BACTERIAL COLONISATION AND DEGRADATION OF GEOLOGICALLY WEATHERED AND DISCARD COAL

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JACOB TAIWO OLAWALE

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Abstract

Bacterial beneficiation of low-grade coal, coal discard, and waste has the potential to mitigate land degradation, water and soil pollution and, be a strategy for mining companies to responsibly extract and process coal with environmental sustainability. This study investigated the colonisation and biodegradation or depolymerisation of coal discard and geologically weathered coal by selected strains of bacteria, and an attempt has been made to describe the mechanisms associated with colonisation and biodegradation of this carbonaceous material. Ten bacterial strains, *Bacillus* strain ECCN 18b, *Citrobacter* strain ECCN 19b, *Proteus* strain ECCN 20b, *Exiguobacterium* strain ECCN 21b, *Microbacterium* strain ECCN 22b, *Proteus* strain ECCN 23b, *Serratia* strain ECCN 24b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b and *Bacillus* strain ECCN 41b, isolated from diesel-contaminated soil and coal slurry and identified using DNA sequencing, were rescreened and their coal biodegradation potential ranked. The ranking of the bacterial strains was undertaken using several indicators including; formation of brown halos on the plate culture (solid), change in colour intensity of the medium in liquid culture, change in culture media pH, and an increase in absorbance at 280nm and 450nm. Although, all the ten strains showed evidence of biodegradation of coal discard and geologically weathered coal based on the ranking employed, and the three strains considered the best candidates were *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. The actions of the three bacterial strains were further studied and characterised in relation to coal degradation. Electron microscopy revealed that *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b attached to the surface of coal discard and geologically weathered coal by a process that appeared to involve extracellular polymeric substances (EPS), and flagella. The presence of flagella for *Citrobacter* strain ECCN 19b and *Serratia* strain ECCN 24b was confirmed by transmission electron microscopy. Bacterial degradation of coal discard and geologically weathered coal by these selected strains resulted in the release of soluble and insoluble products. Ultraviolet/ visible spectrophotometric (UV/VIS) analysis revealed that the soluble products resembled humic acid-like substances, which was confirmed following Fourier Transform Infrared (FTIR) spectroscopy. Analysis revealed that the coal-derived humic acid-like substances were similar to commercial humic acid extracted from bituminous coal. Elemental analysis of the insoluble product residue after bacterial biodegradation revealed the modification of the chemical compositions of the coal discard and geologically weathered coal

substrates. Characterisation of the functional groups of the insoluble product using FTIR spectroscopy indicated changes, with the appearance of new peaks at 1737cm^{-1}, 1366cm^{-1}, 1228cm^{-1}, and 1216cm^{-1} characteristic of aldehyde, ketones, carboxylic acids, esters, amines, and alkanes. Broad spectra regions of 3500 −3200cm^{-1}, characteristic of alcohol and phenol, were also observed. Together, these results were taken as evidence for increased oxidation of the coal substrates, presumably as a consequence of bacterial catalysed biodegradation of coal discard and geologically weathered coal. During bacterial degradation of coal discard and geologically weathered coal, strains produced extracellular protein, which was detected and further investigated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). At least three protein bands with molecular mass 53 kDa, 72 kDa, and 82 kDa were common to the three bacterial strains. Following ammonium sulphate precipitation and gel filtration chromatography, additional bands with molecular mass 16 kDa, 33 kDa, 37 kDa, and 43 kDa were detected. An extracellular laccase activity was detected in cultures of *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. Cytochrome P450 activity was detected in all the bacterial strains in the presence of both coal discard and geologically weathered coal. This is the first time that cytochrome P450 activity has been reported following exposure of these three bacterial strains to a coal substrate. Overall, this research has successfully demonstrated the partial degradation of coal discard and geologically weathered coal by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b and the release of humic acid-like substances. Thus, the biodegradation process involved adherence to and growth of the bacteria on the surface of coal substrate and appeared to require the formation of alkaline substances and the combined activities of extracellular LAC and cytochrome P450. Since bacterial degradation of low-grade coal and discard appears to be viable, the bacteria isolated in this study can potentially be used either for conversion of discard into valuable chemicals or to mitigate the deleterious effects of stockpiled coal discard on the environment.

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List of abbreviations

BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Units
CHNS	Carbon, Hydrogen, Nitrogen and Sulphur
CPR	Cytochrome P450 Reductase
CYT	Cytochrome
DNA	Deoxyribonucleic acid
DPA	Diphenylamine
EA	Enzyme Activity
EBRU	Institute for Environmental Biotechnology, Rhodes University.
EC	Electrical conductivity
ECCN	EBRU Culture Collection Number
EPS	Extracellular polymeric substances
FTIR	Fourier Transform Infrared
G6P	Glucose-6-phosphate
HMDS	Hexamethyldisilazane
kDa	kiloDalton
LAC	Laccase
LB	Luria broth
LiP	Lignin Peroxidase
LPS	Lipopolysaccharide
MnP	Manganese Peroxidase
MSM	Mineral salt medium
NA	Nutrient agar
NADPH	Nicotinamide adenine diphosphate
NCBI	National Centre for Biotechnology Information
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SE	Standard error
SEM	Scanning Electron Microscopy

TEM	Transmission Electron Microscopy
TES	Trace element solution
UV-VIS	Ultraviolet Visible Spectrophotometer

Dedication

This research work is dedicated to my late wife; OLAWALE, Toyin Florence who left this sinful world on 30 May, 2013. May her gentle soul rest in perfect peace. She would have preferred to be alive to witness this success and I guess I owe her a lot. God knows the best and Glory be to him in the highest. Still love you, MUMMY TOLU.

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CHAPTER 1

General Introduction

Most of the word's energy is derived from non-renewable fossil fuel resources including oil, oil shales, tar sands, coal and natural gas (Finnerty, 1992). After crude oil, coal is next in world primary energy consumption, at 30% in 2010 (Thielemann and Schiffer, 2012). Of the fossil fuels, coal is the most important, and thus, a basic energy source as well as a raw material for chemical industries. Not only is it an important energy source that contributes 41% to total world electricity generation (Smuts, 2010), it is also a vital resource that can transform a country technologically and act as a substitute for imported sources of energy (Akpabio *et al.*, 2008). Electricity, apart from providing health benefits worldwide, is a significant contributor to economic development, a higher standard of living, and increased life expectancy (Markanya and Wilkinan, 2007). The nations that possess the largest coal reserves are the United States of America, with reserves of 108950Mt, China (62200Mt), India (54000Mt), Russia (49088Mt), Australia (36800Mt) and South Africa (30408Mt) (DMR, 2009). In South Africa, coal is found as far south as Molteno, but is concentrated in four major coal-producing belts, namely, the Mpumalanga Province around the towns of Emalahleni and Ermelo, in the North West Province near Lephalale, the Free State Province, northern Free State near the town of Sasolburg, and in the KwaZulu-Natal Province stretching from the town of Newcastle to Vryheid (Morgenthau, 2003). Figure 1 shows the distribution of coal producing areas in South Africa.

Figure 1.1 South Africa coal production areas. Notable coalfields are Witbank, Highveld, Ermelo and Waterberg located in the provinces (coloured black). These represent about 85% of coal production in South Africa

Five mining groups control and operate these coal belts: Anglo American Coal, BHP Billiton, Exxaro Resources, Sasol, Glencore plc. Remaining production is carried out by smaller mining companies (junior miners) that are largely Black Economic Empowerment (BEE) enterprises (Mining Weekly, 2010). About 75% of coal produced in South Africa is either used for electricity generation by the national electricity supplier Eskom, or by Sasol for liquid fuels production (DMR, 2009). Currently, coal provides 73% of South Africa's primary energy: 95% of electricity is from coal-fired thermal generation, while coal conversion technology by Sasol provides half of the liquid fuel requirement (Jeffery, 2005). As a result of the modernisation of agriculture, the rapid growth in chemical industries, and the need to generate cheap forms of energy, much pressure is now on coal extraction and utilisation in coal-producing countries of the world, although China and Europe have adopted Renewable Energy Sources (RES) such as wind and solar power. Renewable energy sources mitigate greenhouse gas (GHG) emission, lower environmental pressures associated with conventional energy production, and reduce the reliance on fossil fuel, such as coal, for energy generation (EEA, 2016). Today, coal is primarily used for producing electricity and, to a lesser extent, by the iron and steel industries for smelting (Raven *et al.*, 1993).

