olympus BX-50 light microscope) after staining with lactophenol blue.

Fungal spores were harvested into suspension by washing mature fungal lawns with sterile phosphate buffered saline (PBS) and using the liquid spore suspension for inoculation into basal salts media comprising K₂HPO₄ (1.71 g); KH₂PO₄ (1.32 g); NH₄Cl (1.26 g); MgSO₄·6H₂O (0.011 g); CaCl₂ (0.02 g), 4 ml trace element mixture and 60 ml glutamate media (KH₂PO₄, 12.7 g; NaNO₃, 3.0 g; K₂HPO₄, 3.1 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.5 g; and glutamate 2.0 g, per L) diluted to 1 L and adjusted to pH 6-6.5. For biodegradation studies in liquid media, sterilized powdered waste coal was added and cultures incubated at 30°C on a rotary shaker (130 rpm). Fungal growth was allowed to proceed for 20 d with regular monitoring of pH, biomass harvested every 4 d, and dry weight determined after teasing apart the sclerotia-like structures to remove attached coal particles and drying at 50°C for 24 h.

For plate culture, spores were inoculated on agar prepared using a basal salts medium of KH₂PO₄ (1.0g) NH₄Cl (1.26g); MgSO₄·7H₂O (0.5g); CaCl₂·2H₂O (0.01g); yeast extract (0.01g); CuSO₄·5H₂O (0.001g); Fe₂(SO₄)₃ (0.001g); MnSO₄·H₂O (0.001g), glucose (0.4%, w/v) per L, and solidified with 1.6% (w/v) agar agar.¹⁴⁾ After incubation for 8 d at 30°C, dry sterile powdered discard coal was added and the plates incubated for 7 d prior to analysis.

Light and scanning electron microscopy and energy dispersive x-ray spectroscopy.

The association between the fungal biocatalyst and coal particles was investigated using both light and scanning electron microscopy (SEM) and any metabolic interaction established by energy dispersive x-ray spectroscopy (EDS).

N. fischeri strain ECCN 84 was cultured either in liquid glutamate-containing basal salts media or on agar plates prepared as described above. After addition of sterile waste coal powder, growth was allowed to proceed until sclerotia-like structures were visible.

For light microscopy, sclerotia were carefully removed from the culture media, prepared as wet mounts and examined using an Olympus BX-50 light microscope. For SEM, sclerotia from liquid-glutamate containing basal salts media were collected and fixed using 2.5% buffered glutaraldehyde at 4°C overnight. The buffered glutaraldehyde was decanted and the samples washed twice with phosphate buffer (0.1 M, pH 7.3) and then dehydrated using a graded alcohol series at 30%, 50%, 70%, 80%, 90% and absolute alcohol, the specimens dried in hexamethyldisilazane, gold coated and mounted on metal stubs for examination.¹⁵⁾ Samples were examined using a Vega 3 LMU (TESCAN, Brno, Czech Republic) analytical scanning electron microscope at 30 kV.

Elemental analysis of coal particles trapped within fungal mycelia was by EDS using an INCA Penta FET X3 assembly attached to the scanning electron microscope and was carried out at the intervals specified in Results. Sample preparation was as described for SEM but without gold coating.

Oxidative enzyme screening and LAC assay.

Screening for LAC activity was initially carried out by culturing the fungus ECCN 84 on 2.5% PDA plates with added 2,6-dimethoxyphenol, 2,2',6,6'-tetraabzino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt] (ABTS) as described by Hofrichter et al.¹⁶⁾ The prepared PDA/ABTS (0.02%) and control (without ABTS) plates were inoculated with a 5×5 mm mycelial plug and incubated at 30°C in darkness. The plates were monitored at regular intervals for colour change (green) indicating the production of the ABTS cation radical, ABTS^{•+}, the product of LAC activity.¹⁷⁾ For the screening of MnP, strain ECCN 84 was sub-cultured on agar plates containing 2.5% PDA and increasing concentrations of MnCl₂ (250, 500, 1000 and 2000 µg/mL) and incubated in darkness at 30°C. Formation of a black precipitate (MnO₂) surrounding the fungal colony was taken to indicate oxidation of Mn²⁺ to Mn³⁺.

For analysis of extracellular LAC activity, culture filtrates of ECCN 84 growing in glutamate supplemented basal salt medium with and without added coal or glucose were analysed for enzyme activity by measuring the change in absorbance of ABTS at 420 nm using the molar extinction coefficient of 36.0 mM⁻¹ cm⁻¹ as described by Li et al.¹⁸⁾ Heat-

inactivated ($100^{\circ}\text{C} \times 10 \text{ min}$) filtrates were similarly analysed. Reaction mixtures consisted of a 1.5mL aliquot of culture filtrate added to 1.5mL sodium acetate buffer (1mM, pH5) with 1.5mL ABTS (0.5mM) serving as substrate for enzyme catalysed oxidation. Absorbance of the reaction mixture was monitored at intervals (1 min) over a period of 25 min. A plot of absorbance versus time was used to derive the slope and enzyme activity expressed as $\text{nmol min}^{-1}\text{mL}^{-1}$, calculated by multiplying the slope by the molar extinction coefficient of ABTS.

Statistical analysis.

All data were processed using Sigma Plot version 11.2 (SPSS Inc., Chicago, IL). Lines of best fit were computed using the statistical function of Sigma Plot following non-linear regression using one-way analysis of variance and the Shapiro-Wilks normality test ($P < 0.05$). Data are presented as the mean of at least three determinations \pm standard deviation (SD).

Results and Discussion

Figure 1 shows the growth response of *N. fischeri* strain ECCN 84 in liquid culture either in basal salts medium or basal salts medium supplemented with glutamate. Fungal spores germinated and produced mycelial pellets or sclerotia-like structures in agitated basal salts media and in glutamate supplemented media after several days' incubation at 30°C . However, biomass accumulation was greater in the glutamate supplemented medium (Fig. 1). Mean dry weight of fungal biomass harvested from glutamate supplemented basal salts medium with added waste coal appeared to be proportional to the amount of coal substrate added (Table 1). Thus, cultures of *N. fischeri* strain ECCN 84 supplied 0.1 g of waste coal produced one and a half times the dry weight of biomass than cultures given 0.05g indicative of a classical dose-response effect. Furthermore, formation of pellets or sclerotia-like structures after addition of waste coal substrate suggested active engulfment of the coal particle by fungal mycelia. Since entrapment of

coal particles has been observed previously¹⁹⁾ but not analysed in any detail, it seemed pertinent to examine this phenomenon in *N. fischeri* using both light and SEM.

Figure 1

Table 1

Spores of *N. fischeri* strain ECCN 84 were inoculated into glutamate-containing basal salts media or on agar plates and cultivated for 8 and 1 d respectively prior to enrichment with sterile waste coal powder.

In liquid culture, spores of ECCN 84 germinated and grew to form compact spherical pellets or sclerotia-like structures (Fig. 2A). As described by Singh,²⁰⁾ fungi in liquid culture form clumps from loose hyphal aggregates while spherical pellets form by aggregation of fungal spores, either individually or in clumps, which results in subsequent aggregation of fungal hyphae upon spore germination into non-coagulating pellets. Dissection of these individual pellets revealed some “engulfed” coal forming a nucleus at the centre (arrows, Fig. 2A) while scanning electron microscopy, revealed coal particles entrapped by fungal mycelia (Fig. 2B and C). In situ elemental analysis of the coal particles trapped within fungal mycelia was by EDS and was carried out 1, 3, 5, 7, 14 and 21 d after addition of coal substrate. Results showed a time dependent decline in weight percentage of elemental carbon within the coal particles entrapped by fungal mycelia (Fig.3A) which occurred concomitant with an increase in weight percentage of elemental oxygen (Fig.3B). Together, these results provide strong circumstantial evidence for a direct interaction between strain ECCN 84 and waste coal in which the fungus not only colonises and is attached to the coal particles but is able to extract elemental carbon by oxidative metabolism to sustain growth and proliferation.

Figure 2

Figure 3

When coal particles were added to lawns of strain ECCN 84 growing on solidified basal salt media fungal hyphae covered the coal particles in a mycelial structure reminiscent of sclerotia (Fig. 4A). Light microscope examination of wet mounts of coal covered by fungal mycelium showed hyphae attached to coal particles (arrows, Fig. 4B) and, which on closer scrutiny, revealed the presences of organelles presumably

peroxisomes (arrows, Fig. 4C). These organelles were absent in hyphae from *N. fischeri* strain ECCN 84 cultivated on medium without added coal (Fig. 4D). Typically, peroxisomes are associated with the oxidation of compounds such as fatty acids using naturally occurring peroxide, typically hydrogen peroxide (H₂O₂), which is reduced to water. In fungi, these organelles are known to be important for carbon source utilization, pathogenesis, development, and secondary metabolism.^{21, 22)}

Figure 4

Several studies have indicated that coal degrading fungi are able to accomplish the breakdown of this substrate by synthesizing a suite of ligninolytic enzymes and in particular, enzymes that catalyse peroxidation reactions. Chief amongst these enzymes are LiP, MnP, and the phenol oxidase, LAC.²³⁻²⁹⁾ Preliminary screening by the culturing of strain ECCN 84 on PDA plates in the presence and absence of substrates for MnP (MnCl₂) and LAC (ABTS) indicated a positive response for LAC activity only (data not shown). Consequently, experiments were carried out to confirm the presence of extracellular LAC activity in culture filtrates of ECCN 84 and to determine whether this strain responded to the addition of waste coal by increasing activity of LAC and the results are presented in Figure 5.

Figure 5

Extracellular LAC activity increased to a maximum within 5 d following addition of waste coal to liquid cultures of ECCN 84 (Fig. 5). No induction of LAC activity was evident in filtrates from cultures in which an equal weight of glucose was substituted for waste coal as substrate and, LAC activity was not detectable in heat-inactivated filtrates from cultures supplied waste coal as substrate confirming that the activity detected was the result of an enzyme catalysed reaction. These results together with the recent cloning and characterization of a β -glucosidase from *N. fischeri* which catalyses the hydrolysis of *p*-nitrophenyl- β -D-glucopyranoside and flavone compounds with high levels of catalytic activity,³⁰⁾ coupled with the cloning and sequencing of an endo-1,3(4)- β -glucanase from *N. fischeri* NRRL 181³¹⁾ illustrate the potential and versatility of this biocatalyst to degrade recalcitrant materials and in particular waste coal.

In conclusion, this work has demonstrated for the first time the induction of an extracellular LAC in *N. fischeri* by coal. This coupled with EDS analysis which showed a decline in carbon and concomitant increase in elemental oxygen content, supports fungal mediated oxidative metabolism of waste coal. In addition to searching for other oxidase enzymes that might be involved in coal biodegradation by *N. fischeri*, effort is also focused on the purification and characterisation of the LAC activity detected in this study. Thus, further research will aim to elucidate the role of LAC and other oxidases and hydrolases as part of the mechanism of biodegradation of coal by *N. fischeri*.

Acknowledgements

This research was supported by a grant from Anglo American Thermal Coal and the National Research Foundation (IFR1202220169, Grant No: 80879). Ms. Lerato M. Sekhohola acknowledges financial support from Anglo American Thermal Coal in the form of a doctoral bursary.

References

- 1) Varga J, Vida Z, Toth B, Debets F, and Horie Y, Phylogenetic analysis of newly described *Neosartorya* species. *Antonie van Leeuwenhoek*, 235-239, **77**, (2000).
- 2) Girardin H, Monod M, and Latgé JP, Molecular characterization of a food-borne fungus *Neosartorya fischeri* (Malloch and Cain). *Appl. Environ. Microbiol.*, 1378-1383, **61**, (1995).
- 3) Igbinigie EE, Atkins S, van Breugel Y, van Dyke S, Davies-Coleman MT, and Rose PD, Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri*. *Biotechnol. J.*, 1407-1416, **3**, (2008).
- 4) Hong SB, Kim DH, Park I, Samson RA, and Shin HD, Isolation and identification of *Aspergillus* section *Fumigati* strains from arable soil in Korea. *Microbiol.*, 1-6, **38**, (2010).
- 5) Uribe-Alvarez C, Ayala M, Perezgasga L, Naranjo L, Urbina H, and Vazquez-Duhalt R, First evidence of mineralization of petroleum asphaltene by a strain of *Neosartorya fischeri*. *Microbiol. Biotechnol.*, 663-672, **4**, (2011).
- 6) Amaeze NJ, Ugwuanyi JO, and Obeta JAN, Studies of heat-resistant fungi in the soil: *Talaromyces flavus* isolated in Nigerian soils. *New York Sci. J.*, 8-14, **3**, (2010).
- 7) Taewoo Y, Eun-Hee L, Hyerim P, and Kyung-Suk C, Biodegradation of petroleum hydrocarbons by *Neosartorya* sp. BL4. *J. Environ. Sci. Health Part A: Toxic/Hazardous Substances and Environmental Engineering*, 1763-1768, **46**, (2011).
- 8) Sekhohola LM, Igbinigie EE, and Cowan AK, Biological degradation and solubilisation of coal. *Biodegrad.*, 305-318, **24**, (2013).
- 9) Fakoussa RM, and Frost PJ, In vivo decolorization of coal-derived humic acids by laccase-excreting fungus *Trametes versicolor*. *Appl. Microbiol. Biotechnol.*, 60-65, **52**, (1999).

- 10) Ralph JP, and Catcheside DEA, Transformations of low rank coal by *Phanerochaete chrysosporium* and other wood-rot fungi. *Fuel Process. Technol.*, 79-93, **52**, (1997).
- 11) Garcia TA, Santiago MF, and Ulhoa CJ, Studies on the *Pycnoporus sanguineus* CCT-4518 laccase purified by hydrophobic interaction chromatography. *Appl. Microbiol. Biotechnol.*, 311-318, **75**, (2007).
- 12) Maciel MJM, Silva AC, and Ribeiro HCT, Industrial and biotechnological applications of ligninolytic enzymes of the Basidiomycota: a review. *J. Biotechnol.*, 1-13, **13**, (2010).
- 13) Dwivedi UN, Singh P, Pandey VP, and Kumar A, Structure-function relationship among bacterial, fungal and plant laccases. *J. Mol. Cat. B: Enzymatic*, 117-128, vol. **68**, (2011).
- 14) Pointing SB, Qualitative methods for the determination of lignocellulotic enzyme production by tropical fungi. *Fungal Div.*, 17-33, **2**, (1999).
- 15) Weber WJ, Pirbazari M, and Melson GL, Biological growth on activated carbon: an investigation by scanning electron microscopy. *Amer. Chem. Soc.*, 817-819, **127**, (1978).
- 16) Hofrichter M, Bublitz F, and Fritsche W, Fungal attack on coal I. Modification of hard coal by fungi. *Fuel Process. Technol.*, 43-53, **52**, (1997).
- 17) Steffen KT, Hofrichter M, and Hatakka A, Mineralisation of ¹⁴C-labelled synthetic lignin and ligninolytic enzyme activities of litter decomposing basidiomycetous fungi. *Appl. Microbiol. Biotechnol.*, 819-825, **54**, (2000).
- 18) Li A, Zhu Y, Xu L, Zhu W, and Tian X, Comparative study on the determination of assay for laccase of *Trametes* sp. *Afr. J. Biochem. Res.*, 181-183, **2**, (2008).
- 19) Haider R, Ghauri MA, SanFilipo JR, Jones EJ, Orem WH, Tatu CA, Akhtar K, and Akhtar N, Fungal degradation of coal as a pretreatment for production of methane. *Fuel*, 717-725, **104**, (2013).
- 20) Singh H, "Mycoremediation: fungal bioremediation", Wiley-Interscience, New Jersey, pp. 1-29 (2006).

- 21) Gabaldon T, Peroxisome diversity and evolution. *Phil. Trans. Royal Soc. B: Biological Sciences*, 765-773, **365**, (2010).
- 22) Hynes MJ, Murray SL, Khew GS, and Davis MA, Genetic analysis of the role of peroxisomes in the utilization of acetate and fatty acids in *Aspergillus nidulans*. *Genetics*, 1355-1369, **178**, (2008).
- 23) Bartoszewska M, Opalinski L, Veenhuis M, and Klei I, The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. *Biotech. Lett.*, 1921-1931, **33**, (2011).
- 24) Hatakka A, Ligin modifying enzymes from selected white rot fungi: production and role from lignin degradation. *FEMS Microbiol. Rev.*, 125-135, **13**, (1994).
- 25) Thurston CF, The structure and function of fungal laccases. *Microbiol.*, 19-26, **140**, (1994).
- 26) Silva-Stenico ME, Vengadajellum CJ, Janjua HA, Harrison STL, Burton, SG, and Cowan DA, Degradation of low rank coal by *Trichoderma atroviride* ES11. *J. Indust. Microbiol. Biotechnol.*, 19-26, **34**, (2007).
- 27) Mot AC, and Silaghi-Dumitrescu R, Laccases: complex architectures for one-electron oxidation. *Biochem. (Moscow)*, 1395-1407, **77**, (2012).
- 28) Yadav M, Singh SK, and Yadava S, Purification, characterisation and coal depolymerisation activity of lignin peroxidase from *Lenzites betulina* MTCC-1183. *Appl. Biochem. Microbiol.*, 583-589, **48**, (2012).
- 29) Shevchenko EA, Bessolitsyna EA, and Darmov IV, Identification of genes encoding ligninolytic enzymes in naturally occurring basidiomycete isolates. *Appl. Biochem. Microbiol.*, 280-286, **49**, (2013).
- 30) Ramachandran P, Tiwari MK, Singh RK, Haw J-R, Jeya M, and Lee J-K, Cloning and characterization of a putative β -glucosidase (NfBGL595) from *Neosartorya fischeri*. *Process Biochem.*, 99-105, **47**, (2012).
- 31) Hua C, Yan Q, Jiang Z, Li Y, and Katrolia P, High-level expression of a specific β -1,3-1,4-glucanase from the thermophilic fungus *Paecilomyces thermophila* in *Pichia pastoris*. *Appl. Microbiol. Biotechnol.*, 509-518, **88**, (2010).