Coal is a combustible, sedimentary, organic rock, composed of carbon (C), hydrogen (H), and oxygen (O). In addition to C and H, coal contains the heteroatoms of O, nitrogen (N), and sulphur (S). The heteroatom content varies greatly from coal to coal although O predominates. A considerable proportion of the O in coal exists as phenolic hydroxyl groups with other structures such as ether linkages also known to exist. The N and S are generally present mainly in heterocyclic form. The youngest coals (lignites) have the highest heteroatom content (mainly O) and the lowest C contents.

Coal can be found in four basic forms: lignite, sub-bituminous, bituminous, and anthracite. Lignite is formed from vegetation, which has been consolidated between other rock strata and altered by the combined effects of pressure and heat over millions of years to form coal seams. Coal is a highly complex mineral in nature, due to variations in vegetation origin and subsequent bio- and geochemical transformations that are reflected by the content and constitution of organic and inorganic matter (Neavel, 1981). Lignites are known as brown coals while the remaining coal types namely, sub-bituminous, bituminous, and anthracites are known collectively as hard coals or black coals. Lignite has a more complex structure than hard coal

because it consists of several distinct compound classes; the mainly hydrophobic bitumen, the alkali soluble humic and fulvic acids and an insoluble residue, the matrix or humine (Fakoussa and Hofrichter, 1999).

The formation of coal starts with dehydration, which results in the conversion of wood, cellulose, lignin, and peat to lignite. Decarboxylation is the most important reaction in the conversion of lignite to bituminous coal, and demethanation converts bituminous coal to anthracite coal. These coal ranks are determined by the proportionate amounts of various constituents of coal such as moisture, volatile matter, fixed carbon and ash. Lignite and sub-bituminous coals are low-rank coals and are softer, more friable materials with a dull, earthy appearance. They are characterised by high moisture and low C contents and therefore a low energy content.

Higher rank coals are generally harder and stronger and often have a black vitreous luster. They contain more C, have a lower moisture content, and more energy. Anthracite, at the top of the rank scale, has a correspondingly higher C and energy content with a lower level of moisture. The stages in the formation of coal from vegetable/plant (wood) matter are as follows: plant debris (wood) → peat → lignite → brown coal → sub-bituminous coal → bituminous coal → semi-anthracite → anthracite coal → graphite (Gupta, 2008). Other categories that are present during coal formation, but not generally grouped amongst the coal ranks are peat, formed before lignite, and graphite, which succeeds anthracite (Haenel, 1991).

The coal rank is used to predict many important technological properties of coal, including the behaviour of coal in cooking, combustion, liquefaction and gasification. The organic structure of coal is made up of heterogeneous aromatic structures, with aromaticity increasing from low to high-rank coals, i.e. lignite to anthracite (Balachandran, 2014). Different modes of degradation exist within the four categories and the major difference among them is a function of pressure. It has long been known that some coals are more susceptible to degradation than others by abiotic and biotic factors. For example, lignite and sub-bituminous coal are known to be degraded more easily by weathering and microorganisms than bituminous coal and anthracite (Strapoc *et al.*, 2011: Furmann *et al.*, 2013). Thus, two major ways to convert coal into value-added products exist: long-term oxidation by environmental factors, and biotechnological conversion by microbial and other biological processes.

Both these processes bring about the degradation of coal and contribute to environmental problems prevalent in coal mining areas such as acid mine water production, and air and ground water pollution. Microorganisms play a pivotal role in converting recalcitrant materials such as coal, and are therefore potential candidates for use in bioremediation. The key to the success of microorganisms is the immense biochemical and physiological diversity among species and an ability to adapt to changes in the environment. Studies have reported the biological degradation, solubilisation or transformation of various coal ranks by both bacteria and fungi (Hofrichter and Fakoussa, 2001 and Silva-Stenico, *et al.*, 2007).

Biological degradation, or biodegradation, is defined as the biologically catalysed reduction in the complexity of chemical compounds of a substance (Alexander, 1994). For coal, the rate of biodegradation depends on biological and physicochemical factors, such as the type of microorganism community, soil pH, oxygen, nutrient availability, temperature and water availability (Mannisto *et al.*, 2001). The products of coal biodegradation are believed to include humic and fulvic-like substances (Valero *et al.*, 2014; Park and Kim, 2015) of which humic and fulvic acids are the principal components. These are the major organic constituents of soil (humus), peat, and coal, and are commonly used as a soil supplement in agriculture, and less commonly, as a human nutritional supplement.

An investigation into the fungal biodegradation of coal at the Institute for Environmental Biotechnology, Rhodes University, Grahamstown, South Africa, focused on the isolation of the fungus, *Neosartorya fischeri,* from waste coal dumps, which was subsequently shown to biodegrade weathered, hard coal to humic substances (Igbinigie *et al.*, 2008; 2010).

More recently, it was shown that this fungus readily colonises and degrades coal and that enzyme-mediated oxidation appeared to coincide with increased extracellular laccase activity (Sekhohola *et al.*, 2014). Full-scale commercial trials using *Neosartorya fischeri* in combination with *Arbuscular mycorrhizal* fungi and the grass, *Cynodon dactylon*, confirmed in-situ phyto-biodegradation of coal on coal discard dumps (Cowan *et al.*, 2016). In addition to coal-degrading fungi, a consortium of bacterial strains isolated from diesel-contaminated soil and coal slurry was shown to degrade waste coal to form humic substances (Edeki, 2015). The

research described in this thesis examines in more detail the bacterial colonisation, and bioconversion of geologically weathered coal and coal discard.

1.1 Coal and the environment

Pollution has been one of the major concerns of environmentalists and the general public in recent years (Rivilla *et al.,* 2009). South Africa is confronted with contamination of soils, water resources, and air by hazardous and toxic chemicals released during coal mining, processing, transportation and use. There is growing concern about the danger to human health and the ecosystem in general caused by mining and using coal. As a result of this closer scrutiny, the coal industry has had to address numerous environmental issues such as land degradation, soil erosion, soil depletion, and land escapement. Until 2002, the regulation and management of environmental impacts in South Africa, was governed by the Minerals Act no. 50 of 1991 (South Africa, 1991). This act enjoined prospecting mining companies to submit an Environmental Management Programme (EMP) containing baseline information, impact assessments, and mitigation measures for each stage during the commissioning, operating and decommissioning of the mine.

It is undeniable that mining and the use of coal have pronounced effects on the environment. Coal mining raises a number of environmental challenges such as soil erosion, dust, noise and water pollution, and impacts on local biodiversity**.** The major source of water pollution is the carry-over of suspended solids into the drainage system (Banerjee, 1980). Mining operations, such as drilling, blasting, movement of the heavy earth-moving machinery on haul roads, collection, transportation and handling of coal, and the screening, sizing and segregation units are some of the major sources of such emissions (Jamal and Siddhartha, 2003; Mishra, 1990). Mining activities have differing degrees of environmental impact such as gaseous emission, mine drainage, and the generation of large volumes of solid waste (Vitalis and Kaliampakos, 2006). Handling, transport, and processing coal cause pollution of the plant sites by some xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAH), benzene, toluene, ethylbenzene and xylene (BTEX), phenols, cyanides and heavy metals (Klein *et al.*, 1997).

Coal mining also has a visual impact on the landscape and leads to the disturbance or destruction of natural habitats, sometimes resulting in a loss of biodiversity (Sengupta, 1993; Azapagic, 2004). Mining activities also create extensive geomorphological disturbances such as the

destruction of the geological continuity of the ground surface, soil pollution, hydrological effects on the runoff capacity, and morphological changes of the landscape (Kordas, 1997; IGME, 2001; Foscolos, 2001). Furthermore, coal mining disturbs large areas of land, resulting in organic and inorganic pollution, hydrocarbon contamination, soil erosion, dust, water pollution, and reductions in local biodiversity (Bench, 2010; Bhuiyan *et al.*, 2010). Dust is the major air pollutant in opencast coal mining areas (CMRI, 1998; Vallack and Shillito, 1998). Ninety-three percent (93.3%) of total emissions from coal mines is as a result of dust generated from haul roads of South African coal mines (Amponsah-Dacosta, 1997).

Mining activities generate large amounts of waste materials and tailings that are deposited at the surface in the form of spoil and discard dumps (Juwarkar *et al.*, 2009). Spoil and discard are nutritionally deprived substrates that are characterised as unfertile, having extreme pH values, low cation exchange capacity, low water-holding capacity, low nutrient availability, and are either poor organic matter or lack microbial activity (Gonzalez- Sangregorio *et al.*, 1991). The environmental impact of coal is also very much associated with greenhouse gas (GHG) emission, including carbon dioxide (CO_2) and methane (CH_4) and this has become a concern because of a link to climate change. It is important to note that methane gas generation has gone up to a rate of 0.2 ppm/year (Zeller *et al.*, 1999). Methane emissions from coal mining depend on the mining methods, depth of coal mining, coal quality and entrapped gas content in the coal seams (Chand, 2001).

An investigation by the National Renewable Energy Laboratory estimated the releases of about 1.9 -4.23 g of methane by surface and underground mining activities respectively (Pamela *et al.*, 1999).

Climate change is predicted to lead to the extinction of numerous species on Earth (Thomas *et al.*, 2004) as it has always done and will continue to do. Climate is not static. It is a dynamic entity that changes continuously. According to the Intergovernmental Panel on Climate Change, methane gas has a global warming potential twenty-one times greater than that of CO_2. It is, therefore, reasonable to state here that all plants need CO_2 and most grow well when CO_2 levels are high but to a certain limit. Indeed, plants evolved on this planet at a time when there was substantial CO_2 and little or no O_2. Methane, on the other hand, is a gas that can be utilised as a fuel source (Sudheer *et al.*, 2016). South Africa is among the highest emitters of CO_2 in the

world and burning of coal is one of the primary sources of these emissions (Mukherjee *et al.*, 2010). The perceived negative impact of coal mining and combustion on the environment has forced the implementation of so-called 'clean' coal technologies (Gee *et al.*, 2014), whose aim it is to reduce the environmental impact of coal mining and coal consumption (World Coal Institute, 2008; Schlapfer, 2009). According to Muntambagwe (2009), clean coal technology has developed in three major areas, namely, (1) reduction in coal waste, (2) increased energy value and (3) production of cleaner fuels and value-added products.

1.2 Characterisation of coal

Characterising coals requires the application of multiple analytical techniques to derive various parameters of interest (Karr, 1978). The characterisation of coal provides information about ash content, moisture, volatile matter, minerals, C, H, N, S, and trace elements, among others. This information might help in estimating atmospheric emissions of S, C and N and the contribution of these elements to environmental problems, such as acid rain and global climate change (Finkelman and Gross, 1999). Even so, coal is extremely difficult to characterise; the organic fraction varies based on the starting plant material, the conditions of decomposition, and the physical and chemical changes that occur during the process of coalification.

The chemical and structural characteristics of coal depend on the coal rank, and the organic fraction of coal consists of a complex mixture of aromatic and aliphatic hydrocarbons as well as N, S, and O_2-containing heterocyclic compounds (Kabe *et al.*, 2004). Coal is thought to consist of a large polymeric matrix of aromatic structures commonly called the macromolecule. This macromolecule network consists of clusters of aromatic carbon that are linked to other aromatic structures by bridges. Bridges between the aromatic clusters are formed from a wide variety of structures. Most bridges are thought to be aliphatic in nature, but may also include other atoms such as O and S. There are other attachments to the aromatic clusters that do not form bridges. These attachments are referred to as side chains and are thought to consist of aliphatic and O functional groups (Hambly, 1998).

A number of methods are used to classify coal, based on variations in chemical composition and physical properties; they include factors such as increasing C content, decreasing moisture content, elemental analysis, and atomic ratio distribution (Levandowski and Kalkreuth, 2009). Physical properties in coal vary with coal rank as may be predicted from the C or volatile

content of coal. Properties such as porosity, density, and surface area pass through a more or less well-defined maximum or minimum, usually between 85% and 89% C, and thereby reflect the rank-dependent surface-to-volume ratio of coal, or the transition from bituminous to anthracitic coal (Sharkey and McCartney, 1981).

Coals are heterogeneous in nature and consist of exceedingly complex arrangements of organic and inorganic materials. The organic portion of coal is composed of C, O, H, N, and S. The C content is 65 --95% and increases during coalification with a concomitant decrease in the percentages of O and H, which typically range between 2 --30% and 2 --7%, respectively. Coal is a reservoir of highly reduced C compounds and made up of aromatic rings fused into different smaller polycyclic clusters that are linked by aliphatic structures. The aromatic rings comprise phenolic, hydroxyl, quinone, and methyl substituents. Coal contains a variety of S compounds. Nitrogen (N) and S are minor organic components and usually occur in concentrations of less than 2% by mass (Glick and Davis, 1991). The N in the macromolecular organic network of coal most likely originates from plant debris, microbial residues, and algal remains. The S in coal exists in inorganic and organic forms (Karr, 1978).

The major inorganic S compound in coal is pyrite, while organic S compounds are diverse and contain mainly thiol, sulphide, disulphide, and thiophene rings. Sulphur gasses in the form of SO_2 emitted into the atmosphere during combustion have a detrimental effect on ecosystems. Oxygen is mainly contained in the hydroxyl (-OH) form, but also with some ethers, carboxyl, and carbonyl groups. Nitrogen appears mainly as pyrole and pyridines. The macromolecular entities in coal are probably composed of subunits, some of which may themselves be released more or less intact upon mild dissolution or by other chemical treatments, which only affect the weakest bonds.

1.3 Biological/ biotechnological aspect of clean coal technology

The development of processes to convert coal to energy through biological mechanisms is one of the promising technologies for cleaner coal utilisation (Sudheer *et al.*, 2016). The principal product of the microbial oxidative activity against various lignite and bituminous coals is generally characterised as a heterogeneous, high molecular weight, acid-precipitated, polar material that is partially oxidised and has high oxygen content (Gupta and Birendra, 2000). Thus, bioconversion of coal results in the production of value-added products such as humic

acid, nitrohumic acid and methane. Methane gas can be utilised as a fuel source, while humic acid helps in boosting agricultural poduction.

1.3.1 Coal to methane

Coal can be broken down into simple methane precursors by complex, anaerobic communities of fermentative bacteria, acetogenic bacteria, and other microorganisms (Ferry, 2011). Under strictly anaerobic conditions, coals are degraded stepwise to methane, and CO_2 via a syntrophic interaction of fermentative and methanogenic bacteria. Complex aromatic hydrocarbons in coal such as benzene, toluene, and ethylbenzene degraded by aerobic organisms (Huang *et al.*, 2013), can be used to evaluate the biological potential of coal-derived substrates, aerobically (Aneela *et al.*, 2016). Anaerobic degradation is very slow and not able to degrade all compounds, and the possibility of toxic product(s) creation is high (Prince, 1993; Benoit and Love, 1996). The solubilisation of lignite (low-rank coal) and subsequent breakdown into low molecular weight aromatic and aliphatic compounds can be an indirect option for extracting some material capable of fermentation by anaerobic microorganisms (Gokcay *et al.*, 2001).

However, in all models of microbial production of methane from coal, the rate-limiting steps involves the solubilisation and degradation of coal to substrates that can be utilised by methanogens for methane generation (Strapoc *et al.*, 2011). The amount of methane produced directly relates to coal rank (lignite, sub-bituminous coal, and bituminous coal) (Harris *et al.*, 2008; Jones *et al.*, 2008; Jones *et al.*, 2010). As a result of its complex structure, coal is slowly and only partially biodegraded to methane. At present, a substantial amount of methane is released into the atmosphere as a result of energy harvesting and utilisation. Global methane emission is of concern to environmentalists because it has greater global warming potential than CO_2; it is the second most common greenhouse gas emitted into the atmosphere.

Capturing methane from coal mines prevents it from being vented into the atmosphere and allows the energy to be used to generate electricity. Mechanisms by which bacteria degrade coal to methanogenic substrates and finally into methane for biogenic generation are not completely understood, though in recent years there has been a focus on developing some models for metabolic pathways involved in the biodegradation of coal to methane (Strapoc *et al.*, 2008; Jones *et al.*, 2010). Biogenic processes occur when the organic matter that was once deeply buried and transformed into coal has subsequently undergone uplift and cooling, making

biogenic methane production a favourable process (Faiz and Hendry, 2006). Biogenic process generates 20% methane on the planet particularly when microbes catalyse the degradative process (Rice and Claypool, 1981). Microbial methane production from coal is a natural process that contributes up to 30% of the coalbed methane that is produced worldwide (Srapoc *et al.*, 2011). Methanogens are a group of obligate anaerobic archaebacteria capable of using a variety of simple organic matters to produce methane. According to Jones *et al* (2010), the process of forming biogenic methane from coal can be divided into three events: (i) the release of soluble organic intermediates from the coal geopolymer, (ii) the degradation of soluble intermediates into substrates utilisable by methanogens, and (iii) methanogenesis.