Tables

Table 1. Effect of substrate on accumulation of biomass by *N. fischeri* strain ECCN 84 cultured in glutamate supplemented basal salts media with either glucose (0.05g) or coal (0.05 and 0.1g). Data are the mean \pm SD of three determinations.

Substrate	Biomass (g)
+ 0.05g gluc	0.15 \pm 0.02
+ 0.05g coal	0.12 \pm 0.01
+ 0.10g coal	0.18 \pm 0.01

Figure Headings

Fig. 1. Growth curves of *Neosartorya fischeri* strain ECCN 84 in liquid culture. Spores were inoculated into either basal salt medium or glutamate supplemented basal salt medium (GluM) containing sterile powdered waste coal and incubated for 20 d at 30°C. At the specified intervals biomass accumulation was determined after drying at 50°C for 24 h. Data are the mean of at least three determinations \pm SD.

Fig. 2. Entrapment of coal particles by *Neosartorya fischeri* strain ECCN 84 cultured in liquid media containing basal salts medium and waste coal. (A) Light micrograph of a section through a fungal pellet (sclerotia) showing mycelial entrapment of coal particles (arrows); (B & C) scanning electron micrographs of hyphae engulfing and ramifying coal particles embedded within the fungal mycelial pellet.

Fig.3. Change in elemental carbon and oxygen content of coal particles engulfed by fungal mycelia within sclerotia-like structures. Spores of *Neosartorya fischeri* strain ECCN 84 were inoculated in glutamate medium with added coal, biomass harvested after 21 d at 30°C, and analysed by energy dispersive x-ray spectroscopy as described in Materials and methods. Data are the mean of at least three determinations \pm SD.

Fig. 4. Light micrographs of *Neosartorya fischeri* strain ECCN 84 cultured as a lawn on agar containing basal salts medium illustrating attachment to coal particles. (A) Typical mycelial pellet or sclerotia-like structure formed after addition of sterile powdered waste coal; (B) attachment of hyphae to coal particles within the mycelial pellet or sclerotia-like structures; (C) high resolution image showing the presence of peroxisome-like organelles in hyphae from cultures supplied waste coal; and (D) hyphae from cultures in the absence of waste coal substrate lacking peroxisome-like organelles.

Fig. 5. Waste coal induced changes in extracellular LAC activity in culture filtrates from *Neosartorya fischeri* strain ECCN 84. Filtrates were assayed for extracellular LAC

activity by monitoring the oxidation of ABTS spectrophotometrically at 420 nm and data represent the mean \pm SD of three experiments.

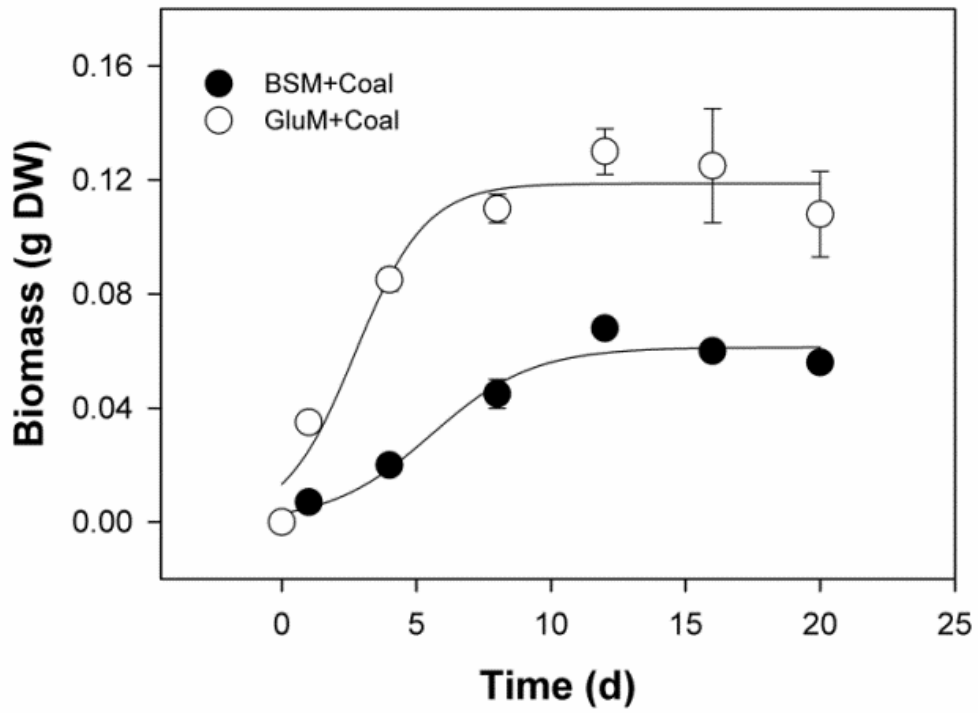


Fig. 1, Sekhohola et al.

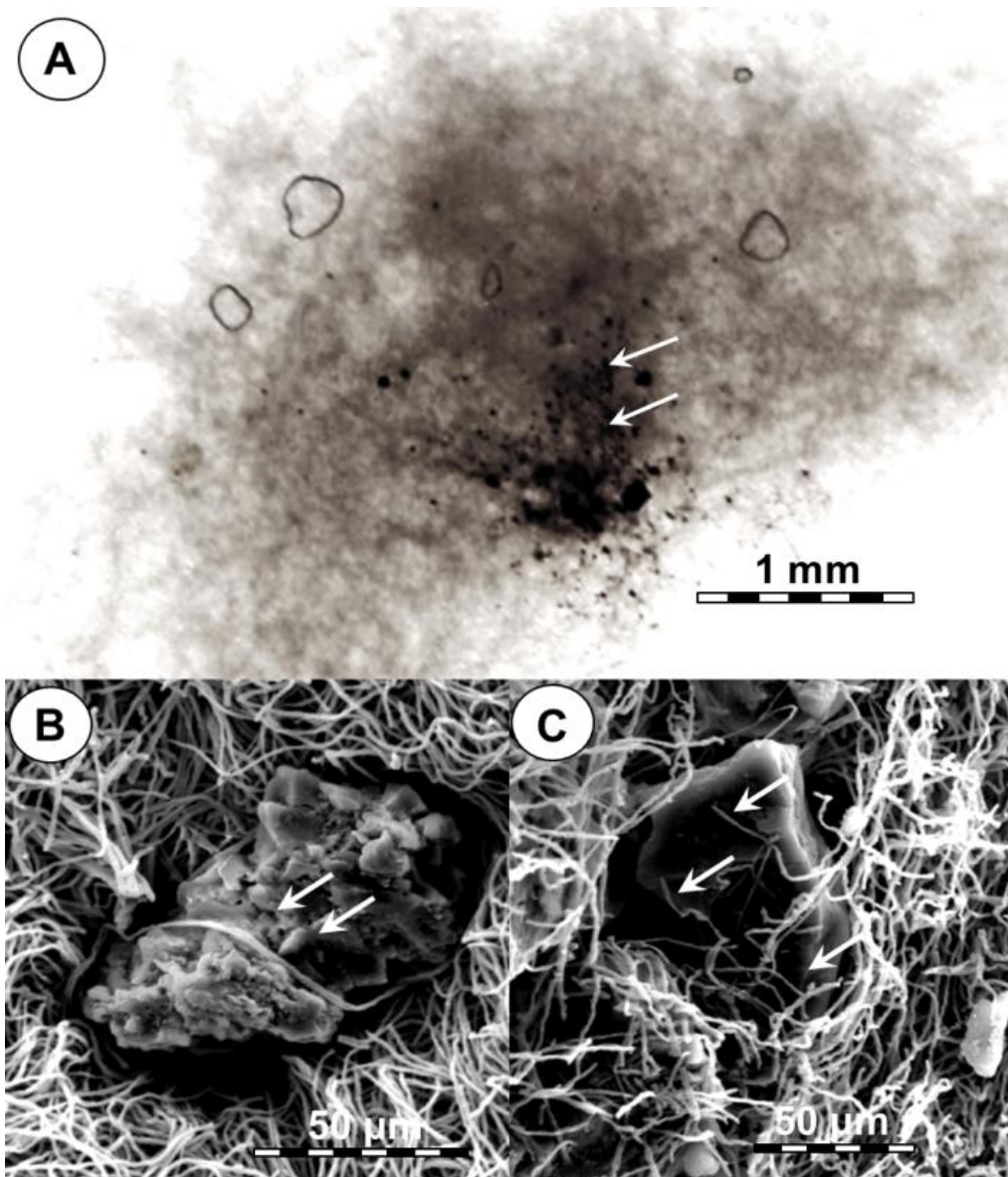


Fig. 2, Sekhohola et al.

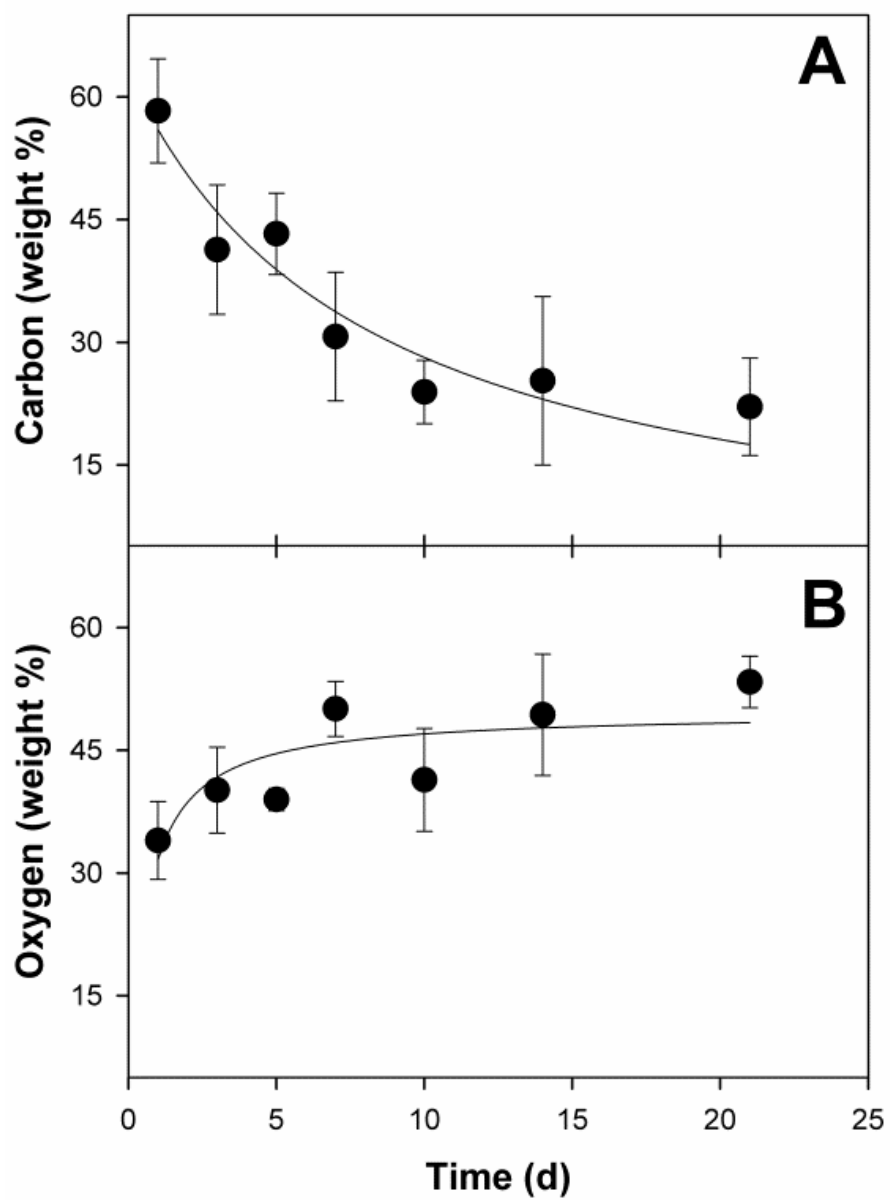


Fig. 3, Sekhohola et al.

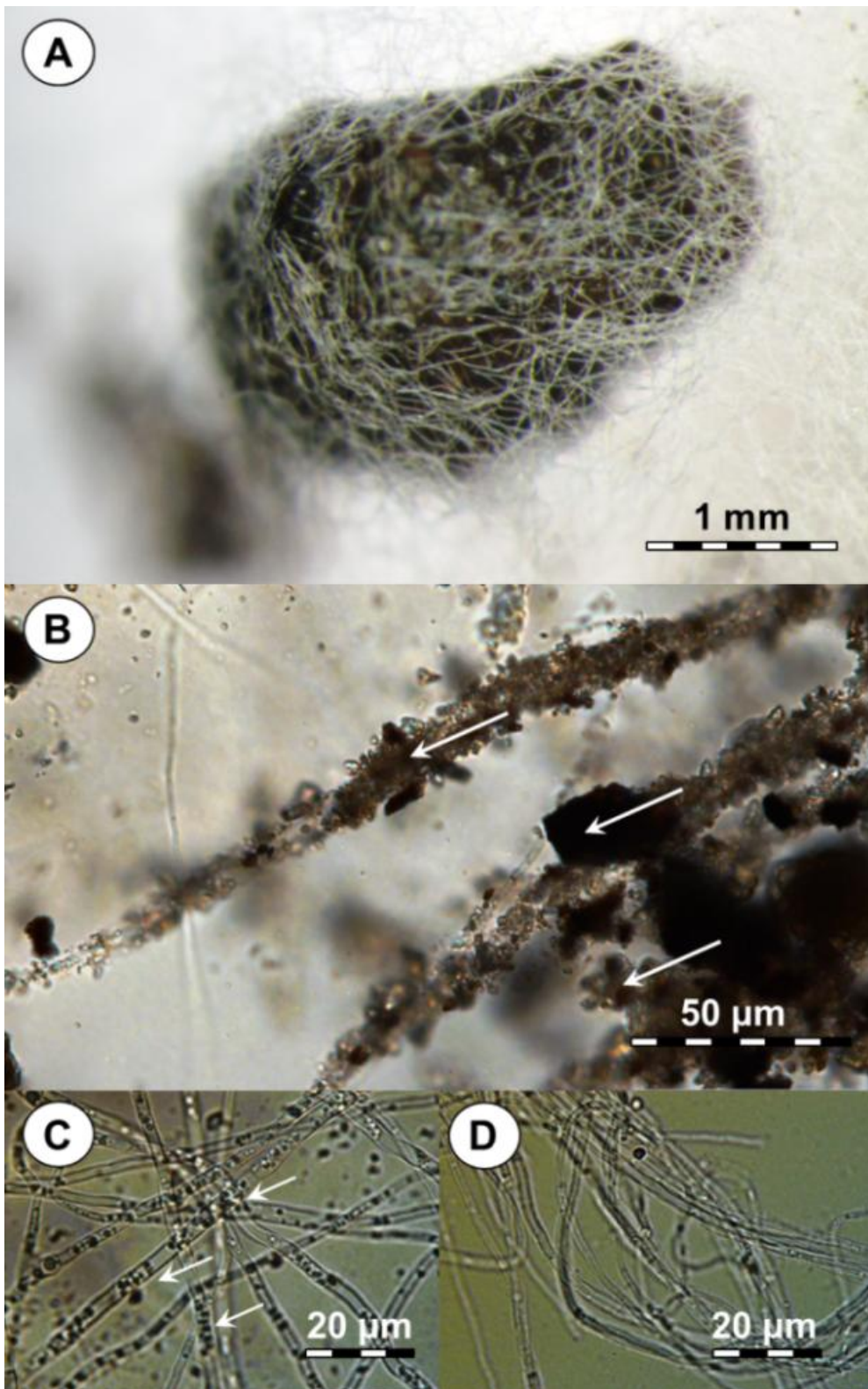


Fig. 4, Sekhohola et al.