Microorganisms such as methanogenic archaea, which carry out the process of methanogenesis, are able to use coal as a sole carbon source to produce biogenic methane (Harris *et al.*, 2008; Jones *et al.*, 2010), generating methane from precursors such as acetate. In order to break down a complex substrate such as coal, a metabolically diverse microbial community is necessary to generate these precursors from the macromolecules that make up the coal structure (Gallagher *et al.*, 2013).

1.3.2 Coal to value-added products

Biologically derived value-added products from coal that are of biotechnological importance include humic substances which are complex and heterogeneous mixtures of polydisperse materials formed by biochemical and chemical reactions during the decay and transformation of plant and microbial remains in a process called humification. Humics are irregular polymers of aromatic polyhydroxy carbonic acids and comprise the major component of soil organic matter and sediments (Clapp *et al.*, 1993). The classical definition of humic acids is a category of naturally occurring heterogeneous organic substances that can generally be characterised as refractory, yellow to black in colour, and of high molecular weight (Aiken *et al.*, 1985). Humics contain carboxyl (-COOH), amine (-NH_2), and phenol (Ar-OH) functional groups, and are negatively charged in weakly acidic to basic media because of deprotonation (Evangelou *et al.*, 2002). The predominant functional groups in humic acids are carboxyl, phenolic hydroxyls and alcoholic hydroxyls (Shen, 1999), which affect reactivity. Therefore, their reactivity with organic and inorganic contaminants depends on structure.

Humic-like substances can be obtained by heating mineral coal at relatively low temperatures (180^0 C) followed by alkalisation with a process resembling an inversion of the natural diagenetic transformation (Bernacchi *et al.,* 1996). The compounds obtained reveal physical and chemical properties quite similar to those of natural humic and fulvic acids (Calemma and Rausa, 1987: Rausa *et al.,* 1989). Plant lignin and its transformation products, as well as polysaccharides, melanin, cutin, proteins, lipids, nucleic acids, fine char particles, etc., are important components in this process. Humic substances can be fractionated into fulvic acids, brown humic acids, grey humic acids, and humin, as a function of solubility and pH (Alvarez-Puebla and Garrido, 2005). Based on solubility in acids and alkalis, humic substances can be divided into three main fractions; humic acids which are soluble in alkali and insoluble in acid; fulvic acids which are soluble in alkali and acid; and humins which are insoluble in both alkali and acid (Grinhut *et al*., 2007).

Elemental analysis of humic acids, fulvic acid, and humins range from 40 to 50% C and 40 to 50% O for fulvic acid; 50 to 65% C and, 30 to 40% O for humic acid. The H, N and S content also ranges from 3 to 7%, 0.8 to 4.3% and 0.1 to 3.6%, respectively in all of the fractions (Stevenson, 1994). Although, humic acids are found in various environments such as soils, natural waters, rivers, lakes, sea sediments, plants, peat and compost, none of these resources are as abundant as low-grade coal. The low-grade coal recorded 40 —85% of humic substances compared to black peat (10 —40%), sapropel peat (10 —20%), brown coal (10 —30%), compost (2 —5%), soil and sludge (1 —5%) (Fong *et al*., 2006).

Humic acids are active components of soil and aquatic organic matter and are important to agriculture (Carthy *et al*., 1990). Humic acids are universally considered of great importance in determining extracellular enzyme activity and stability of soil via association with essential soil enzymes such as ureases, proteases, phosphatases, hydrolases, laccases, and peroxidases, which have been detected in soil extracts as complexes with humic substances (Masciandro and Ceccanti, 1999: Benitez *et al.,* 2005). Humic acids in coal depend on the degree of maturity (rank) of the coal, and the coalification process of coal formation. As with 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).

The release of humic substances is achieved in the laboratory by using oxidising agents such as nitric acid (Machnikowka *et al.,* 2002; Elbeyli *et al.,* 2006), and hydrogen peroxide (Hofrichter *et al.*, 1997; Holker *et al.,* 1997). Humic substances may be used to improve soil fertility, replenish water, and neutralise munitions, converting them into organic fertiliser. In fact, they are one of the main environmental factors controlling the fate and behaviour of deleterious effects of metals on plant growth (Scnitzer, 1986; Kulikova *et al.*, 2013), improving plant health, and providing resistance to pathogen attack (Schisler and Linderman, 1989). Humic substances modify cellular activity through stimulation of oxygen transport, accelerate respiration, and promote efficient utilisation of nutrients in plants (Visser, 1985; Islam *et al.*, 2005). The use of humics, in combination with inorganic fertilisers, to maximise plant nutrient uptake and yield, may also have a tremendous impact on increasing the economic efficiency of fertilisers and protecting the environment from pollution caused by excessive use of fertilisers (Piccolo, 2001).

1.4 Biological degradation of coal

There has been substantial interest in the biological process of converting coal to value-added, energy-related products. The basic principle of biological coal degradation relies solely on the use of microorganisms, as this method possesses a number of advantages over the use of chemical methods of coal breakdown, and it opens new possibilities of economical production of new coal-derived products (Tripathi *et al.*, 2010). Thus, biological coal conversion has received greater attention in recent years (Dong *et al.,* 2006). Microbial treatment of coal is a cost-effective and environmentally friendly way to produce value-added products (Ralph and Catcheside, 1994; Elbeyli *et al.*, 2006; Yuan *et al.*, 2006).

Because of its hydrophobicity, heterogeneity, and recalcitrance, the degradation of coal requires a community of microorganisms with a range of metabolic strategies (Strapoc *et al.*, 2011). Microorganisms interact with coal in different ways and these are solubilisation, depolymerisation and utilisation (Sudheer *et al.*, 2016). Coal solubilisation consists of a non-enzymatic breakdown of coal and occurs at an alkaline pH as a result of the release of alkaline materials by microorganisms on coal (Romanowska *et al.*, 2015).

Coal is considered a complex and biologically recalcitrant materials (Strapoc *et al.*, 2011), and thus susceptible to increased solubility and microbial degradation, first by pre-treatment using

nitric acid and strong bases (sodium hydroxide and peroxide) (Sudheer *et al.*, 2016). In addition, chemical oxidants such as potassium permanganate have been used for the pre-treatment of coal prior to microbial biosolubilisation (Huang *et al.*, 2013). However, many studies have been carried out on microbial degradation of coal without chemical oxidation (pre-treatment), using microbial oxidative and hydrolytic enzymes, alkaline substances, surfactants and chelators (Ghani *et al.*, 2015).

During the process of solubilisation, microorganisms first attack side chains of aromatic rings in the macromolecule of coal, followed by cleavage of crosslinks between aromatic rings (Aneela *et al.*, 2016). However, a *Bacillus* sp. was reported to break down coal by secreting an alkaline substance that resulted in an increase in pH as well as coal solubility (Yuan *et al.*, 2013). The depolymerisation of coal is also mediated by enzymes (Xiu-xiang *et al.*, 2009), and usually at pH below 6. Specific enzymes involved in depolymerisation of coal are chiefly extracellular oxidative enzymes, that is, peroxidases (MnP, LiP) and laccases (Sudheer *et al.*, 2016). These lignin-degrading enzymes have been investigated in the microbial conversion of coal (Fakoussa and Hofrichter, 1999). Recently, bacteria have received considerable attention for their coal biodegradation potential, which is attributed to the enzymes they produce. The utilisation, also known as chelation, involves microorganisms using components of the mobile part of coal as a carbon source (Hofrichter and Fakoussa, 2001).

1.4.1 Fungal degradation of coal

The role of fungi in the biological breakdown of coal has long been established (Cohen and Gabriel, 1982; Sekhohola *et al.*, 2014). Several authors have demonstrated the potential of mostly filamentous fungi, Basidiomycetes and Ascomycetes (Hofrichter *et al.*, 1999; Hofrichter and Fritsche, 1997) in coal biodegradation. Several species of these fungi can solubilise low-rank coals and convert them into complex and heterogenous products such as humic acid substances (Willmann and Fakoussaa, 1997; Ghani *et al.*, 2015). Basidiomycetes involved in coal degradation are: *Bjerkandera adusta* (Belcarze *et al.*, 2005; Huang *et al.*, 2013), *Trametes* sp. (Veith, 2004 ; Klein *et al.*, 2013; Klein *et al.*, 2014), *Phanerochaete chysosporium* (Gokcay *et al.*, 2001 ; Oboirient *et al.*, 2008; Malik *et al.*, 2016). Many Basidiomycetes, or white rot fungi, have non-specific oxidising enzymes such as Mn-dependent peroxidase (MnP, E.C.1.11.1.13), lignin peroxidase (LiP, E.C 1.11.1.14), laccases (LAC, E.C.1.10.3.2) (Ghani *et al.*, 2015) and produce alkaline metabolic products (Quigley *et al.*, 1989).