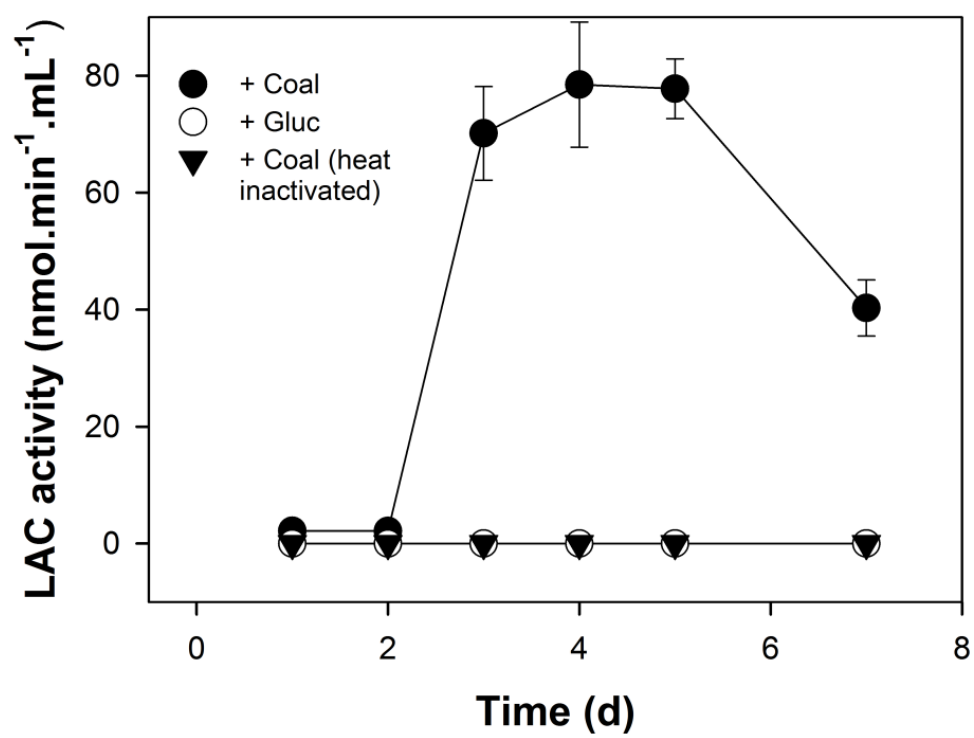


Fig. 5, Sekhohola et al.

Paper 3

Biological degradation of waste coal and seedling establishment in the resultant humic-like material

Lerato M. Sekhohola and A. Keith Cowan

In revision for CLEAN, Soil, Air and Water

Title:

Biological degradation of waste coal and seedling establishment in the resultant humic-like material

Authors:

Lerato Mary Sekhohola and Ashton Keith Cowan

Affiliation:

Institute for Environmental Biotechnology, Rhodes University (EBRU), P.O. Box 94, Grahamstown 6140, South Africa

Running Title:

Waste coal biodegradation

Corresponding author:

Professor Ashton Keith Cowan

Institute for Environmental Biotechnology, Rhodes University (EBRU),

P.O. Box 94

Grahamstown 6140

South Africa

email: a.cowan@ru.ac.za

Tel: +27 46 6222 656

Fax: +27 46 6222 656

Abbreviations:

ABTS – 2,6-dimethoxyphenol, 2,2’[azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt]

ECCN - EBRU Culture Collection Number

FTIR – Fourier Transform Infrared spectra

HS – Humic-like substances

LAC – Laccase

LiP – Lignin peroxidase

MnP – Manganese peroxidase

TOC – Total organic carbon

Key words:

Biodegradation, *Cynodon dactylon*, humic-like substances, *Neosartorya fischeri*, waste coal

Abstract

Biological degradation of coal results in formation of humic-like substances and these coal-derived humics have been reported to improve soil structure in land rehabilitation and enhance plant growth. In this paper bioconversion of waste coal within the rhizosphere of *Cynodon dactylon* and in the presence of coal degrading and mycorrhizal fungi was investigated. Spinach, maize, bean and pea seedling establishment on the resultant substrate was also determined. After 47 weeks of *Cynodon dactylon* growth on waste coal the concentration of humics within the rhizosphere of this grass species increased from 63.1 ± 1.3 to 112.2 ± 6.2 wt %. FTIR spectroscopy of the extracted humic-like substances confirmed both product identity and the increase in oxidation of the waste coal substrate. Results therefore support the proposal that biological oxidative degradation of waste coal leads to the increase in humic-like substance concentration. Successful seedling establishment of spinach, maize, bean and pea showed that the residue after treatment with coal degrading and mycorrhizal fungi in the presence of *Cynodon dactylon* cultivation, supports emergence and early development of agronomic plants, a phenomenon that has not previously been demonstrated. Taken together, these results have profound implications for the beneficiation of waste coal as a material for use as a soil amendment and more importantly, in the development of bioremediation strategies for sustainable rehabilitation post mine closure.

1. Introduction

Waste coal is low calorific residual material generated as a by-product of coal mining and processing and is not considered marketable by the coal mining industry. The proliferation of unsightly and potentially hazardous waste/discard coal dumps and tailings dams in particular, requires that the mining industry find new ways of treating waste. Indeed, several strategies do exist and include; 1) re-mining of dumped waste coal; 2) allowing waste coal dumps to be ameliorated naturally or by accelerating the process through re-vegetation; and 3) disposing of waste coal in landfills [1]. The disadvantage of such strategies is the capital investment and time required to achieve successful rehabilitation and an inability to guarantee elimination of waste coal.

Phytoremediation is widely practiced by the industry and directed towards restoration of land that has either been disturbed by coal mining or transformed into waste dumps [2, 3, 4]. Moreover, establishment of vegetation cover has been an effective and socially acceptable implementation strategy for rehabilitation of coal dumps in South Africa. Current practices rely heavily on the import of topsoil to stabilize land disturbed by coal mining, clad waste coal dumps, and to support re-vegetation. Topsoil in South Africa is a limited resource and is particularly limiting on land that has been mined. Consequently, topsoil must be sourced elsewhere and transported to sites for rehabilitation. Furthermore, about 58% of soils contain less than 0.5% organic carbon and only 4% contain more than 2% organic carbon [5, 6]. Rapid mineralization of the imported topsoil together with the excessive cost of this practice has necessitated the search for an alternative technology for use in rehabilitation of disturbed mining land and waste coal dumps.

Recent studies in our laboratory on the biodegradation of waste coal within the rhizosphere of *Cynodon dactylon* led to a novel beneficiation strategy for rehabilitation of waste coal dumps in

South Africa [7,8]. *Cynodon dactylon* was observed growing intermittently on bituminous hard coal dumps that had not undergone any form of rehabilitation [7, 8, 9]. Further study revealed the potential of weathered coal to sustain plant growth while investigation of the root zone indicated the presence of a diverse fungal flora. An extensive screening exercise followed and resulted in the isolation of the deuteromycete, *Neosartorya fischeri* that was shown to actively degrade coal *in vitro* and in a perfusion fixed-bed bioreactor used to simulate the coal dump environment [7, 10]. Further research suggested that phyto-bioconversion of hard coal involved interaction between plants and rhizospheric microbes that together promoted growth of *Cynodon dactylon* [8]. Based on these findings, an integrated biotechnological process was proposed to describe some of the interactions within the rhizosphere that result in biodegradation of waste coal, production of humic-like substances, and waste dump self-cladding [11] This model proposes, in no particular order, that; 1) grasses exude organic acids into the rhizosphere through the root system; 2) arbuscular mycorrhiza in association with roots utilize these organic acids to facilitate uptake of nutrients by the plant; 3) deuteromycetes which may occur within the coal environment also produce and/or utilize organic acids to degrade coal; and, 4) complex organics in the resulting humus material are broken down by coal-degrading microorganisms and made available to the plant.

The concept of fungal depolymerization and solubilization of low rank coal is not novel and has previously been investigated and reported by many authors [7, 11, 12-16]. However, most of the early work on coal bio-solubilization was carried out in the laboratory where processes are constrained by conditions that do not necessarily simulate what happens in the field. Consequently coal bio-solubilization has not been fully explored as a viable commercial scale rehabilitation methodology. This notwithstanding, focus is shifting towards bridging the gap between testing the process *in situ* and assessing its full potential in the mining environment. Observations from recent studies clearly show that direct contact between metabolically active biocatalysts and coal facilitates the transformation of this recalcitrant substrate into soil-like material, which lessens the burden on the environment through provision of a more beneficial resource [9, 19-21].

In the present work the proposed mutualistic relationship between grasses and fungi in the phyto-biodegradation of waste coal was explored. A waste coal substrate was inoculated with a consortium of laccase- and manganese peroxidase-positive fungi and used as a medium for the cultivation of *Cynodon dactylon*. The formation of humic-like substances and consequently, soilification was monitored, and the resultant humic-like residue was tested for its ability to support germination and seedling establishment of agronomic species.

2. Materials and Methods

Fungal culture maintenance and preparation of the inoculum

A consortium of six coal degrading fungi from the EBRU culture collection was prepared using strains ECCN 178, ECCN 187, ECCN 224, ECCN 226, ECCN 241 and ECCN 243, which had been stored as mycelial plugs on 2.5% potato dextrose agar (PDA) in 50% glycerol (v/v) at -20°C. *Neosartorya fischeri*, strain ECCN 84, isolated from different coal environments around South Africa [7, 8] and strains PPRI 5328 (*Phanerocheate chrysosporium*) and PPRI 4835 (*Coriolus*

versicolor), obtained from the Agricultural Research Council (ARC, South Africa), were re-cultured on plates of 2.5% PDA at 26°C. All fungi used as inoculum tested positive for production of the ligninolytic enzymes laccase (LAC, EC 1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13) on PDA with added 2,6-dimethoxyphenol, 2,2'-[azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt](ABTS) at 37°C using the protocols described by Pointing [22]. The fungal inoculum was prepared by washing and collecting spores from fungal mats (5 plates per strain) as described elsewhere [9].

Substrate preparation

Waste coal and topsoil were sourced from mines in Witbank, Mpumalanga Province, South Africa. The waste coal, typical of coal dump material, comprised a mixture of low-grade roof coal and discard (calorific value of 8-10 MJ.kg⁻¹) from the void following extraction of high-grade coal. Topsoil and waste coal were milled, pulverized and sieved to obtain a particle size of between 1-2 mm and where specified, combined 3:1 (v/v) to yield a homogeneous “mixed” substrate.

Characterization of waste coal, topsoil, and waste coal/topsoil substrates

The basic physical and chemical characteristics of topsoil, the prepared waste coal/topsoil mix and waste coal were determined and are presented in Table 1. Water holding capacity, electrical conductivity and pH of these substrates were determined as described by Rayment and Higginson [23] after oven drying (50°C for 24 h). For each substrate, a saturated paste was prepared by adding de-ionized water to 100 g dry material and after 24 h the weight determined. Water holding capacity was calculated as percentage moisture content of the saturated paste where; % moisture content = (weight after water addition/weight of dry sample) × 100. For electrical conductivity and pH, 100 mL de-ionized water was added to 20 g dry material which was mixed for 1 h and settled for 30 min. Conductivity and pH were measured at 25°C respectively without disturbing the settled solids using a EUTECH conductivity meter (EUTECH instruments) and pH meter (HANNA instruments HI 8314).

Total organic carbon (TOC) was determined by direct combustion of 10 µg aliquots of material in a combustion furnace at 680°C using an Apollo 9000 Total Organic Carbon Analyser fitted with a boat sampler (Model 183 Teledyne Tekmar, Mason, OH). For measurement of ash content, pre-weighed aliquots of dry substrate were placed into crucibles and heated at 900°C for 5 h using a Carbolite CE muffle furnace (Carbolite, Hope Valley, UK), the residue weighed, and ash content expressed as weight percentage.

Plant material, cultivation and sampling

To 5L black plastic potting bags was added a layer of stones and each bag then filled with 2 kg of substrate to which was applied 10 g arbuscular mycorrhizal fungi (Mycoroot Supreme, Mycoroot Pty Ltd., Grahamstown, South Africa) and 10 mL of coal-degrading fungal suspension (0.031 ± 0.002g spore biomass/mL). The content of each potting bag was thoroughly mixed and irrigated. Control pots were filled with untreated waste coal material. Subsamples of substrate from each pot were retained for characterization and for determination of initial humic acid levels.

Seed of *Cynodon dactylon* L. (0.06 g \approx 200 seeds) was sown and lightly covered with moistened substrate (untreated waste coal control pots were not seeded), irrigated every 2 d for the first 8 wks, and thereafter twice weekly using rain water. Potting bags were arranged in a complete randomized block design in a polycarbonate-covered tunnel (Ulma Agricola, Spain) and growth allowed to proceed under ambient conditions. After 24 wks (experiment initiated on 23 February 2010), the above ground plant material was harvested from half of the potting bags of the first pot trial and aliquots of the topsoil, waste coal/topsoil (3:1, v/v), and waste coal substrates sampled using a PVC auger (30 \times 2 cm) inserted to a depth of 10 cm and the cored samples oven dried (50°C \times 24 h) prior to analysis. The residual topsoil, waste coal/topsoil, and waste coal substrate in each potting bag was thoroughly mixed and allowed to lie fallow for 23 wks at temperatures ranging from 25 to 30°C. Growth in the remaining potting bags was allowed to proceed for a further 23 weeks under the conditions already described after which the substrate and plant material was harvested and analyzed. A duplicate pot trial was allowed to proceed for 47 wks after which the above ground biomass was harvested and the substrate analyzed.

Seedling emergence and establishment were monitored using both non-remediated substrate (i.e. topsoil; waste coal/topsoil, 3:1; waste coal) and the residual substrate that previously supported growth of *C. dactylon* after lying fallow for 23 wks. Plastic pots (20 cm caliber diameter \times 18 cm height \times 16 cm bottom diameter) were filled with 2 kg of either fresh substrate or previously used substrate, irrigated and sown with seeds of *Zea mays* (maize), *Spinacia oleraceae* (spinach), *Phaseolus vulgaris* (bean), and *Pisum sativum* (pea). The experiment consisted of 3 replicates per plant species and substrate, resulting in a total of 60 pots arranged in a complete randomized block design. The experiment was conducted in a polycarbonate-covered tunnel (Ulma Agricola, Spain) and growth allowed to proceed under ambient conditions for 8 wks. Seedling establishment was determined by monitoring both germination and survival at 2 and 8 wks after planting.

Humic substance extraction and analyses

Humic-like substances (HS) were extracted and analyzed using a method adapted from that described by Janos [24]. To 2.5 g of oven dried material was added 0.1 M NaOH to a final volume of 100 mL and the mixture extracted on a rotary shaker for 24 h, centrifuged (Eppendorf Benchtop Centrifuge 5810 R, Drücken, Germany) at 1252 \times g for 90 min at 10°C, and the supernatant and pellet separated. The pH of the supernatant was adjusted to <1 using HCl and the HS precipitated by centrifugation and the pellet re-suspended in 0.1 M NaOH. HS in the re-suspended pellet were quantified by interpolation from standard curves for Leonardite-derived humic acids and peat-derived fulvic acids (purchased from the International Humic Substance Society, St. Paul, MN) after determining the absorbance using a Thermo Spectronic Aquamate UV-Vis scanning spectrophotometer (ThermoFisher Scientific, Waltham, MA) at 240 nm and 250 nm respectively.

Functional groups in HS extracted from mine topsoil, waste coal/topsoil, and waste coal were determined by Fourier Transform Infrared (FT-IR) spectroscopy using a PerkinElmer Spectrum 100 instrument (PerkinElmer, Waltham, MA) equipped with an attenuated total reflectance (ATR) accessory eliminating the need for mixing of samples with KBr. The ATR accessory, fitted with a

diamond top-plate, has spectral range of 25 000-100 cm^{-1} and refractive index of 2.4 and 2.01 μ depth of penetration.

Statistical analyses

All data were processed using Microsoft Office Excel 2010 and are presented as the mean of at least three determinations \pm standard deviation (SD). Graphs were computed using either STATISTICA (Ver.12; StatSoft, Inc., Tulsa, OK) or SigmaPlot (Ver. 11; Systat Software, Inc., San Jose, CA).

3. Results

Humic-like substance production in the rhizosphere: Soilification

Table 2 shows the humic-like substance concentration of the topsoil, waste coal/topsoil (3:1, v/v), and waste coal substrates detected prior to inoculation and seeding with *Cynodon dactylon* to study fungal-induced soilification. As expected, the waste coal substrate contained the highest amount of HS at 19.4 wt %, followed by the waste coal/topsoil mix which yielded 15.6 wt % HS. Topsoil contained the least amount of HS at 1.9 wt %.

Analysis by FTIR spectroscopy of HS extracted from topsoil, waste coal/topsoil mix and waste coal prior to inoculation with coal degrading fungi and cultivation of *C. dactylon* yielded the spectra shown in Figure 1. As anticipated, HS extracted from topsoil and waste coal/topsoil (3:1, v/v) showed that these substrates were indeed more oxidized than waste coal. This is reflected by a broad band between 2793-2888 cm^{-1} , indicative of the presence of free and hydrogen-bonded O-H and COOH groups that were not detected in waste coal-derived humic acids (Fig. 1a and b). On the other hand, the peak at 1578 cm^{-1} indicated the presence of alkene (C=C) groups in waste coal- and waste coal/topsoil-derived HS that was absent in topsoil-derived HS extracts (Fig. 1a and c). There were some similarities in the spectra of HS from the three extracts, shown by small sharp peaks detected at 3640 cm^{-1} and 3571 cm^{-1} indicating the presence of O-H group and a peak at 1429 cm^{-1} indicating the presence of methylene (CH_2) groups.

<Figure 1>

Following cultivation of *C. dactylon* for 23 and 47 wks respectively, on waste coal and waste coal/topsoil (3:1, v/v) inoculated with coal-degrading fungi, the substrates were transformed into soil-like material and the results are shown in Figure 2.

< Figure 2 >

Concentration of HS increased within the rhizosphere of *C. dactylon* cultivated on substrates of either waste coal or waste coal/topsoil (3:1, v/v) and this increase was quite dramatic after 47 wks (Fig. 2). Humics increased by 8.6 and 25.6% respectively in waste coal and waste coal/topsoil (3:1, v/v) after 23 weeks (Fig. 2a) and by 77.8 and 59.5% in waste coal and waste coal/topsoil (3:1, v/v) following 47 weeks of grass cultivation (Fig. 2b). The HS concentration of topsoil declined by approximately 40% at 23 weeks and thereafter, remained unchanged presumably due to rapid

utilization and/or leaching (Fig. 2a and b). A very slight increase in HS content of the untreated waste coal control indicated that natural weathering of the waste coal was not a major contributor to the soilification process during the course of this experiment.

Figure 3 shows a representative FTIR spectrum of HS extracted from waste coal before inoculation with coal-degrading fungi and cultivation of *C. dactylon* compared to the spectrum of HS extracted after 47 weeks of treatment. Prior to fungal inoculation and cultivation of *C. dactylon*, HS extracted from waste coal showed the presence of alkenes and methylene moieties (Fig. 3a). By comparison, the HS extracted after 47 weeks after inoculation and grass cultivation were highly oxidized. Increased oxidation of the waste coal substrate was confirmed by the FTIR data that revealed a broad band from 1800 cm^{-1} to 3600 cm^{-1} indicative of the abundance of O-H and COOH groups coupled with peaks at 1702 cm^{-1} and 1219 cm^{-1} confirming C=O stretching of various carbonyl groups including COOH and C-O stretching and O-H bending of COOH groups respectively (Fig. 3b). These findings confirmed that the soil-like residue contained in the rhizosphere of *C. dactylon* cultivated on waste coal inoculated with coal degrading fungi had indeed arisen as a consequence of increased biological weathering.

< Figure 3 >

Seedling establishment on HS-containing residue derived by *Cynodon dactylon* cultivation on waste coal

In order to demonstrate the suitability of the oxidized, HS-containing residue, remaining after inoculation of waste coal with coal-degrading fungi and cultivation of *C. dactylon*, to support plant growth the emergence and survival of spinach, maize, bean and pea seedlings was evaluated. The results in Figure 4 show that maize, a C4 grass species, perhaps not surprisingly was unaffected by the various substrates. Similarly seedling emergence and survival of bean, a legume characterized by epigeal (above ground) germination, was apparently unaffected by substrate. However, pea which displays hypogeal germination, and spinach had mean emergence values of 8.3 and 11.7% respectively (Fig. 4a) and seedling survival of <10% on all but one of the coal-containing substrates (Fig. 4b). When waste coal/topsoil-derived HS residue was used to support seedling emergence and survival values of 100% for bean and pea were routinely obtained while for maize and spinach these were 77.8 and 20% respectively (Fig. 4a) and of the seedlings that emerged, all survived (Fig. 4b). Spinach seedlings, which are susceptible to damping-off, were generally unable to survive and performed poorly on the coal-containing substrates (Fig. 4b).

< Figure 4 >

4. Discussion

In the present study, cultivation of *C. dactylon* on waste coal and waste coal/topsoil inoculated with coal-degrading fungi resulted in the transformation of these substrates to a highly oxidized dark soil-like material with increased HS content. FTIR spectroscopy of the extracted HS confirmed both product identity and the increase in oxidation of the waste coal substrate. These results thus support

the occurrence of oxidative degradation and/or biological weathering of waste coal by coal-degrading fungi in the *C. dactylon* rhizosphere.

Early studies demonstrated the ability of ligninolytic fungi to degrade low rank coals *in vitro* through production of extracellular oxidative enzymes, predominantly lignin peroxidase (LiP), MnP and LAC [16, 25, 26]. The coal-degrading fungal strains used in the present study tested positive for the ligninolytic enzymes (i.e. LAC and MnP) which have been reported in several studies to be responsible for fungal breakdown of coal [16, 26, 27, 28]. Furthermore, studies in our laboratory on the fungal metabolism of waste coal confirmed that coal degradation by *Neosartorya fischeri* ECCN 84, occurs coincident with elevated extracellular LAC [10]. It was previously postulated that fungal interaction with coal particles results in bioconversion of this substrate into a mixture of heterogeneous macromolecules that are mainly humic acids [12, 30, 31].

Soil humic substances support plant growth and appear to do so by stimulating development of soil microbial populations and enhancing uptake of essential nutrients and trace elements [32, 33]. This leads to enhanced plant growth, crop performance and ultimately increased crop quality and yield [32, 34, 35]. Indeed, FTIR analysis of the HS extracted from the residue after inoculation of waste coal and cultivation of *C. dactylon* revealed that these were similar to naturally occurring humic acids from compost which were shown to benefit germination and growth of switchgrass [35]. Despite generally good seedling emergence and survival data (i.e. seedling vigor) for maize and bean on the various coal substrates used, pea and spinach seedlings performed poorly. It is distinctly possible that compaction of waste coal-containing substrates could have compromised pea seedling emergence and survival particularly as this species displays hypogeal germination. For spinach, the situation is somewhat different. This species is sensitive to several soil-borne fungi including; *Fusarium*, *Pythium*, and *Rhizoctonia* which can cause poor seedling performance, due to damping-off during or immediately after germination. Thus, in this study, residual coal-degrading fungi may have exerted a damping-off effect on the emergence and survival of spinach seedlings. Further research is needed to examine this aspect in more detail particularly as other members of the Amaranthaceae (formerly Chenopodiaceae) are used in land restoration. Interestingly, several bacterial species are known to suppress damping-off disease [36] and may assist in developing more holistic phyto-bioremediation strategies.

In conclusion, fungal-coal degradation has previously been demonstrated *in situ* in the *C. dactylon*/hard coal rhizosphere and an increase in HS concentration was the purported outcome [7, 8, 9]. These findings are in accordance with data from the present study that demonstrates phyto-biodegradation of low-grade waste coal. Furthermore, results from the current work lend support to the proposal that *in situ* phyto-biodegradation of coal is driven by associations and mutualistic interaction between plant roots, and ligninolytic and mycorrhizal fungi [7, 11]. Taken together, these results demonstrate the potential of biologically weathered waste coal to serve as a substrate for plant cultivation and as cladding of waste coal dumps thus potentially eliminating the need for topsoil and its associated negative effects.

Acknowledgements: This research was supported by a grant from Anglo American Thermal Coal and the National Research Foundation (IFR1202220169, Grant No: 80879). Ms. Lerato M. Sekhohola

acknowledges financial support from Anglo American Thermal Coal in the form of a doctoral bursary.

Conflict of interest: The authors have declared no conflict of interest.

5. References

- [1] N. L. Nemerow, F. J. Agardy, *Strategies of Industrial and Hazardous Waste Management*, John Wiley & Sons, New York **1998**.
- [2] H. M. Leung, Z. H. Ye, M. H. Wong, M. H. Survival Strategies of Plants Associated with Arbuscular Mycorrhizal Fungi on Toxic Mine Tailings, *Chemosphere* 2007, 66, 905-915.
- [3] S. K. Maiti, Bioreclamation of Coalmine Overburden Dumps - With Special Emphasis on Micronutrients and Heavy Metal Accumulation in Tree Species, *Environmental Monitoring and Assessment* 2007, 125, 111-122.
- [4] A. A. Juwarkar, H. P. Jambhulkar, Phytoremediation of Coal Mine Spoil Dump Through Integrated Biotechnology Approach, *Bioresource Technology* 2008, 99, 4732-4741.
- [5] C. C. Du Preez, C. W. van Huyssteen, P. N. S. Mnkeni, Land Use and Soil Organic Matter in South Africa 1: A Review on Spatial Variability and the Influence of Rangeland Stock Production, *South African Journal of Science* 2011, 107(5/6), Art. #354, 8 pages.doi:10.4102/sajs. v107i5/6.354
- [6] C. C. Du Preez, C. W. Van Huyssteen, P. N. S. Mnkeni, Land Use and Soil Organic Matter in South Africa 2: A Review on the Influence of Arable Crop Production, *South African Journal of Science*, 2011, 107(5/6), Art. #358, 8 pages.doi:10.4102/sajs. v107i5/6.358
- [7] E. E. Igbini, S. Aktins, Y. van Breugel, S. van Dyke, M. T. Davies-Coleman, P. D. Rose, Fungal Biodegradation of Hard Coal by a Newly Reported Isolate, *Neosartorya fischeri*, *Biotechnology Journal* 2008, 3, 1407-1416.
- [8] E. E. Igbini, C. Z. Mutambanengwe, P. D. Rose, Phyto-bioconversion of Hard Coal in the *Cynodon dactylon*/Coal Rhizosphere, *Biotechnology Journal* 2010, 5, 292-303.
- [9] T. T. Mukasa-Mugerwa, J. F. Dames, P. D. Rose, The Role of a Plant/Fungal Consortium in the Degradation of Bituminous Hard Coal, *Biodegradation* 2010, 1, 1-13.
- [10] L. M. Sekhohola, M. L. Isaacs, A. K. Cowan, Fungal colonization and enzyme-mediated metabolism of waste coal by *Neosartorya fischeri* strain ECCN 84, *Bioscience, Biotechnology and Biochemistry* 2014, 78, 1797-1802.
- [11] L. M. Sekhohola, E. E. Igbini, A. K. Cowan, Biological Degradation and Solubilization of Coal, *Biodegradation* 2013, 24, 305-318.
- [12] M. S. Cohen, P. D. Gabriele, Degradation of Coal by the Fungi *Polyporus versicolor* and *Poria monticola*, *Applied and Environmental Microbiology* 1982, 44, 23-27.
- [13] J. P. Ralph, D. E. A. Catcheside, Transformations of Low Rank Coal by *Phanerochaete chrysosporium* and other Wood-rot Fungi, *Fuel Processing and Technology* 1997, 52, 79-93.
- [14] M. Hofrichter, W. Fritsche, Depolymerization of Low-Rank Coal by Extracellular Fungal Enzyme Systems. I. Screening for Low-Rank-Coal-Depolymerizing Activities, *Applied Microbiology and Biotechnology* 1996, 46 (3), 220-225.

- [15] M. Hofrichter, W. Fritsche, Depolymerization of Low-Rank Coal by Extracellular Fungal Enzyme Systems. II. The Ligninolytic Enzymes of the Coal-Humic-Acid-Depolymerizing Fungus *Nematoloma frowardii* b19, *Applied Microbiology and Biotechnology* 1997, 47 (4), 419-424.
- [16] M. Hofrichter, D. Ziegenhagen, S. Sorge, R. Ullrich, F. Bublitz, W. Fritsche, W. Degradation of Lignite (Low Rank Coal) by Ligninolytic Basidiomycetes and their Manganese Peroxidase System, *Applied Microbiology and Biotechnology* 1999, 52, 78-84.
- [17] G. Willmann, R. M. Fakoussa, Biological Bleaching of Water-Soluble Coal Macromolecules by a Basidiomycete Strain, *Applied Microbiology and Biotechnology* 1997, 47, 95-101.
- [18] G. Willmann, R. M. Fakoussa, Extracellular Oxidative Enzymes of Coal-Attacking Fungi, *Fuel Processing and Technology* 1997, 52, 27-41.
- [19] R. C. Tripathi, V. K. Jain, P. S. M. Tripathi, Fungal Biosolubilization of Neyveli Lignite into Humic Acid, *Energy Sources* 2010, 32, 72-82.
- [20] O. I. Klein, N. A. Kulikova, A. I. Konstantinov, T. V. Fedorova, E. O. Landesman, O. V. Koroleva, Transformation of Humic Substances of Highly Oxidized Brown Coal by Basidiomycetes *Trametes hirsute* and *Trametes maxima*, *Applied Biochemistry and Microbiology* 2013, 49, 287-295.
- [21] B. O. Oboirien, T. V. Ojumo, S. O. Obayopo, Fungi Solubilization of Low Rank Coal: Performances of Stirred Tank, Fluidized Bed and Packed Bed Reactors, *Fuel Processing Technology* 2013, 106, 295-302.
- [22] S. B. Pointing, Qualitative Methods for the Determination of Lignocellulotic Enzyme Production by Tropical Fungi, *Fungal Diversity* 1999, 2, 17-33.
- [23] G. E. Rayment, F. R. Higginson, *Australian Laboratory Handbook of Soil and Water Chemical Methods*, Inkata Press, Melbourne 1992.
- [24] P. Janos, Separation Methods in the Chemistry of Humic Substances, *Journal of Chromatography A* 2003, 983, 1-18.
- [25] R. M. Fakoussa, M. Hofrichter, Biotechnology and Microbiology of Coal Degradation, *Applied Microbiology and Biotechnology* 1999, 52, 25-40.
- [26] A. G. Zavarzina, A. A. Leontievsky, L. A. Golovleva, S. Y. Trofimov, Biotransformation of Soil Humic Acids by Blue Laccase of *Panus tigrinus* 8/18: An *In vitro* Study, *Soil Biology and Biochemistry* 2004, 36, 359-369.
- [27] T. Grinhut, Y. Hadar, Y. Chen, Degradation and Transformation of Humic Substances by Saprotrophic Fungi: Processes and Mechanisms, *Fungal Biology Reviews* 2007, 21, 179-189.
- [28] X. X. Tao, H. Chen, K. Y. Shi, Z. P. Lv, Identification and Biological Characteristics of a Newly Isolated Fungus *Hypocrea lixii* and its Role in Lignite Bioconversion, *African Journal of Microbial Research* 2010, 4, 1842-1847.
- [29] K. Henning, H. Steffes, R. M. Fakoussa, Effects on the Molecular Weight Distribution of Coal-derived Humic Acids Studied by Ultrafiltration, *Fuel Processing Technology* 1997, 52, 225-237.
- [30] D. E. A. Catcheside, J. P. Ralph, Biological Processing of Coal, *Applied Microbiology and Biotechnology* 1999, 52, 16-24.
- [31] L. H. Dong, Q. Yuan, H. L. Yuan, Changes of Chemical Properties of Humic Acids from Crude and Fungal Transformed Lignite, *Fuel* 2006, 85, 2402-2407.

- [32] A. Piccolo, G. Celano, G. Pietramellara, Effects of Fractions of Coal-derived Humic Substances on Seed Germination and Growth of Seedlings (*Lactuca sativa* and *Lycopersicon esculentum*), *Biology and Fertility of Soils* 1993, 16, 11-15.
- [33] A. Piccolo, G. Pietramellara, J. S. C. Mbagwu, Reduction in Soil Loss from Erosion-susceptible Soils Amended with Humic Substances from Oxidized Coal, *Soil Technology* 1997, 10, 235-245.
- [34] H. A. van de Venter, M. Furter, J. Dekker, I. J. Cronje, Stimulation of Seedling Root Growth by Coal-derived Sodium Humate, *Plant and Soil* 1991, 138, 17-21.
- [35] A. Traversa, E. Loffredo, C. E. Gattullo, A. J. Palazzo, T. L. Bashore, L. Sinesi, Comparative Evaluation of Compost Humic Acids and their Effects on Germination of Switchgrass (*Panicum vigatum* L.), *Journal of Soils and Sediments* 2014, 14, 432-440.
- [36] Md. T. Islam, Y. Hashidoko, A. Deora, T. Ito, S. Tahara, Suppression of Damping-Off Disease in Host Plants by the Rhizoplane Bacterium *Lysobacter* sp. Strain SB-K88 Is Linked to Plant Colonization and Antibiosis against Soilborne Peronosporomycetes, *Applied and Environmental Microbiology* 2005, 71 (7), 3786-3796.

Table 1. Physicochemical properties of topsoil, waste coal/topsoil (3:1, v/v), and waste coal used as substrate for cultivation of *Cynodon dactylon*.

Characteristic	Topsoil	Waste coal/topsoil (3:1, v/v)	Waste coal
Water retention (%)	26.4 ± 0.2	32.6 ± 0.2	33.6 ± 0.2
pH	6.4 ± 0.1	4.3 ± 0.1	4.2 ± 0.0
Electrical conductivity (mS.m ⁻¹)	27.7 ± 0.6	56.0 ± 2.0	35.7 ± 2.5
Ash content (wt %)	93.7 ± 0.1	71.2 ± 0.1	55.5 ± 0.3
Total Organic Carbon (mg kg ⁻¹)	1.4 ± 0.3	7.2 ± 1.1	10.3 ± 2.0

Table 2. Humic-like substance concentration in topsoil, waste coal/topsoil (3:1, v/v), and waste coal detected prior to inoculation and seeding with *Cynodon dactylon*.

Substrate	Humic substances (wt %)
Topsoil	1.9 ± 0.1
Waste coal/topsoil (3:1, v/v)	15.6 ± 0.2
Waste coal	19.4 ± 0.3

Figure Headings

Figure 1. Fourier Transform Infrared spectra of humic-like substances extracted from (a) waste coal/topsoil (3:1, v/v), (b) topsoil and (c) waste coal.

Figure 2. Biodegradation induced changes in humic-like substance concentration of the waste coal, waste coal/topsoil (3:1, v/v) and topsoil substrates. Humic-like substances were extracted from untreated waste coal (control) and waste coal, waste coal/topsoil and topsoil following inoculation with mycorrhizal fungi, coal degrading fungi and cultivation of *Cynodon dactylon* after 23 (a) and 47 weeks (b) respectively.

Figure 3. Fourier Transform Infrared spectra of humic-like substances extracted from waste coal before (a) and after (b) inoculation with coal-degrading fungi and cultivation *Cynodon dactylon* for 47 weeks.