As a consequence, white rot fungi have been shown to be the most efficient degraders of humic substances using non-specific oxidising enzymes which react with a variety of aromatic substrates present in coal (Ghani *et al.,* 2015; Yuan *et al.*, 2016).

Ascomycetes, by contrast, biodegrade coal to release clean fuels and chemical feedstocks (Yuan *et al.*, 2006), using hydrolytic and ligninolytic enzymes for coal solubilisation (Holker *et al.,*1999). According to Ghani *et al* (2015), Ascomycetes secrete oxalate ions and phenol oxidase to biosolubilise coal substates. Ascomycetes includes; *Aspergillus* sp. (Acharya *et al.*, 2005), *Neosartorya fischeri* (Igbinigie *et al.*, 2008; Sekhohola *et al.*, 2016), *Penicillium* sp. (Jinshui *et al.*, 2004; Yin *et al.*, 2009; Malik *et al.*, 2016), *Fusarium* sp. (Ratin *et al.*, 2000; Etemadzadeh *et al.*, 2016), *Trichoderma* sp. (Oboirient *et al.*, 2008; Tao *et al.*, 2009 ; Malik *et al.*, 2016).

1.4.2 Bacterial degradation of coal

The use of bacteria for bioconversion of coal to useful, value-added products is an option in clean coal technology. Bacterial degradation of coal proceeds in either the presence or absence of molecular oxygen to yield value-added products such as humic acid and methane respectively. Bacteria have developed the ability to degrade different kinds of coal using a variety of mechanisms, i.e. enzymatic and non-enzymatic mechanisms such as chelation, alkaline production and production of surfactants (Hofrichter and Fakoussa, 2004). Some filamentous bacteria, including member of Actinomycetes and occasionally Eubacteria, have been implicated in coal bioconversion (Catcheside and Ralph, 1999). Table 1.1 summarises the studies on different ranks of coal with the bacteria and the mechanisms involved.

Table 1.1 Bacteria and the mechanisms used in coal biodegradation and biosolubilisation

Bacteria species	**Mechanisms**	**Substrates**	**References**
Streptomyces viridosporous and *Streptomyces setonii*	Non-enzymatic	Lignite coal	Strandberg and Lewis, (1987)
Arthrobacter sp.	Non-enzymatic	Lignite coal	Torzilli and Isbister, (1994)

Bacillus sp.	Alkaline solubilising agent	Lignite coal	Yuan *et al.,* (2013)
Bacillus licheniformis	Enzymatic, non-enzymatic and alkaline solubilising agent	Leonardite coal	Gao *et al.,* (2012)
Pseudomonas cepacia	Enzymatic action	Lignite coal	Gupta and Crawford, (1991)
Pseudomonas fluoroscens	Enzymatic action	Sub-bituminous coal	Hazrin-Chong *et al.,* (2014)
Pseudomonas putida	Alkaline solubilising agent	Lignite and sub-bituminous coal	Machnikowska *et al*, (2002); Huang *et al,* (2013); Yperman *et al*., (2013)
Pseudomonas stutzeri	Coal solubilising agent	Lignite, bituminous and Anthracite coal	Triphati and Singh, (2013)
Rhizobium and *Chelatococcus* sp.	Akaline solubilising agent	Lignite coal	Triphati *et al*., (2011)
Gordonia alkanivorans and *Bacillus mycoides*	Enzymatic and alkaline solubilising agent	Lignite coal	Romanowska *et al*., (2015)
Cupriavidus nectar, Sphingopyxis ginsengisolis and *Sphingomonas* sp.	Alkaline solubilising agent	Lignite coal	Baylon *et al*., (2017)

1.4.2.1 Colonisation/adherence to the coal substrate

Bacteria-degrading coal have been isolated from coal environments; they include species such as, *Bacillus lichenifromis and Stenotrophomonas maltophilia* (Gao *et al*., 2012), *Pesudomonas* sp., *Bacillus* sp. (Malik *et al*., 2016), *Bacillus* sp. (Yuan *et al*., 2013), on the basis of their ability to either depolymerise, solubilise or remove the metal ions that form the integral structural components of the coal substrates. The degradation of coal leads to disruption in the structural

matrix of the coal, resulting in its distribution to lower molecular weight aromatic and aliphatic organic moieties (Haider *et al.*, 2015). The ability of these microorganisms to metabolise or degrade coal depends on their ability to adhere to the coal surface; this is considered essential in order to initiate coal degradation. The adherence of bacteria to coal substrate is, therefore, an important step in any biotechnological application of coal.

Much of thestudies carried out on bacteria attachment to coal has demonstrated that adherence of bacteria such as *Sulfolobus acidocaldarius* (Chen and skidmore, 1988; Vitaya and Toda, 1991) and *Pseudomonas fluoroscens* (Hazrin-Chong *et al.*, 2014) to coal substrate is the initiation process of coal metabolism. Bacteria utilise the carbon compounds in coal as a substrate for energy, cell growth and proliferation. As a sequence, attachment of microorganisms on the material surface has profound effects on the material performance as well as the metabolic activity of the attached cells (Beech *et al.,* 2005; Videla and Herrera, 2005). Adhesion of bacteria to coal is controlled by a number of factors such as the properties of the coal surface, bacteria species and the pH of the medium (Raichur and Vijayalakshmi, 2003). The mechanisms which facilitate adhesion of bacteria to coal also depends on physicochemical properties such as van der Waals and electrostatic forces, and hydrophobic interactions (Fletcher 1996; Busscher *et al.*, 1999), as well as nutrient availability.

The main components of the cell surface that may contribute to the adhesion properties of the microorganisms are extra polymeric substances (EPS), lipopolysaccharides (LPS) and outer membrane and extracellular proteins (Lugtemberg *et al.*, 1983). Extra polymeric substances are produced during colonisation by bacteria and typically support the proliferating biofilm. Exopolymeric substances (EPS) are high molecular weight compounds secreted by microorganisms that are composed of polysaccharides, protein, lipids and humic substances (Bramhachari and Dubey, 2006), and are produced during the process of adherence to a particular substrate (Geesey and White, 1990). The percentage composition of each of these substances is; exopolysaccharide (40 –95%), protein (1 – 60%), nucleic acids (1 –10%), and lipids (1 – 40%) (Davey and O'Toole, 2000; Flemming and Wingende, 2001).

Bacteria EPS are responsible for the interaction of bacteria with each other as well as with interfaces (Neu *et al.*, 2001; Flemming, 2002) thereby promoting or aiding attachment of cells to substrates. Lipopolysaccharide (LPSs), a major component of the outer membrane of

bacteria, play a role in the interaction of some microbial cells with a solid surface (Labishchinski *et al.*, 1985). During the process of attachment, the organisms are brought into close proximity to the surface, propelled either randomly or in a directed fashion via chemotaxis and mobility (Prakash *et al.*, 2003). These processes of attachment lead to release of some metabolites, which are of biotechnological importance. These processes can only be observed using the well-established method of scanning electron microscopy of observing the morphology of bacteria adherence to a material surface, the morphology of the material surface, and the relationships between them (Peters *et al.*, 1982). Scanning electron microscopy has also been used to analyse initial cell attachment and colonisation at a high resolution and magnification, with 3D imaging in a 2D projection (Manefield and Hazrin-Chong, 2012).

1.4.2.2 Growth of bacteria using coal as substrate

The role of bacteria and other microorganisms in biotechnological field applications calls for extensive characterisation of their growth pattern on coal substrate. Microorganisms show a large variation with regard to physiological and morphological activities on substrates and this has been of recurring interest in biotechnological circles. The utilisation of coal as the sole carbon source requires bacteria to associate closely with coal components, hence, its ability to grow on coal. The brown colour supernatant of a coal culture medium inoculated with bacteria species indicates that substances are being released, and is also an indication of the growth and increased bacteria biomass.