Figure 4. Emergence and survival of bean, pea, spinach and maize seedlings at 2 (a) and 8 (b) weeks on topsoil, waste coal, waste coal/topsoil (3:1, v/v) and the biologically weathered residue from waste coal and waste coal/topsoil inoculated with coal-degrading fungi and which had supported cultivation of *Cynodon dactylon* for 23 weeks before lying fallow for 24 weeks.

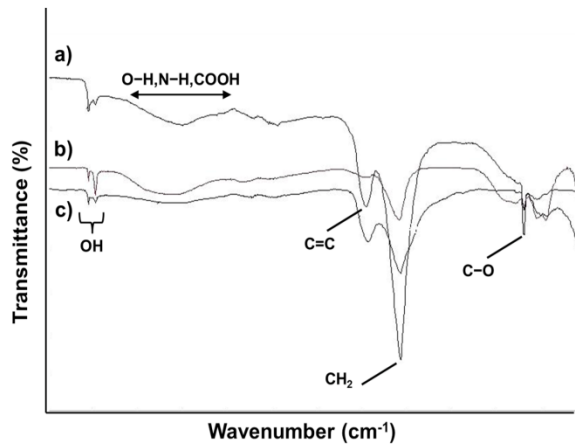


Figure 1.

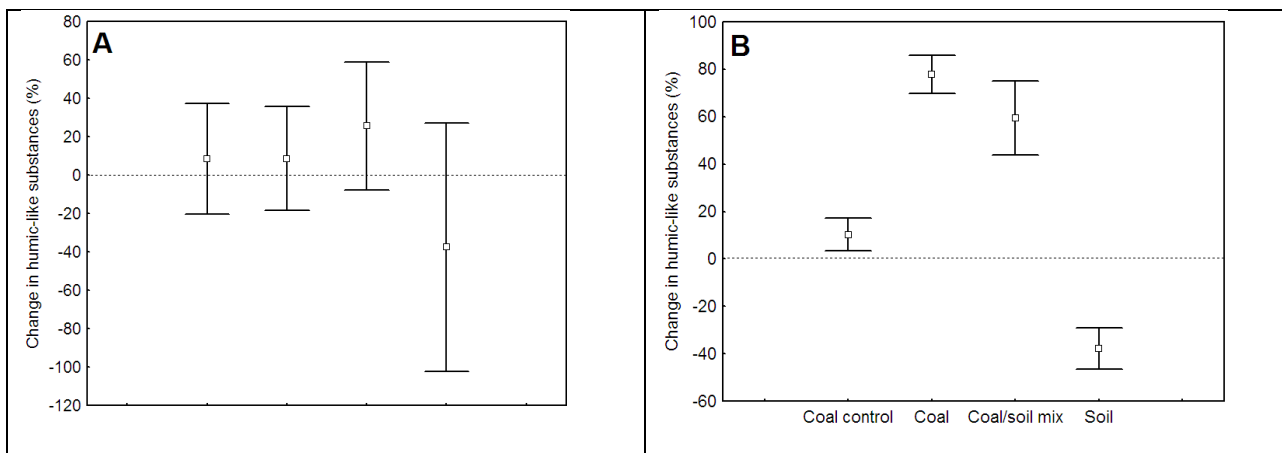


Figure 2.

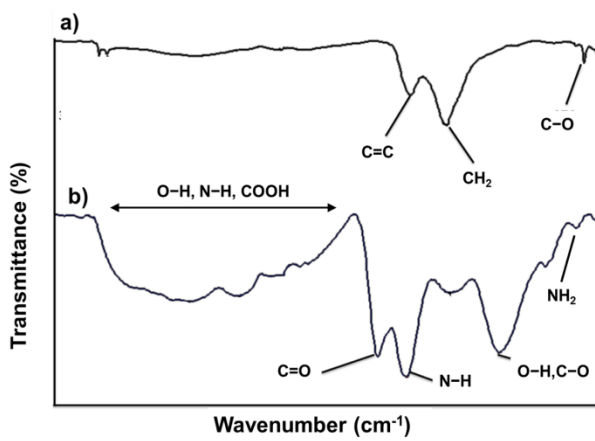


Figure 3.

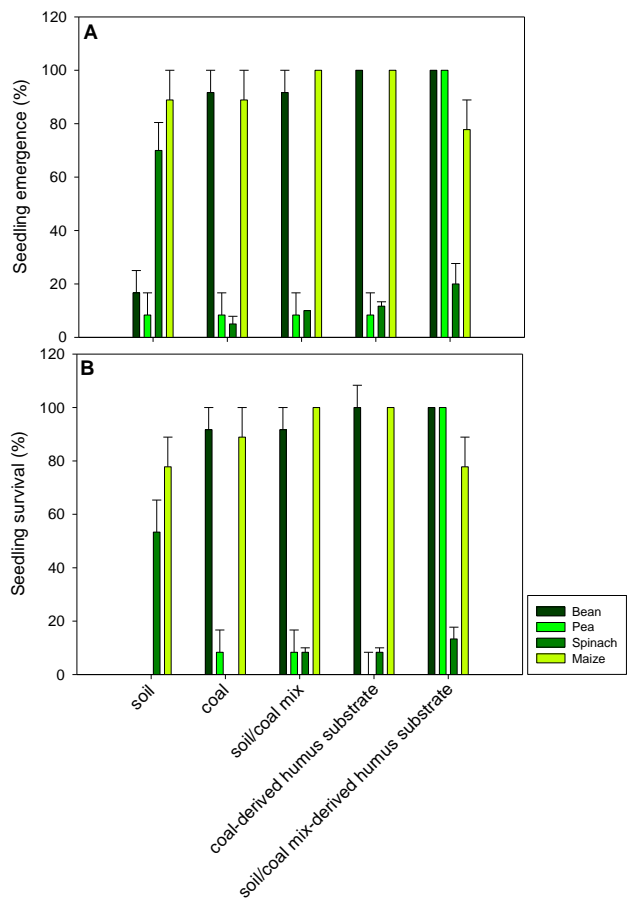


Figure 4.

Paper 4

In situ bioremediation and self-cladding of South African coal discard dumps

Lerato Mary Sekhohola, O.G. Edeki and Ashton Keith Cowan

In revision for The Journal of the South African Institute of Mining and Metallurgy

Title:

In situ bioremediation and self-cladding of South African coal discard dumps

Authors:

L.M. Sekhohola¹, O.G. Edeki¹, H. Lodewijks², and A. K. Cowan^{1,*}

Affiliations:

¹Institute for Environmental Biotechnology, Rhodes University, Grahamstown, 6140, South Africa

²Safety & Sustainable Development: Coal, Anglo Operations Proprietary Limited

PO Box 61587, Marshalltown, 2107, South Africa

Corresponding author:

*Professor A. K. Cowan

Institute for Environmental Biotechnology, Rhodes University (EBRU)

P.O. Box 94, Grahamstown, 6140

South Africa

Email, a.cowan@ru.ac.za

Tel, +27 (0)46 6222 656

Fax, +27 (0)46 6222 656

Synopsis

Current rehabilitation of waste coal dumps remains a challenge due to reliance on topsoil for establishment of vegetation. Fungcoal has been proposed as a viable and alternative strategy for rehabilitation of discard dumps, overburden and spoils. Fungcoal exploits fungi-plant mutualism to achieve biodegradation of waste coal, which in turn, promotes reinvigoration of soil components, grass growth and re-vegetation. The main objective of the present study was to determine the effect of different cladding materials as carbon donor to support Fungcoal-induced rehabilitation of waste coal dumps at commercial scale. This was achieved by monitoring vegetation establishment and changes in physicochemical properties of the substrate after Fungcoal application over a three-year period. Results show that dump self-cladding with annual and perennial grasses was successfully achieved where Fungcoal was applied with weathered coal as the carbon donor. Fungcoal suppressed acidification of the waste coal substrate, reduced substrate electrical conductivity, and promoted humic-like substance enrichment to support growth and establishment of annual and perennial grasses. In the absence of cladding or where oxidized waste coal was used as cladding poor and sporadic grass growth and ineffective rehabilitation was the outcome. The potential of an *in situ* bioremediation strategy like Fungcoal as an alternative to topsoil is discussed.

Keywords

Coal discard dumps, Fungcoal, myco-remediation, phyto-stabilisation, rehabilitation, weathered coal

Introduction

South Africa is a leading coal-producing country and with its large reserves, coal mining is likely to continue for the foreseeable future. While other dominant players in coal production such as China have advanced to clean coal technology (Chen and Xu, 2010) South Africa still lags in efforts to “clean up” waste generated from coal production and processing. Alternative uses of so-called waste coal have been explored although large amounts are still disposed of in a form of unsightly dumps that pose an environmental hazard. Coal mining companies are obliged by South African legislation governing mine closure to rehabilitate and return mined land to a viable post-mining state (Limptlaw *et al.*, 2005). The conventional approach to rehabilitation of waste coal dumps in South Africa involves use of topsoil for establishment of vegetation cover. Common practice is to deposit a soil layer of at least 100cm as a clad, followed by addition of fertilizer prior to seeding using a predetermined mix of grasses (Anglo American Thermal Coal Rehabilitation Tool Box). Unfortunately the practice requires excavation and transportation of many tonnes of fertile soil, leaving behind bare land that is susceptible to erosion. In some cases low nutrient content of the soil coupled with accelerated mineralization of soil organic matter reduces the efficiency of mineral fertilizers leading to failure of rehabilitation process (Glaser *et al.*, 2002).

Rehabilitation of dumped solid mine waste follows the phyto-remediation principle (Jawarkar and Jambhulkar, 2008; Jawarkar *et al.*, 2009; Arocena *et al.*, 2010; Kappes *et al.*, 2012; Finkenbein *et al.*, 2013). In a review, Salt *et al.* (1998) described the different aspects of phyto-remediation and it appears “phyto-remediation ex planta” remains the most widely applied strategy on waste coal dumps. The principle is based on plant-driven bio-stimulation of naturally occurring rhizospheric microorganisms through mineralization of root exudate, which serves as a simple carbon source, followed, by degradation of the carbonaceous waste material. Establishment of a healthy vegetative cover on fairly new coal dumps however remains a big challenge due to hostile conditions such as lack of the macro minerals nitrogen and phosphorus, acidity, high temperatures and toxic metal concentrations which are characteristic of coal dumps (Maiti, 2007). It is therefore not surprising that remediation strategies in South Africa have traditionally relied on creating a soil microenvironment on the dump surface to facilitate plant growth. The deposited layer, usually of topsoil, provides a medium conducive for seed germination, seedling establishment and subsequent plant colonization.

Soil formation remains the most important aspect in restoration of functional ecosystems in mining landscapes (Sourkova *et al.*, 2005). As pointed out by Singh *et al.* (2002), the land restoration process should focus on improving physicochemical properties and vegetation cover to accelerate natural recovery. Rehabilitation of discard coal dumps therefore focuses on two aspects; 1) vegetative cladding of the dump, and 2) physicochemical transformation of dumped coal material through abiotic and biotic weathering. Successful establishment of the former can positively influence the rate at which the latter occurs. Modern techniques have shifted towards integrated biotechnological approaches that combine phyto-remediation (establishing plant cover on the dump) and bio-stimulation while exploring the use of alternative cost effective carbon substrates. For example, Jawarkar and Jambhulkar (2008) used sludge obtained after wastewater treatment as a carbon source and biofertilizers together with mycorrhizal fungi to facilitate growth of native tree species on coal mine spoil. After three years, the following were observed; increased microbial populations, improved physicochemical properties, increased organic and nutrient contents that resulted in higher fertility and productivity. Jawarkar *et al.* (2009) also developed an integrated

biotechnological rehabilitation method which incorporated the use of microorganisms known to benefit plant growth such as free living soil bacteria, nitrogen fixing bacteria and vesicular arbuscular mycorrhizal fungi, in combination with ‘pressmud’ (the residue from filtration of sugarcane juice) as organic carbon source to enhance plant growth on manganese mine overburden. The technology resulted in successful restoration in terms of soil formation, accumulation of organic carbon and nutrients as well as microbial activity within an 18-year period. Rapid plant growth coupled with improved biogeochemical changes attributed to the presence of ‘pressmud’ and microbial inoculants respectively, were observed.

A number of studies have emphasized that the more oxidized the carbonaceous waste (as in the case of low rank weathered coal) the easier it is for fungi and other microorganisms to utilize as substrate (Laborda *et al.*, 1999; Klein *et al.*, 2013). Earlier work on the myco-remediation of waste coal carried out in our laboratory showed, albeit under defined conditions, that *Neosartorya fischeri* can catalyse degradation of coal in vitro and in fixed-bed bioreactors (Igbini *et al.*, 2008; 2010; Sekhohola *et al.*, 2014). Furthermore, unpublished data from small scale field studies on the Kleinkopje Roofcoal Dump (Mpumalanga, South Africa) indicated the in situ “manufacture” of an organic rich topsoil-like material when *Neosartorya fischeri*, mycorrhizal fungi, and the grass *Cynodon dactylon* were introduced together with weathered coal as a carbon donor and that transformation of coal discard to a depth of 650 mm occurred within six years. Based on these results and other laboratory-based studies a bioprocess for the self-cladding of waste coal dumps was developed and patented (Rose *et al.*, 2010). This bioprocess, known as Fungcoal, exploits the mutualistic interaction between the coal degrading biocatalyst *Neosartorya fischeri*, and several mycorrhizal fungi and grass species. The process is dependent on a supply of suitable weathered coal and/or suitable weathered discard and/or suitable carbonaceous weathered spoils which act as the major carbon donor to achieve myco-remediation of dumped waste coal. Evaluation of the technology and field trial data indicated that it might be a suitable method with which to rehabilitate not only waste coal dumps by facilitating self-cladding but also opencast mined ground.

The present study therefore set out to investigate Fungcoal as a bioprocess technology for large-scale rehabilitation on discard coal dumps at three mines in Mpumalanga, South Africa. The aims of the study were; 1) to determine how different cladding materials contribute to the Fungcoal process, and 2) to determine changes in physicochemical properties that reflect soilification of the coal material as a result of Fungcoal application.

Materials and methods

Study site

Waste coal dumps located at Kleinkopje Colliery (Klippan Dump, latitude 26° 48’S; longitude 28° 77’E), Greenside Colliery (Greenside Dump, latitude 26° 48’S; longitude 28° 77’E) and Landau Colliery (Kromdraai opencast, latitude 25° 98’S; longitude 27° 77’E), in the Witbank coalfield of South Africa were used in this study (Fig. 1). Dumps are typically 1V: 5H in size with maximum height of 50 m and cover a ground surface area of 166 ha. The region receives mean annual rainfall of 696-1032 mm with mean daily minimum and maximum temperatures of 15 and 27°C respectively. Strong winds are experienced in spring (August to September) and intermittent hailstorms from October to December.

< **Figure 1** >

The Greenside and Klippan waste dumps are composed of compacted low grade coal and discard (a mix of coarse material of varying particle sizes between 150µm to 150mm) left in the void following extraction of the high grade coal seam while Kromdraai opencast consists of a mix of overburden, spoil and highly weathered coal material.

Preparation of fungal inoculum

The fungal inoculum was prepared by culturing *Neosartorya fischeri* strain ECCN 84 in malt extract broth at 25°C for 7 d. Fungal mycelia were then homogenized, filtered through a Bucher funnel using Whatman No. 1 filter paper and air dried to 1% moisture content. A mixture of ECCN 84 and Mycoroot in the ratio 0.5 g ECCN 84:25 kg Mycoroot (Mycoroot Pty Ltd., Grahamstown, South Africa) was prepared using an inert clay carrier and the inoculum packaged and shipped to the trial sites.

Trial design and layout

Four 1 ha blocks on the south-facing aspect of each waste coal dump were cleared, demarcated, the surface rip ploughed and lime (Pistorius & Co, Mpumalanga, South Africa) applied at a rate of 25 tonnes/ha.

No cladding was applied to the trial site on Klippan discard dump. Highly oxidized waste coal, obtained from the Clydesdale waste dump, was applied as a clad (10-15 cm; 50 tonnes per 100 m² or 2.5 twenty tonne truck loads) to the Greenside discard dump. For Kromdraai opencast, weathered coal (sourced from B Seam, Kromdraai Mine, Landau Colliery) was applied as a 10-15 cm clad. Cladding material was then disked into the waste coal substrate using a disc set at 200 mm. Where specified, the fungal inoculum prepared as described above was applied at rates of either 37.5 (50% Fungcoal) or 75 kg/ha (100% Fungcoal) followed by LAN (Omnia, Bryanston, South Africa) unless otherwise indicated, which was applied at rate of 300 kg/ha using a fertilizer (lime) spreader.

Untreated 1 ha controls (i.e. without cladding and the fungal inoculum) and 1 ha mine rehabilitation standards (i.e. the current rehabilitation protocol specific for that mine) were included for each trial as references. Typically, the mine rehabilitation standard involved applying a 1 m clad of topsoil, followed by fertilization using either LAN or Humus Mix (AGRON Co. Marble Hall, South Africa). All experimental blocks and controls were then seeded using a mix of *Cynodon dactylon*, *Eragrostis tef*, *Chloris gayana*, *Pennisetum clandestinum* and *Paspalum notatum*. Irrigation was by natural precipitation (Fig. 2). Due to mine regulation and existing contracts all operations relating to trial layout were carried out by either Fraser Alexander Bulk Mech. Pty. Ltd. or Dust and Erosion Control CC.

< **Figure 2** >

Sampling

Sampling was by complete randomized design. For sampling, at each of the intervals specified in the Results, the 1 ha blocks were divided into 10×10 m quadrants and a total of twenty allocated for sampling using a random number generator. Similarly, each of the twenty 10×10 m blocks was divided further into 1×1 m units and sampling points assigned using a random number generator. Soil and coal samples of approximately 500 g and from a depth of 30 cm were obtained using an auger, thoroughly mixed and transported to the laboratory in sealed zip-lock bags. Prior to analysis,

the soil and coal samples were dried at 50°C for 24 h to a constant weight and pulverized (HERZOG Maschinenfabrik GmbH Co., Osnabrück, Germany).

Substrate physicochemical analysis

Electrical conductivity (EC) and pH were determined using a conductivity meter (EUTECH instruments) and pH meter (HANNA instruments HI 8314) according to protocols 3A1 and 4A1 respectively as described by Rayment and Higginson (1992). Humic acid-like substances were extracted using the method described by Janos (2003) with minor modifications. Briefly, soil and coal samples (each 2.5 g) were extracted in 0.1M NaOH by agitation for 24 h at 150 rpm. The suspension was centrifuged (2000×g for 90 min) at 10°C, the pellet discarded, the pH of the resultant supernatant adjusted to <1 using HCl, and precipitate formation allowed to proceed for 1 h. Following centrifugation (2000×g for 90 min at 10°C), the pellet was re-suspended in 0.1M NaOH and the concentration of humic acid-like substances determined spectrophotometrically at 280 nm by interpolation from a standard curve for authentic humic acid (Sigma-Aldrich, St Louis MO).

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using GenStat (VSN International, Hemel Hempstead, UK). Differences between means were tested using Duncan's multiple range test ($P < 0.05$).

Results

Vegetation establishment

The effect of cladding material and Fungcoal (100%, 75 kg/ha), as a rehabilitation strategy, on vegetation growth and cover establishment on waste coal dumps is shown in Figure 3. Figure 3A illustrates the response to Fungcoal in the absence of dump cladding. The effect of a highly oxidized waste coal clad on Fungcoal performance is shown in Figure 3B while the effect of a weathered coal clad on Fungcoal on opencast is presented in Figure 3C.

Soon after application of Fungcoal to the Klippan and Greenside discard dumps (September 2011) and Kromdraai opencast (September 2012), and as expected, there was no evidence of grass growth (Figure 3). Following the summer hydrological cycle (March 2012), vegetation establishment on the Klippan (Fig 3A) and Greenside dumps (Fig 3B) although poor and sporadic was dominated by the warm season annual C4 grass *Eragrostis tef*. Similarly, onset of summer rainfall (Fig. 2; Nov 2012 - Feb 2013) stimulated growth and establishment of vegetation on opencast cladded with weathered coal which was again dominated by *E. tef*, (Fig 3C). No or very poor plant growth was evident on the untreated plots whereas the mine rehabilitation standards displayed significant vegetation establishment and cover (data not shown). Establishment of the perennial species *Cynodon dactylon*, *Chloris gayana*, *Pennisetum clandestinum* and *Paspalum notatum* followed winter dieback of *E. tef*, and provision of 100% cover. This occurred only where a weathered coal clad had been applied to support Fungcoal i.e. Kromdraai opencast (Fig 3C). In the absence of cladding i.e. Klippan dump (Fig 3B), establishment of the perennial grasses in the subsequent summers (Feb 2013 and 2014) was stunted and consequently, only insignificant cover was achieved. Establishment of perennial grass species in the subsequent summer of 2013/2014 failed completely

where Fungcoal-treated plots were cladded with highly oxidized waste coal i.e. Greenside discard dump (Fig 3B).

<Figure 3>

Substrate physicochemical characteristics

Changes in the physicochemical characteristics of the coal dump substrate as a consequence of cladding material with and without the addition of Fungcoal, applied at either 37.5 (50%) or 75 kg/ha (100%), is summarized in Figures 4 and 5.

<Figure 4>

Substrate for determination of pH and EC was sampled following the summer hydrological cycles in 2012, 2013 and 2014 to minimize any confounding effect of seasonality. Data are presented as the cumulative mean and the respective bars represent the amount of variation within each treatment (Fig. 4). Results show that in the absence of cladding and where a highly oxidized waste coal clad was used substrate pH declined while EC increased (Fig. 4A and B). By comparison, use of a carbon-rich weathered coal clad near neutral substrate pH was sustained while electrical conductivity was in the range 1-100 mS.m⁻¹ typical for substrate materials such as sand or silt (Fig 4C). Interestingly, although substrate pH and EC appeared to be inversely related, low variation in substrate pH was associated with a high degree of variation in substrate EC and visa versa (Fig. 4A and B). The data for Klippan dump suggests therefore a substrate with inherently high salinity (Fig. 4A) whereas for Greenside dump, the results indicate increased acidity (Fig. 4B). Neither could be mitigated by Fungcoal treatment and the effect of inherent acidity was presumably exacerbated in the case of Greenside dump, by applying a 10-15 cm clad of ash-rich oxidized waste coal. Both scenarios would be expected to either retard or inhibit vegetation growth and establishment, which was indeed the observed outcome (see Fig. 2).

< Figure 5>

The results presented in Figure 5 illustrate the outcome of analysis of the substrate carried out to determine humic substance concentration. It is evident that on Klippan dump and in the absence of cladding, very low concentrations of humic substances were quantified (Fig 5A). Similarly, on Greenside dump cladded using high ash containing oxidized waste coal, low amounts of humic substances were present (Fig. 5B). Relatively high amounts of humic substance were extracted from the substrate sampled from mine control and the 0% Fungcoal treatment on Kromdraai opencast which contrasts with the very low humic content of substrate following 50 and 100% Fungcoal treatment (Fig. 5C). As mentioned in the Materials and Methods, the standard rehabilitation strategy adopted for Kromdraai opencast includes the use of a propriety fertilizer, rich in humic substances known as Humus Mix. Use of this commercial fertilizer would indeed contribute to the elevated levels of humic substances determined in the present study for both mine control and 0% Fungcoal. Furthermore, this result indicates that contrary to prescription, no Humus Mix was applied by the contractor during establishment of the 1 ha 50 and 100% Fungcoal treatments. Nonetheless, 50 and 100% Fungcoal reduced substrate acidification, suppressed any increase in electrical conductivity, and facilitated vegetation growth and establishment of both annual and perennial grass species.

Discussion

Coal mining visibly affects the aesthetics of a landscape and disrupts soil components including soil horizon and structure, soil microflora survival and proliferation, and nutrient recycling, which are essential to sustain functional ecosystems. After exposure to air and water, oxidation of metal sulphides within the surrounding rock generates acidity. The resultant soil water is often highly acidic, high in sulphate and may contain various heavy metals (Johnson and Hallberg, 2005). The metals originate from the dissolution of sulphide minerals such as pyrite, arsenopyrite, chalcopyrite, sphalerite, and marcasite, which produce sulfuric acid-rich solutions, that contain high concentrations of these toxic metals (Baker and Banfield, 2003). These harmful substances contaminate surrounding ecosystems and cause massive damage to the natural flora and fauna.

Results presented in this study confirm that the current protocol practiced on coal mines in South Africa for rehabilitation of land disturbed by mining and for reclamation of waste coal dumps does indeed prevent soil acidification, maintain apparent soil electrical conductivity in the appropriate range, lead to humic substance enrichment, and support re-vegetation using annual and perennial grass species. Unfortunately, availability of topsoil is limited and for rehabilitation purposes must be transported over long distances at great cost suggesting that the current approach is unsustainable. Thus, there is a need to seek alternative but viable rehabilitation strategies that are less reliant on the importation of topsoil. A potential solution is the adoption of an *in situ* bioremediation strategy for the self-cladding of land disturbed by mining and for use on waste coal dumps. One such strategy, currently being developed for soil restoration and land rehabilitation is Fungcoal. This technology exploits fungi-plant mutualism to achieve biodegradation of the waste coal substrate, which in turn, promotes reinvigoration of soil components, grass growth and re-vegetation (Igbini *et al.*, 2008; 2010; Mukasa-Mugerwa *et al.*, 2010; Sekhohola *et al.*, 2014). Thus, the addition of fungi and other microbes facilitates degradation of the carbonaceous substrate, microbial utilization of the products of waste coal degradation, and proliferation of soil microbial populations (Sekhohola *et al.*, 2013). The outcome is, improvement in soil aggregation due to increased polysaccharide and humic substance content (i.e. increased soil organic matter) that positively affect plant growth (Sekhohola *et al.*, 2013). Typically an active soil microbial community leads to stable soil aggregation, whereas without microbial activity soils compact and are poorly aggregated (Edgerton *et al.* 1995).

The present study has shown that application of the coal-degrading fungus *Neosartorya fischeri* together with mycorrhizal fungi (i.e. Fungcoal) in the presence of a suitable carbon donor such as weathered coal and without addition of topsoil mitigates substrate acidification and salinity to support growth and establishment of annual and perennial grasses. Where no cladding was applied (i.e. Klippan) or where a highly oxidized waste coal clad was used (i.e. Greenside), Fungcoal treatment was less able to mitigate substrate acidification and increased salinity. Indeed, it is very likely that acidic conditions and elevated salinity of the substrate on the uncladded Klippan waste dump and oxidized waste coal cladded Greenside dump exacerbated plant deterioration (Limptlaw *et al.*, 2005). High soil acidity is known to inhibit plant root growth and appears to do so by limiting nutrients availability (Kidd and Proctor, 2001; Zu *et al.*, 2014). The use of an appropriate carbon-rich cladding substrate appears therefore to be crucial for successful performance of Fungcoal technology.

Application of Fungcoal at 100% suppressed substrate acidification on uncladded (i.e. Klippan) and weathered coal cladded (i.e. Kromdraai) waste dumps suggesting that use of this rehabilitation strategy can mitigate acid mine drainage which is a characteristic of waste coal dump

surface exposure and oxidation (Limptlaw *et al.*, 2005). Increased electrical conductivity (i.e. salinity) following application of Fungcoal to waste dumps either uncladded or cladded in oxidized waste coal while not desirable might nevertheless be indicative of fungal-catalyzed waste coal biodegradation. Oxidative breakdown of coal by coal-degrading fungi increases the number of negatively charged sites on the coal surface that bind cationic species forming coal-metal ion complexes, which can disassociate in solution contributing to increased salinity (Willmann and Fakoussa, 1997; Skodras *et al.*, 2014). In addition, humic substances, the purported products of biological degradation of coal, function to give the soil structure, porosity, water holding capacity, cation and anion exchange, and are involved in the chelation of mineral elements, and support microbial activity and proliferation (Valdrighi *et al.*, 1996; Moliszewska and Pisarek, 1996; Anesio *et al.*, 2004; Gogala, 2005). In the long term, introduction of coal degrading fungi through application of Fungcoal is expected to stimulate transformation of waste coal into humic substance-like material as demonstrated in laboratory studies (Igbini *et al.*, 2008; 2010; Sekhohola *et al.*, 2014). Similar large-scale rehabilitation studies have shown that a period in excess of 2-3 years is necessary to confirm successful soil restoration and rehabilitation (Jawarkar and Jambhulkar, 2008; Jawarkar *et al.*, 2009). Thus, monitoring of vegetation establishment and substrate physicochemical characteristics will be extended as part of the evaluation process of Fungcoal as a rehabilitation strategy for use on discard dumps, overburden and spoils.

In conclusion, work described herein confirms the current protocol used by South African coalmines to establish vegetation on disturbed land and waste coal dumps. Whether this strategy affords (bio) conversion of the underlying waste/discard to an innocuous soil-like material is unknown. Nevertheless, dependence on topsoil suggests that this protocol is unlikely to be sustainable and cost effective in the long run. Self-cladding was achieved within one season after application of Fungcoal to waste dumps cladded in carbon-rich weathered coal and occurred coincident with suppression of acidification and salinity, and humic substance enrichment. Also, vegetation establishment, grass growth and cover were comparable to that achieved using the current mine rehabilitation protocol. This outcome illustrates the potential of replacing topsoil with an alternative high carbon-containing substrate such as weathered coal to achieve successful and sustainable rehabilitation. Thus, microbial degradation of dumped waste coal and humic substance enrichment coupled with re-vegetation may indeed provide a rehabilitation strategy that can potentially lead to full land restoration post mine closure.

Acknowledgements

This research was supported by a grant from Anglo American Thermal Coal and the National Research Foundation (IFR1202220169, Grant No: 80879). Ms. Lerato M. Sekhohola acknowledges financial support from Anglo American Thermal Coal in the form of a doctoral bursary. Dave Render, Yvonne van Breugel and Michelle Isaacs are thanked for assistance with sampling and Nomaindia Toto for the laboratory analysis. The cooperation of mine personnel (Greenside, Kleinkopje and Landau Collieries) is appreciated.

References

- ALLARDICE, D.J., CLEMOW, L.M., FAVAS, G., JACKSON, W.R., MARSHALL, M., and SAKUROVS, R. 2003. Characterization of different forms of water in low rank coals and some hydrothermally dried products. *Fuel*, vol. 82, no. 6. pp. 661-667.
- ANESIO, A.M., HOLLAS, C., GRANALI, W., and LAYBOURN-PARRY, J. 2004. Influence of humic substances on bacterial and viral dynamics in fresh waters. *Applied and Environmental Microbiology*, vol. 70, no. 8. pp. 4848-4854.
- AROCENA, J.M., VAN MOURIK, J.M., SCHILDER, M.L.M., and CANO, A.F. 2010. Initial soil development under pioneer plant species in metal mine waste deposits. *Restoration Ecology*, vol. 18, no. 2. pp. 244-252.
- BAKER, B.J., and BANFIELD, J.F. 2003. Microbial communities in acid mine drainage. *FEMS Microbiology Ecology*, vol. 44, no. 2, pp. 139-152.
- CHARRIERE, D., and BEHRE, P. 2010. Water sorption on coals. *Journal of Colloid and Interface Science*, vol. 344, no. 2. pp. 460-467.
- CHEN, W., and XU, R. 2010. Clean coal technology development in China. *Energy Policy*, vol. 38, no. 5. pp. 2123-2130.
- CHOI, H., THIRUPATHIRAJA, C., KIM, S., RHIM, Y., LIM, J., and LEE, S. 2011. Moisture re-adsorption and low temperature oxidation characteristics of upgraded low rank coals. *Fuel Processing Technology*, vol. 92, no. 10. pp. 2005-2010.
- EDGERTON, D. L., HARRIS, J. A., BIRCH, P., and BULLOCK, P. 1995. Linear relationship between aggregate stability and microbial biomass in three restored soils. *Soil Biology and Biochemistry*, vol. 27, no. 11, pp. 1499-1501.
- FAKOUSA, R.M., and HOFRICHTER, M. 1999. Biotechnology and Microbiology of coal degradation. *Applied Microbiology and Biotechnology*, vol. 52, no. 1. pp. 25-40.
- FINKENBEIN, P., KRETSCHMER, K., KUKA, K., KLOTZ, S., and HEILMEIER, H. 2013. Soil enzyme activities as bioindicators for substrate quality in revegetation of a subtropical coal mining dump. *Soil Biology and Biochemistry*, vol. 56. pp. 87-89.
- GIANNOULI, A., KALAITZIDIS, S., SIAVALAS, G., CHATZIAPOSTOULOU, A., CHRISTIANIS, K., and PAPAZISIMOU, S. 2009. Evaluation of Greek low-rank coals as potential raw material for the production of soil amendments and organic fertilizers. *International Journal of Coal Geology*, vol. 77, no. 3-4. pp. 383-393.
- GLASER, B., LEHMANN, J., and ZECH, W. 2002. Ameliorating physical and chemical properties of highly weathered soils in the tropics with charcoal - a review. *Biology and Fertility of Soils*, vol. 35, no. 4. pp. 219-230.
- GOODARZI, F. 2002. Mineralogy, elemental composition and modes of occurrence of elements in Canadian feed coals. *Fuel*, vol. 41, no. 9. pp. 119-121.
- GOGALA, N. 2005. Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. *Cellular and Life Science*, vol. 47, no. 4. pp. 331-340.
- 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*, vol. 3, no. 11. pp. 1407-1416.
- IGBINIGIE, E.E., MUTAMBANENGWE, C.Z., and ROSE, P.D. 2010. Phyto-bioconversion of hard coal in the *Cynodon dactylon*/coal rhizosphere. *Biotechnology Journal*, vol. 5, no. 3. pp. 292-303.