The diversity of chemical environments that will support the growth of organisms is a fundamental corpus of scientific knowledge (Makie *et al.*, 2014). Although, the growth conditions for microorganisms differ from one environment to another, it is imperative that bacteria growth characteristics are measured under conditions that are similar to their natural environment (Novak *et al.*, 2009). There are four standard phases of bacteria culture growth observable within a growth curve. These include the lag phase, log phase, stationary phase and death phase, with the log phase recognised as the point where bacteria divide as rapidly as possible (Cadwel, 1995; McKeller and Lu, 2004). The shape of a microbial growth curve is a reflection of events at the cellular level that are regulated by biochemical and biophysical processes inside and outside the individual cells (Peleg and Corradini, 2011). Since turbidity is proportional to the concentration of bacteria, its measurements are a standard method for determining the specific growth rate of bacteria (Welch *et al.*, 2012).

Culturing microorganisms on petri dishes is still the most widely used method; however, the cultivability of bacteria determines their growth potential on any medium (Joubert *et al.*, 2014). The traditional methods of quantifying bacteria growth are usually carried out by growing bacteria on nutrient agar, incubating the cells for 18 -24 hours, scoring as viable bacteria that grow and form colonies (Salaimeh *et al.,* 2011), followed by microscopic observation and cell counting (Ju *et al.*, 1983; Damino and Wang, 1985). Also used are dry weight, turbidity measurement, bioluminescence (Brock *et al.*, 1994) and optical density (Aydin *et al.*, 2011). Bioluminescence measurement is sensitive, but demands expensive equipment while absorbance measurement is easier to perform, but less sensitive and only valid within a limited concentration interval (Gabriel *et al.*, 2002).

However, based on the limitation of the aforementioned methods of measuring bacteria growth, and since coal components create problems in measuring bacteria growth on the basis of turbidity and absorbance at 600nm (Triphati *et al.*, 2011), an approach using selected cellular components, DNA quantification, was developed as a more accurate method of measuring bacteria growth (Zhao *et al.*, 2013). This method involves using a diphenylamine colorimetric assay. The colorimetric method is a very attractive approach since it enables rapid visual detection, on the spot, without the need for any detection equipment (Niemeyer, 2001; Liu and Lu, 2006; Jung *et al.,* 2008). Although various colorimetric assays for DNA detection have been proposed and demonstrated (Liand Rothberg, 2004; Li *et al.*, 2008; Deng *et al.*, 2012), the use of diphenylamine reagent is reliable (Zhao *et al.*, 2013) as it does not involve DNA extraction, as was done by some researchers.

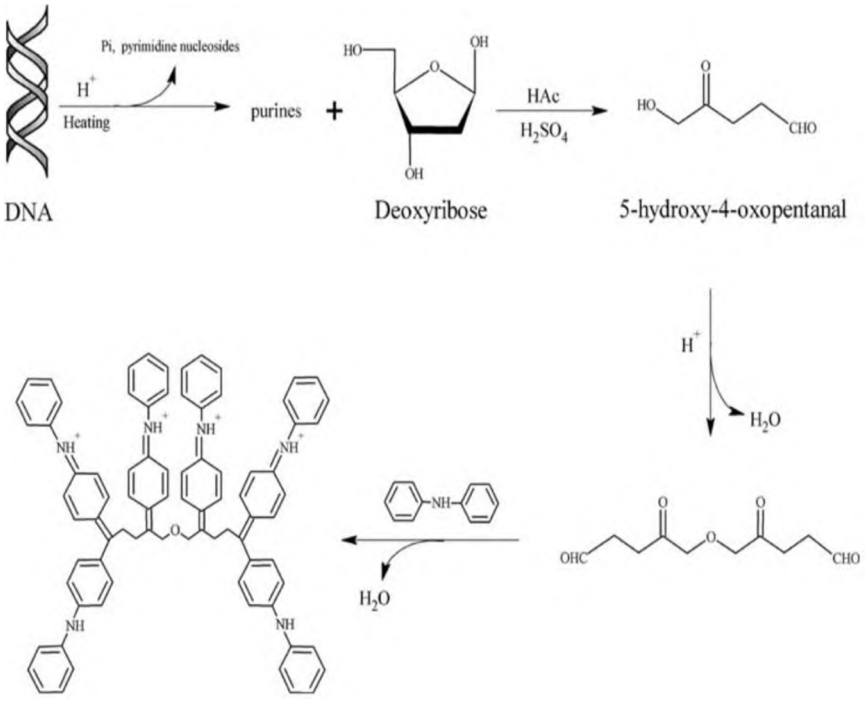


Figure 1.2 Mechanism of diphenylamine colorimetric reaction (Zhao *et al*., 2013).

1.5 Mechanisms of coal biodegradation

There are many mechanisms responsible for microbial coal degradation; oxidative enzymes (peroxidases, laccases), hydrolytic enzymes (esterases), alkaline metabolites and natural chelators (Fakoussa, Hofricheter, 1999), among others. The most rapid and complete degradation of coal is brought about under aerobic conditions. Aerobic degradation is faster and results in non-toxic products of CO_2 and water (Prince, 1993). Huesemann *et al* (1993) showed that polynuclear aromatic compounds degraded faster in oxygen-sparged than in air-sparged laboratory reactors. Casal *et al* (2003) reported that experimental conditions play a crucial role in the course of oxidation reactions. Aerobic bacteria are of particular relevance for the bioconversion of coal because they bring about rapid degradation of coal, thereby achieving complete breakdown in the presence of oxygen.

Although many bacteria are able to metabolise organic pollutants, a single bacterium does not possess the enzymatic capability to degrade most of the inorganic and organic compounds in coals. A consortium of bacteria communities has the most powerful bio-degradative potential because the genetic information of more than one organism is necessary to degrade the complex mixtures of organic and inorganic compounds in coal. In the natural environment, it is more likely that degradation is carried out by mixed microbial communities and it is equally likely

that bacteria in the natural environment would be exposed to more than one organic compound (Clarke, 1984).

Enzymes are biological catalysts that facilitate the conversion of substrates into products in order to provide favourable conditions that reduce the activation energy of the reaction (Karigar and Rao, 2011). The enzymology for bacteria coal degradation is much less studied than that of fungi, yet there are many indications for coal degradation from the studies on *Pseudomonas putida* and *Bacillus subtilis*. Some microorganisms grow on coal and modify it with both enzymatic and non-enzymatic processes (Cohen and Gabriele, 1982). Both oxidative and non-oxidative enzymes are considered to be coal-degrading agents (Crowford and Gupta, 1991). Microorganisms produce enzymes that are capable of reacting with chemicals different from those being used as primary carbon and energy sources. Fakoussa and Troper (1983) reported that based on spectroscopic evidence, a strain of *Pseudomonas fluorescence*, isolated from a mixed population cultured on coal slurry, attacked and solubilised coal by means of an enzyme coupled with a surface-active agent that the organism produced.

1.5.1 Effect of pH changed during coal biodegradation

The pH of the coal culture medium is proportional to the mechanisms used by microorganisms to degrade coal. The solubilisation of coal by microorganisms using secreted alkaline substances results in an increase in the pH (Romanowska *et al.*, 2015). Biodegradation of coal carried out by *Streptomycete viridosporus, Bacillus sp.* (Gao *et al.*, 2012; Jiang *et al.*, 2013), *Pseudomonas putida* (Machnikowska *et al.*, 2002), has been reported to be caused by secretion of alkaline materials that result in an increase in pH as well as coal solubility (Sudheer *et al.*, 2016). The transformation of nitrogen-rich compounds produced during the metabolism of peptones present in the nutrient broth generate alkaline substances (Hofrichter *et al.,* 1997). Meanwhile, depolymerisation of coal mediated by enzymatic activity occurs at pH below 6 (Romanowska *et al* ., 2015).

1.5.2 Role of chelation in coal degradation

Rendering the coal substrate to smaller particle size increases the surface area of coal and provides microorganisms with more chance of access to coal-degrading substances, such as multivalent metals cations bridging the coal matrix (Sudheer *et al.*, 2016). Among the chelating agents of coal degradation substrates are; ammonium oxalate released by *Trametes versicolor*

(Cohen *et al.*, 1990), *Penicillium* sp. (Larboda *et al.*, 1999) and, citrate. Fungi produce oxalic acid and ubiquitous organic acids for metabolic activities. Chelating agents break the ionic linkages of the polar coal macromolecules and pave the way for solubilisation and depolymerisation of coal for most bacteria species (Malik *et al.*, 2016). It has been shown that high absorbance at 450 nm, observed for the supernatant of the coal culture medium enriched with ammonium oxalate, indicates that this chelating agent aids the biodegradation of coal (Romanowska *et al.*, 2015).