- JANOS, P. 2003. Separation methods in the chemistry of humic substances. *Journal of Chromatography A*, vol. 983, no. 1-2, pp. 1-18.
- JOHNSON, B.D., and HALLBERG, K.B. 2005. Acid mine drainage remediation options: a review. *Science of the Total Environment*, vol. 338, pp. 3-14.
- JUWARKAR, A.A. and JAMBHULKAR, H.P. 2008. Phytoremediation of coal mine spoil dump through integrated biotechnology approach. *Bioresource Technology*, vol. 99, no. 11, pp. 4732-4741.
- JUWARKAR, A. A., YADAV, S.K., THAWALE, P.R., KUMAR, P., SINGH, S.K. and CHAKRABARTI, T. 2009. Developmental strategies for sustainable ecosystem on mine spoil dumps: a case study. *Environmental Monitoring and Assessment*, vol. 157, no. 1-4, pp. 471-481.
- KAPPES, H., CLAUSIUS, A., and TOPP, W. 2012. Historical small scale surface structures as a model for post-mining land reclamation. *Restoration Ecology*, vol. 20, no. 3, pp. 322-330.
- KIDD, P.S., and PROCTOR, J. 2001. Why plants grow poorly on very acid soils: are ecologists missing the obvious? *Journal of Experimental Botany*, vol. 52, no. 357, pp. 791-799.
- KLEIN, O.I., KULIKOVA, N.A., KONSTANTINOV, A.I., FEDOROVA, T.V., LANDESMAN, E.O., and KOROLEVA, O.V. 2013. Transformation of humic substances of highly oxidized brown coal by Basidiomycetes *Trametes hirsute* and *Trametes maxima*. *Applied Biochemistry and Microbiology*, vol. 49, no. 3, pp. 287-295.
- LABORDA, F., MONISTROL, I.F., LUNA, N., and FERNANDEZ, M. 1999. Processes of liquefaction/solubilization of Spanish coals by microorganisms. *Applied Microbiology and Biotechnology*, vol. 52, no. 1, pp. 49-56.
- LEYVAL, C., TURMAU, K., and HASELWANDTER, K. 1997. Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza*, vol. 7, no. 3, pp. 139-153.
- LIMPITLAW, D., AKEN, M., LODEWIJKS, H. and VILJOEN, J. Post mining rehabilitation, land use and pollution at collieries in South Africa. Proceedings of the colloquium: sustainable development in the life of coal mining, Boksburg, South Africa, 13 July 2005. *The South African Institute of Mining and Metallurgy*, vol. 1, pp. 1-10.
- MAITI, K.S. 2007. Bioreclamation of coalmine overburden dumps with special emphasis on micronutrients and heavy metals accumulation in tree species. *Environmental Monitoring and Assessment*, vol. 125, no. 1-3, pp. 111-122.
- MOLISZEWSKA, E. and PISAREK, I. 1996. Influence of humic substances on the growth of two phytopathogenic soil fungi. *Environment International*, vol. 22, no. 5, pp. 579-584.
- MUKASA-MUGERWA, T.T., DAMES, J.F. and ROSE, P.D. 2010. The role of a plant/fungal consortium in the degradation of bituminous hard coal. *Biodegradation*, vol. 1, no. 1, pp. 1-13.
- PICCOLO, A., PIETRAMELLARA, G., and MBAGWU, J.S.C. 1997. Reduction in soil loss from erosion-susceptible soils amended with humic substances from oxidized coal. *Soil Technology*, vol. 10, no. 3, pp. 235-245.
- PONE, J.D., HEIN, K.A., STRACHER, G.B., ANNEGARN, H.J., FINKELEMAN, R.B., BLAKE, D.R., MCCORMACK, J.K., and SCHROEDER, P. 2007. The spontaneous combustion of coal and its by-products in the Witbank and Sasolburg coalfields of South Africa. *International Journal of Coal Geology*, vol. 72, no. 2, pp. 124-140.
- PROCHAZKA, J., BROM, J., STASTNY, J., and PECHAROVA, E. 2011. The impact of vegetation cover on temperature and humidity properties in the reclaimed area of a brown coal dump. *International Journal of Mining, Reclamation and Environment*, vol. 25, no. 4, pp. 350-366.

- RAYMENT, G.E. and HIGGINSON, F.R., 1992. Australian laboratory handbook of soil and water chemical methods. Melbourne, Inkata Press.
- 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*.
- SALT, D.E., SMITH, R.D. and RASKIN, I. 1998. Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology*, vol. 49, pp. 643-668
- SEKHOHOLA, L.M., IGBINIGIE, E.E., and COWAN, A.K. 2013. Biological degradation and solubilisation of coal. *Biodegradation*, vol. 24, no. 3, pp. 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*, vol. 78, no. 10, pp. 1797-1802.
- SINGH, A.N., RAGHUBANSHI, A.S., and SINGH, J.S. 2002. Plantations as a tool for mine spoil restoration. *Current Science*, vol. 82, no. 12, pp. 1436-1441.
- SKODRAS, G., KOKOROTSIKOS, P., and SERAFIDOU, M. 2014. Cation exchange capability and reactivity of low-rank coals and chars. *Central European Journal of Chemistry*, vol. 12, no. 1, pp. 33-43.
- SOURKOVA, M., FROUZ, J., and SANTRUCKOVA, H. 2005. Accumulation of carbon, nitrogen and phosphorus during soil formation on alder spoil heaps after brown-coal mining, near Sokolov (Czech Republic). *Geoderma*, vol. 124, no. 1-2, pp. 203-214.
- TAYLOR, L.L., LEAKE, J.R., QUIRK, J., HARDY, K., BANWART, S.A. and BEERLING, D.J. 2009. Biological weathering and the long term carbon cycle: integrating mycorrhizal evolution and function into the current paradigm. *Geobiology*, vol. 7, no. 2, pp. 171-191.
- VALDRIGHI, M.M., PERA, A., AGNOLUCCI, M., FRASSINETTI, S., LUNARDI, D. and VALLINI, G. 1996. Effects of compost-derived humic acids on vegetable biomass production and microbial growth within a plant (*Cichoriumintybus*) – soil system: comparative study. *Agriculture, Ecosystems and Environment*, vol. 58, no. 2-3, pp. 133-144.
- WILLMANN, G. and FAKOUSSA, R.M. 1997. Extracellular oxidative enzymes of coal-attacking fungi. *Fuel Processing Technology*, vol. 52, no. 1-3, pp. 27-41.
- YU, J., TAHMASEBI, A., HAN, Y., YIN, F., and LI, X. 2013. A review on water in low rank coals: the existence, interaction with coal structure and effects on coal utilization. *Fuel Processing Technology*, vol. 106, pp. 9-20.
- Zu, C., Li, Z., Yang, J., Yu, H., Sun, Y., Tang, H., Yost, R., and Wu, H. 2014. Acid soil is associated with reduced yield, root growth and nutrient uptake in blackpepper (*Piper nigrum* L.). *Agricultural Sciences*, vol. 5, pp. 466-473.

Figure Headings

Figure 1–The location of the study area in the Witbank coalfields of South Africa. Inset: Google Earth image showing position of the trial sites at Greenside Dump (Greenside Colliery), Klippan Dump (Kleinkopje Colliery) and Kromdraai Dump (Landau Colliery)

Figure 2–Average monthly rainfall from December 2010 to February 2014 in relation to trial establishment (arrows) and sampling intervals (*)

Figure 3–Time course illustrating the effect of cladding material on vegetation and cover in the presence of Fungcoal (100%; 75 kg/ha). (A) Klippan dump (Kleinkopje Colliery) without cladding; (B) Greenside dump (Greenside Colliery) with highly oxidized waste coal cladding; and, (C) Kromdraai opencast (Landau Colliery) with weathered coal cladding

Figure 4–Changes in pH and electrical conductivity (EC) of mine control (MC) and the coal dump sites treated with 0, 50 and 100% Fungcoal. (A) Klippan dump; (B) Greenside dump; and (C) Kromdraai opencast. Bars are standard errors and a different letter indicates significant difference at $P < 0.05$

Figure 5–Changes in humic substance content of mine control (MC) and the coal dump sites treated with 0, 50 and 100% Fungcoal. (A) Klippan dump; (B); Greenside dump; and (C) Kromdraai opencast. Bars are standard errors and a different letter indicates significant difference at $P < 0.05$

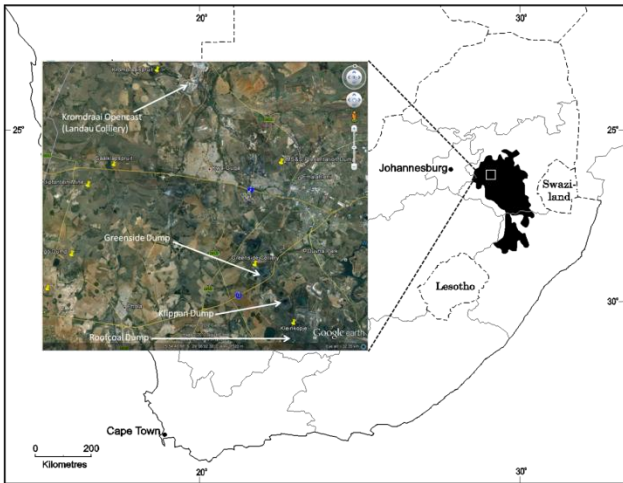


Fig 1

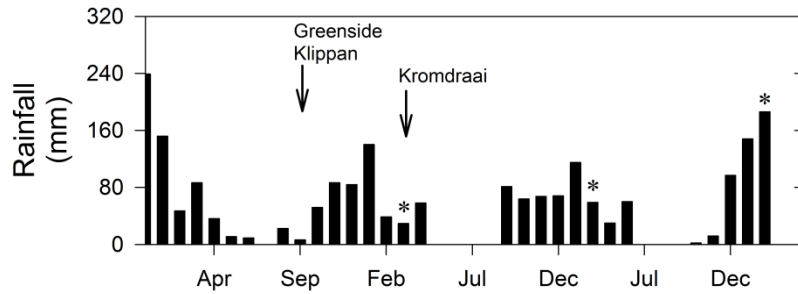


Fig 2



Fig 3

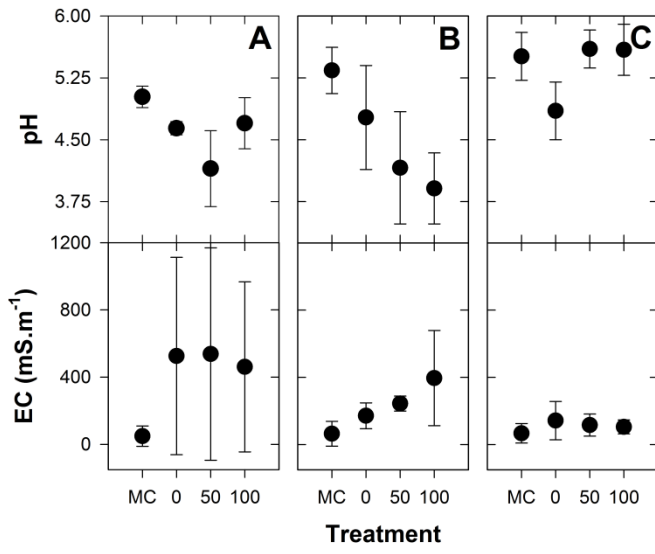


Fig 4

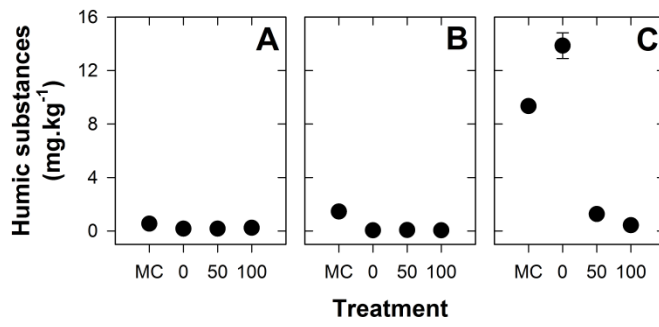
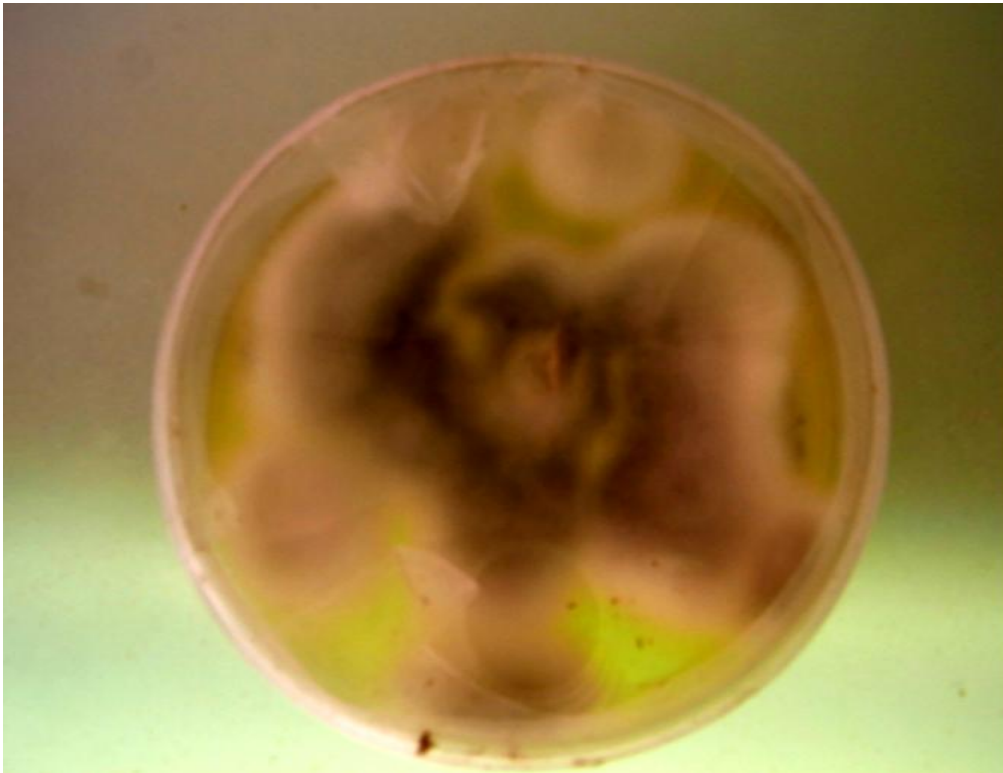


Fig 5

Supplementary Information

1. Preparation of fungal cultures and inoculation in liquid media (glutamate and trace elements supplemented basal salt medium)

Fungus ECCN strain 84 was cultured on potato dextrose agar and incubated at 30°C. Fungal spores were harvested into suspension by washing mature fungal lawns with sterile phosphate buffered saline before inoculation into basal salts medium supplemented with either trace elements or glutamate.



Spore formation of mature ECCN strain 84 culture growing on potato dextrose agar seen as black patches on the fungal lawn.

Basal salt medium preparation

Basal salts	Added amount (g/L)
K_2HPO_4	1.71
KH_2PO_4	1.32
NH_4Cl	1.26
$MgSO_4 \cdot 6H_2O$	0.011
$CaCl_2$	0.02

Trace elements solution

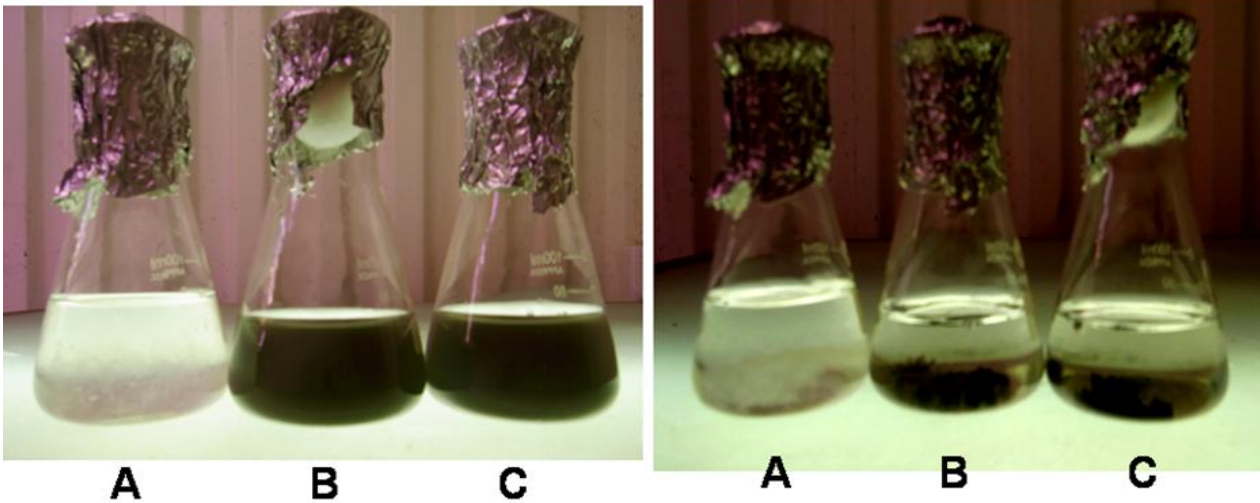
Mineral salt	Added amount (mg/L)
NaCl	10.0
MnSO ₄ ·H ₂ O	5.0
FeSO ₄ ·H ₂ O	5.0
CaCl ₂	1.0
CoCl ₂	1.0
ZnSO ₄ ·7H ₂ O	1.0
NaMoO ₄ ·2H ₂ O	0.9
AlK(SO ₄)·12H ₂ O	0.8
CuSO ₄ ·5H ₂ O	0.1
H ₃ BO ₃	0.1

4 mL trace element solution was mixed with basal salt medium and sterilised by autoclaving at 121°C for 15 minutes then allowed to cool at room temperature before inoculating with fungal spores.