1.5.3 Role of enzymes in coal degradation

Coal biodegradation is a naturally complex process and appears to be driven by a number of extracellular enzymes in the presence of various chelators and supporting enzymes released by a different microorganism that co-inhabits the coal environment (Cowan *et al.*, 2013). It has also been shown that up to 40% of the weight of some coals can be dissolved using extracted microbial enzymes (Scott *et al.*, 1994). Because lignin and lignite coal are similar in structure (Hayatsu *et al.*, 1979; Olson and Brinckman, 1986), lignin-degrading microorganisms might be likewise expected to have the ability to degrade lignite coal. *Citrobacter freundii, Serratia* sp. and *Klebsiella* sp. are among the bacteria species that degrade lignin-derived aromatic compounds (Chandra *et al.*, 2006; Chandra and Abhishek, 2011). The enzymes implicated in lignin degradation are: (1) lignin peroxidase, which catalyses the oxidation of both phenolic and non-phenolic units, (2) manganese-dependent peroxidase, (3) laccase, which oxidises phenolic compounds to give phenoxy radicals and quinones; (4) glucose oxidase and glyoxal oxidase for H_2O_2 production, and (5) cellobiose-quinone oxidoreductase for quinone reduction (Thakker *et al.*, 1992) .

The major groups of enzymes that are involved in lignin degradation are heme peroxidases and laccases (Abdel-Hamid *et al.*, 2013), and Cytochrome P450 (Doddapaneni *et al.*, 2005; Matsuzaki and Wariishi, 2005). Enzymes, specifically oxidoreductases, have also been implicated in this activity both *in vivo* and in cell-free systems (Faison *et al.*, 1990). Oxidoreductases participate in the humification of various phenolic substances that are produced from the decomposition of lignin in the soil environment, and at the same time, detoxify phenolic or anilic compounds through polymerisation, co-polymerisation with other substrates or binding to humic substances (Park *et al.*, 2006). Oxygenases belonging to the oxidoreductases group of the enzymes oxidise reduce substrates by transferring oxygen from

molecular oxygen utilising FAD/NADH/NADPH as a co-substrate. These can be either mono-oxygenases or di-oxygenases and are identified based on the number of oxygen atoms used for oxygenation.

1.5.3.1 Laccases (EC. 1.10.3.2)

Laccase, benzenediol oxygen oxidoreductase (EC 1.10.3.2), is a blue copper enzyme that oxidises aromatic compounds using molecular oxygen as an electron acceptor, resulting in the formation of reactive radicals and water (H_2O) from the reaction. Laccase substrates are carbohydrates, unsaturated fatty acids, phenols, and thio-containing proteins (Adinarayana *et al.*, 2008). They belong to an interesting group of ubiquitous, oxidoreductases that show promise of offering great potential for biotechnological and bioremediation application (Gianfreda *et al.,* 1999). This enzyme represents the largest subgroup of blue multicopper oxidases (MCO) that use the distinctive redox ability of copper ions to catalyse the oxidation of a wide range of aromatic substrates concomitantly with the reduction of molecular oxygen to water (Solomon *et al.*, 1996, Messerschmidt, 1997), and are widely distributed within plants, insects and fungi (Zhongyang *et al.*, 2012) .

With the current rapid progress in genome analysis, there have been widespread reports of cases of laccase in bacteria (Claus, 2003). Bacterial laccase was first reported in *Azospirillum lipoferum* (Givaudan *et al.,* 1993). The enzymes have also been characterised from *Bacillus subtilis* (Martins *et al.*, 2002; Shah, 2015), *Bacillus* sp. (Shukur, 2015), *Streptomyces lavendulae* (Suzuki *et al.*, 2003), *Streptomyces coelicolor* (Dube *et al.,* 2008), *Streptomyces ipomea* (Molina-Guijarro *et al.*, 2009), *Streptomyces cinnamomensis* (Jing and Wang, 2012), *Pseudomonas putida* (Ramtek *et al.*, 2013), *Pseudomonas stutzeri* (Kumar *et al.,* 2005), *Halobacillus halophilus* (Bhuvaneshwari *et al.*, 2015), *Serratia marscens* (Kaira *et al.*, 2015), *Serratia* sp. (Park *et al.*, 2006), *Exiguobacteria aurantiacum* (Hosseini *et al.*, 2012).

In contrast to fungal laccases, bacterial laccases are active and much more stable at high temperatures, pH as well as high concentrations of chloride and copper ions (Upendra *et al.*, 2011). In addition, bacterial laccase is capable of catalysing the oxidation of organic compounds in the absence of hydrogen peroxide (Leonowicz *et al.*, 2001), and this has given laccase an advantage over peroxidase enzyme. Laccase is used for bleaching purposes (Oudia *et al.*, 2008), removal of herbicides from cereal crops (Coelho *et al.,* 2010) and the biodegradation and

bioconversion of agricultural wastes (Sanchez, 2009; Haddadin *et al.*, 2002; Strong and Burgess, 2008). Laccases have many possible applications in bioremediation (Xu, 1999) because of their ability to degrade various substances such as undesirable contaminants, by-products, or discarded materials. Bioremediation application of laccase is achieved in the detoxification of phenolic pollutants such as o-chlorophenol, 2, 4-dichlorophenol 2, 4, 5-TCP, and 4-chloro-2-methylphenol (Bollag *et al.*, 1988). Xenobiotic compounds present in polycyclic aromatic hydrocarbon (PAH) in oil deposits and fossil fuels are known to be easily degraded by laccases (Pointing, 2001; Anastasi *et al.*, 2009; Murugan *et al.*, 2014).

1.5.3.2 Peroxidases

Peroxidases are also ubiquitous enzymes that catalyse the oxidation of lignin and other phenolic compounds at the expense of hydrogen peroxide (H_2O_2) in the presence of a mediator. Lignin peroxidase (EC1.11.1.14) is an extracellular heme-containing peroxidase, which is dependent on hydrogen peroxide (H_2O_2) and has an unusually high redox potential and low optimum pH (Bonugli-Santos *et al.*, 2010). Lignin-degrading peroxidases include, lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and dye-decolorising peroxidase (DyP). LiP, MnP, and VP are class II extracellular fungal peroxidases that belong to the plant and microbial peroxidases superfamily**.** Extracellular manganese peroxidase is the crucial enzyme in the depolymerisation process of both coal-derived humic substances and native coals (Hofrichter *et al.*, 1999).

The depolymerisation of coal by manganese peroxidase is catalysed via chelated Mn (III) acting as a diffusible mediator with a high redox potential and can be enhanced in the presence of additional mediating agents. The depolymerisation process results in the formation of a complex mixture of lower molecular mass fulvic acid-like compounds (Hofrichter *et al.*, 1999). The substrates of this enzyme include, for example, phenolic and non-phenolic compounds (Mongkolthanaruk *et al.*, 2012). Some non-phenolic compounds require mediators to catalyse the oxidation of the enzyme (Sharma *et al.*, 2007). Phenolic compounds are present in wastes from several industrial processes, such as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others (Aggelis *et al.*, 2003) .

1.5.3.3 Cytochrome P450 enzymes

Cytochrome P450s are a superfamily of protein with a maximum absorption at 450nm and are characterised by the presence of cysteine (Cys) residues (Li *et al*, 2013) with Cys distinguishing cytochrome P450s from other oxygen-activating enzymes such as globins and peroxidases that utilise histidine during the reaction with hydrogen peroxide (Ost *et al.*, 2003) . Cytochrome P450s are *b*-heme-containing enzymes that are able to introduce oxygen atoms into a wide variety of organic substrates. They are widespread in nature with diverse functions at both biochemical and physiological level (Alvarez *et al.*, 2013). Cytochrome P450 has been detected in fungi and bacteria followed by their catabolic/enzymatic activity in the metabolism of polycyclic aromatic hydrocarbons (Masaphy *et al.*, 1996), lignin degradation (Doddapaneni *et al.*, 2005; Matsuzaki and Wariishi, 2005) and ability to degrade petrol additives i.e. methyl tert-butyl ether, ethyl tert-butyl ether and tert-amyl butyl ether (Malandain *et al.*, 2010). Cytochrome P450 has been discovered in *Rhodococcus rhodochrous* (Karlson *et al.*, 1993), *Bacillus megaterium* (Nahri and Fulco, 1986), *Mycobacterium* sp. (Aly, 2011), *Actinoplanes* sp. (Prior *et al.*, 2010), expressed in high levels in recombinant *Escherichia coli* and shown to turn over different substrates (Gilardi and Nardo, 2012). The characteristic absorbance peak at approximately 450 nm of the CO-difference spectrum of the reduced P450 provides a ready means to screen for the presence of these enzymes in whole cells and crude extracts (Peterson and Lu, 1991).