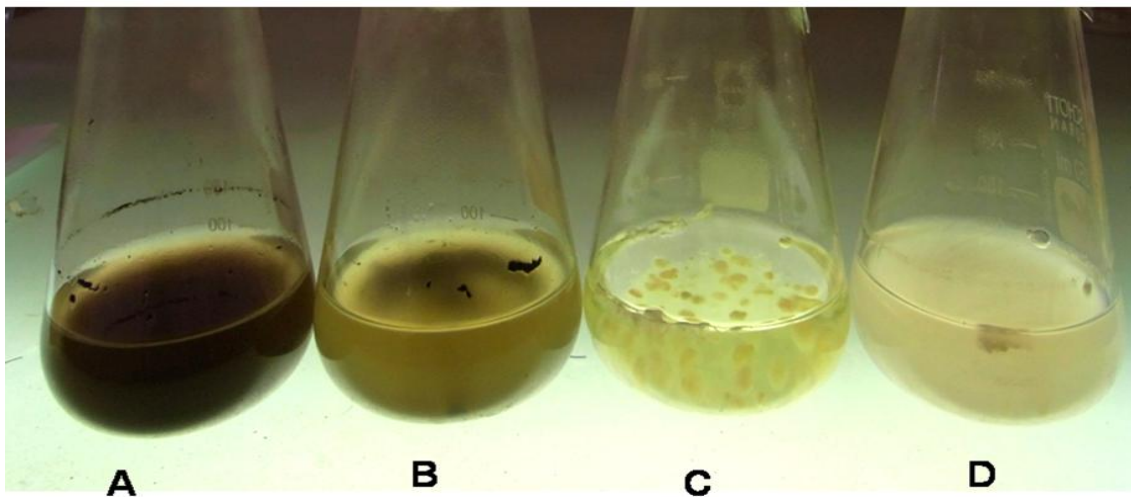
Glutamate supplemented basal salt medium preparation

Content	Added amount (g/L)
KH ₂ PO ₄	12.7
NaNO ₃	3.0
K ₂ HPO ₄	3.1
MgSO ₄ ·7H ₂ O	0.5
KCl	0.5
Glutamate	2.0

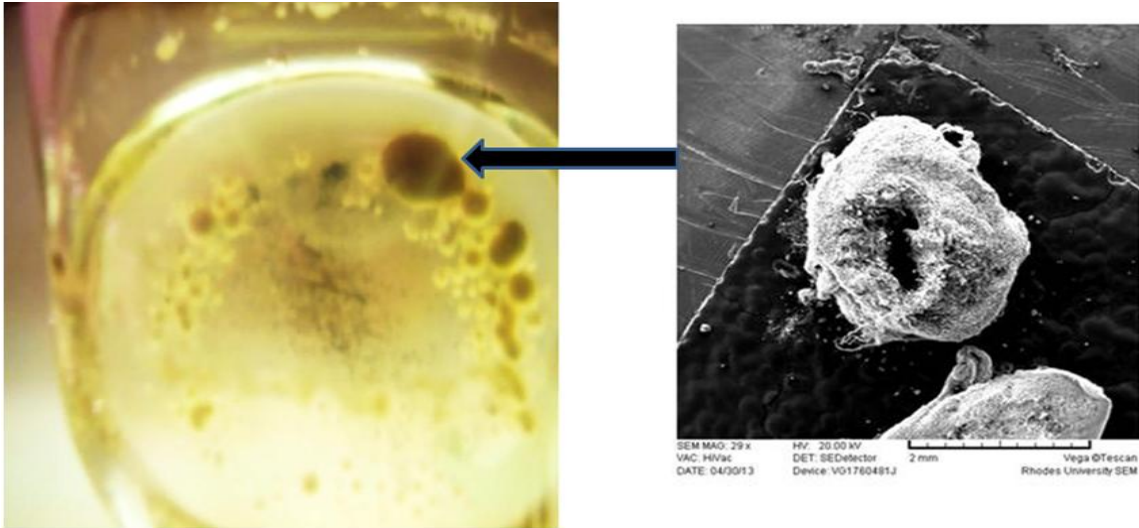
Media was sterilised by autoclaving at 121°C for 15 minutes, allowed to cool at room temperature and adjusted to pH 6-6.5 before inoculating with fungal spores.



Flasks containing glutamate supplemented basal salt medium inoculated with ECCN strain 84 supplied with glucose (A) and waste coal (B and C) before 24 hours of incubation at 30°C (left) and after (right).



Flasks containing glutamate supplemented basal salt media with; waste coal (A), waste coal inoculated with ECCN strain 84 (B), glucose inoculated with ECCN strain 84 (C) and no coal nor fungus after 24 hours of incubation at 30°C.

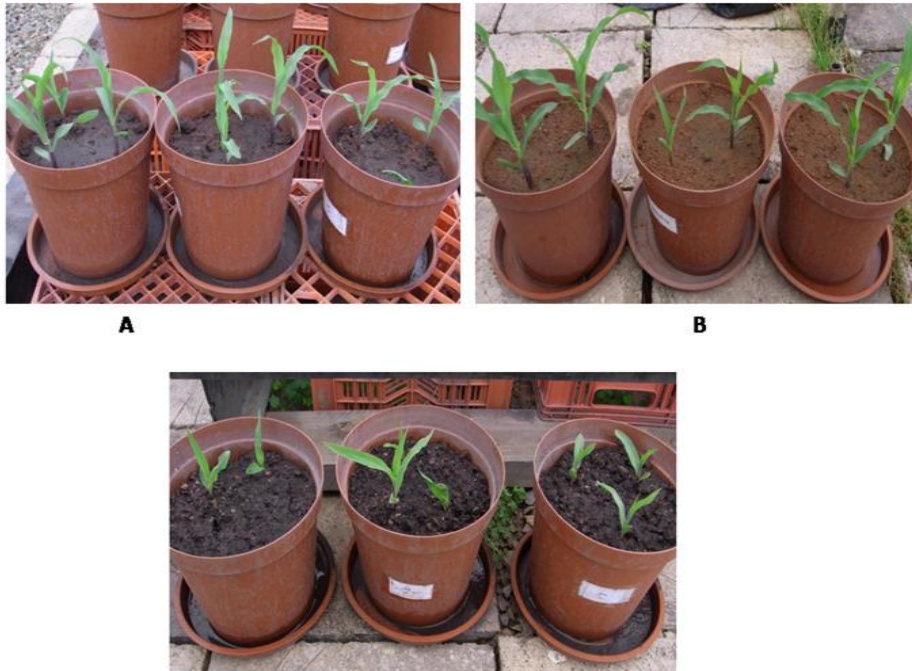


ECCN strain 84 growing and “engulfing” coal particles in glutamate supplemented basal salt medium forming sclerotia-like fungal pellets (left) and scanning electron micrograph of the fungal pellet open (dark spot at the centre) to remove engulfed coal particles (right).

2 Crop seedling establishment on waste coal substrates

Seedling establishment on top soil, waste coal, waste coal/ top soil mix, as well as waste coal and waste coal/ top soil mix remediated substrates.

Plastic pots (20 cm caliber diameter × 18 cm height × 16 cm bottom diameter) were filled with 2 kg of either fresh substrate or substrate previously used to grow *Cynodon dactylon*. The substrate was irrigated and sown with seeds of *Zea mays* (maize), *Spinacia oleraceae* (spinach), *Phaseolus vulgaris* (bean), and *Pisum sativum* (pea).



Maize seedlings growing on waste coal (A), top soil (B) and waste coal/ top soil mix (C).



A

B



Bean and pea seedlings growing on waste coal (A) and waste coal/ top soil mix (C). No seedling growth was observed on top soil (B).



A



B



C

Spinach seedlings growing on top soil (B) and waste coal/ top soil mix (C). No seedling growth was observed on waste coal (A).



A



B

Maize seedlings growing on previously used waste coal (A) and waste coal/ top soil mix (B).



A



B

Bean and pea seedlings growing on previously used waste coal (A) and waste coal/ top soil mix (B).

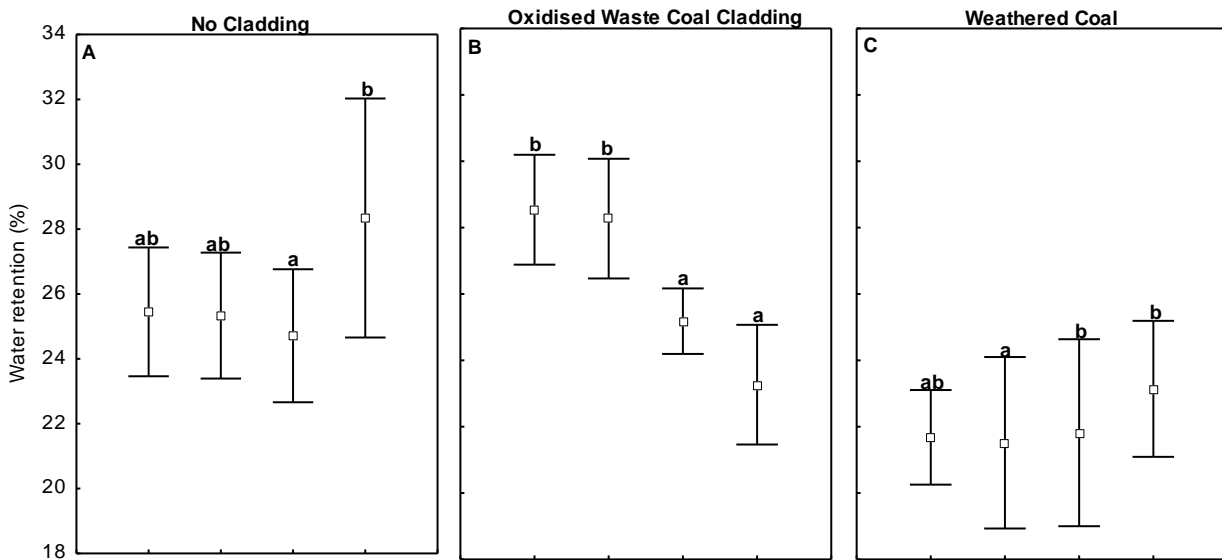
Cynodon dactylon grass establishment on waste coal

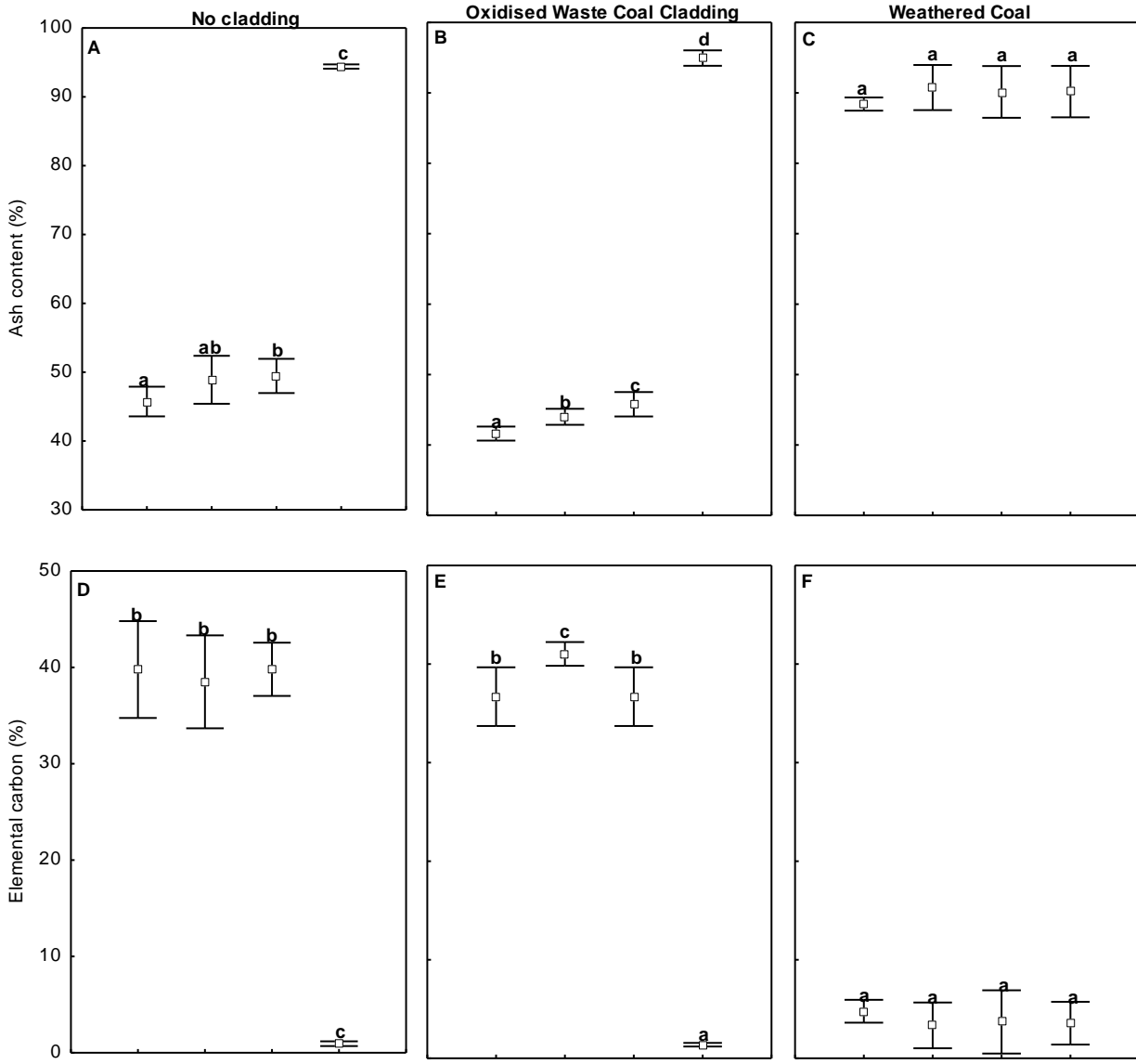


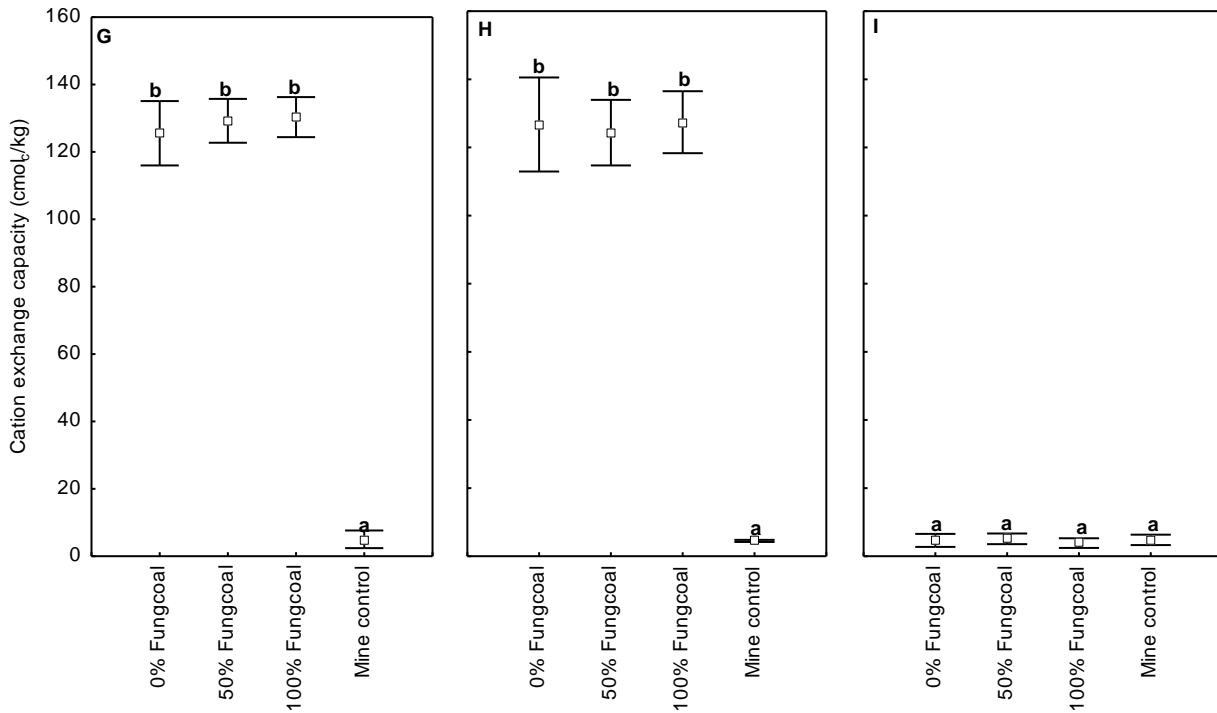
Cynodon dactylon grass growing on fresh waste coal substrate

3 Physico-chemical changes in soil and waste coal substrates obtained from large scale trials on the coal dumps

- Coal and soil samples were obtained from the plots by complete randomized design as outlined in Appendix 4. Prior to analysis, the soil and coal samples were dried at 50°C for 24 h to a constant weight and pulverized
- Moisture content and cation exchange capacity (CEC) were determined as described by Rayment and Higginson (1992) and Ross (1995) respectively.
- For determination of ash content, soil and coal samples (1 g) were combusted at 900°C for 5 h using a Carbolite muffle furnace and the residue expressed as weight percentage of the sample.
- Elemental carbon was determined using an elemental analyzer (Vario MICRO cube V1.6.2) according to the manufacturer's specifications.







Changes in water retention, ash content, elemental carbon, and cation exchange capacity of mine control and the coal dump sites treated with 0, 50 and 100% Fungcoal at Klippan dump (without cladding); Greenside dump (with highly oxidized waste coal cladding); and, Kromdraai opencast (with weathered coal cladding). Bars are standard errors and a different letter indicates significant difference at $P < 0.05$.