1.6 Aims and objectives

A previous study at the Institute for Environmental Biotechnology, Rhodes University, (EBRU) resulted in the isolation and identification of a suite of some selected bacteria with coal biodegradation potential (Edeki, 2015). Bacteria were bio-prospected from diesel-contaminated soil and coal slurry and studies revealed effective coal-degrading activity when assembled as consortia. Unfortunately, no scientific basis was provided for the choice of organisms for the consortia; colonisation of the coal substrate by the various consortia was only partially demonstrated, and the mode of biodegradation was not investigated. The aim of the present study was therefore to:

- confirm the coal degradation potential of the isolated strains;
- investigate in detail the colonisation, and biodegradation of coal discard and geologically weathered coal using the most active bacterial strains; and,
- attempt to describe the mechanism used by these bacteria, either individually or as a consortium, in the breakdown of coal.

CHAPTER 2

Screening of bacteria for potential to degrade coal discard and geologically weathered coal

2.1 Introduction

Several bacterial species have been reported to grow on and degrade coal, particularly when the coal substrate has been pre-treated with oxidising agents, e.g. nitric acid (Romanowska *et al.*, 2015). Among these bacterial species are; *Cupriavidus nectar, Sphingopyxis ginsengisoli* and *Sphingomonas* sp. (Baylon *et al.,* 2017), *Bacillus* sp. *and Pseudomonas* sp. (Malik *et al.,* 2016), *Bacillus mycoides* and *Gordonia alkanivorans* (Romanowska *et al.*, 2015), *Bacillus lichenifromis* (Yuan *et al.*, 2012), *Bacillus* sp. (Yuan *et al.*, 2013), *Pseudomonas putida* (Machnikowska *et al.*, 2002), *Rhizobium* sp. and *Chelatococcus* sp. (Triphati and Singh, 2011), *Pseudomonas cepacia* (Gupta *et al.*, 1990; Crawford and Gupta, 1991), *Pseudomonas fluoroscens* (Fakoussa and Troper, 1983; Hazrin-Chong *et al.,* 2014), *Escherichia freundi* and *Pseudomonas rathonia* (Korburger, 1964), *Escherichia coli* and *Streptococci* sp., (Sharma *et al.*, 1992), *Pseudomonas stutzeri* (Singh and Triphati, 2011), *Bacillus cereus* (Maka *et al.*, 1989), and *Streptomyces viridosporous* (Strandberg and Lewis, 1987).

In an effort to understand coal solubilisation, oxidative degradation studies were carried out using different oxidising agents (pre-treatment) such as nitric acid, sodium hydroxide, ozone or radiation (Hofrichter *et al.*, 1997), since the naturally occurring oxidation (weathered coal) takes many years. Researchers have found that coal oxidised using nitric acid enhances the susceptibility of coal substrates to bacteria degradation (Maka *et al.*, 1989); this has been demonstrated using *Pseudomonas putida* (Machnikowska *et al.*, 2002; Huang *et al.*, 2013) and *Acinetobacter baumannii* (Valero *et al.*, 2011) in the degradation of acid pre-treated coal. Bacteria involvement in coal degradation has been traced to the related research in fungi biodegradation ability (Jiang *et al.*, 2013), which was the earliest study on the biosolubilisation of coal. Bacteria isolated from coal environments have been implicated in coal degradation (Malik *et al.*, 2016) based on the idea that these native bacteria are very well adapted to using coal as a substrate (Zheng *et al.*, 2017).

A previous bioprospecting study yielded ten bacterial strains; *Bacillus* strain ECCN 18b, *Citrobacter* strain ECCN 19b, *Proteus* strain ECCN 20b, *Exiguobacterium* strain ECCN 21b, *Microbacterium* strain ECCN 22b, *Proteus* strain ECCN 23b, *Serratia* strain ECCN 24b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b and *Bacillus* strain ECCN 41b that

were isolated from diesel-contaminated soil and coal slurry, and shown to possess coal-degrading potential without the necessity of pre-treating with nitric acid (Edeki, 2015). Although various consortia of these strains were shown to degrade coal, the ability of the individual strains was overlooked. Thus, the rationale for strain selection of each consortium appears to be without foundation.

In order to address the oversight, these ten bacterial strains were screened for coal biodegradation capacity and ranked accordingly in an effort to derive the most active bacterial strains for more detailed study and, perhaps, for the development of novel applications within the coal industry. The screening was carried out using coal discard and geologically weathered coal as substrates. Geologically weathered coal was chosen because of its greater oxidation state which eliminated the need to pre-treat substrates with acids.

2.2 Materials and methods

2.2.1 Bacterial strains and culture conditions

The bacterial strains used in this study were *Bacillus* strain ECCN 18b, *Citrobacter* strain ECCN 19b, *Proteus* strain ECCN 20b, *Exiguobacterium* strain ECCN 21b, *Microbacterium* strain ECCN 22b, *Proteus* strain ECCN 23b, *Serratia* strain ECCN 24b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b, and *Bacillus* strain ECCN 41b. These strains were previously isolated from diesel-contaminated soil and from coal slurry at coal mines in eMalahleni, Witbank, Mpumalanga Province, South Africa and identified by PCR following amplification of 16S ribosomal deoxyribose nucleic acid (DNA) using the universal primer 907R (5'- CCCCGTCAATTCCTTTGAGTTT-3') and the bacterial primer GMF5 (5'-CCTACGGGAGGCAGCAG-3') (Edeki, 2015). Stock cultures in 30% (v/v) glycerol at -80 ^{o}C, maintained by sub-culturing every three months, were from the culture collection centre of the Institute for Environmental Biotechnology, Rhodes University, Grahamstown, South Africa.

2.2.1.1 Confirmation of bacterial strain identity

Bacterial strains were grown on solid nutrient agar (31g/L) plates for 24 h and morphological features such as colony shape, colony texture and colour were noted.

2.2.1.2 Genomic deoxyribose nucleic acid (DNA) extraction

To determine strain purity, a sterile loop was used to transfer stock cultures to sterile nutrient agar (31 g in 1 L of MilliQ water) plates that were incubated at 37 °C for 24 h. Individual colonies were picked and grown in 100 mL Luria broth (tryptone 10 g, yeast extract 5 g, NaCl 10 g in 1 L of MilliQ water), at 37 °C for 18 –24h and, harvested by centrifugation at 14 000 rpm for 30 s in a bench-top micro-centrifuge. The supernatant was carefully removed so as not to dislodge the pellets, and total genomic DNA from the bacteria cells was extracted using a Norgen Biotek bacteria genomic DNA isolation kit (3430 Schmon Parkway Thorold, ON, Canada. Cat. #17900) according to the manufacturer's instructions. After extraction, the concentration of DNA was determined using a 2000 Nano spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) and the samples stored at 4 °C.

2.2.1.3 16S rDNA gene sequence analysis

The extracted DNA was submitted to Inqaba Biotech (South Africa) for pyrosequencing of the 16S rDNA gene. The 16S target region was amplified using DreamTag™ DNA polymerase (Thermo Scientific™) and the universal primers 27F and 1492R for amplification (Lane *et al.*, 1991; Turner *et al.*, 1999). PCR products were gel-extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit), and sequenced in the forward and reverse directions using a ABI PRISMTM 3500xI Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analysed using CLC main Workbench 7 followed by a BLAST search (NCBI).

* 16S-27F 5'AGAGTTTGATCMTGGCTCAG3' (Forward primer)

* 16S-1492R 5'CGGTTACCTTGTTACGACTT3' (Reverse primer)

PCR products were purified and subsequently sequenced using an ABI 3130XL sequencer (Inqaba Biotech, Pretoria, South Africa). Chromatograms were converted to text format using Chromas, and entered into the NCBI BLAST database (http:/www.ncbi.nlm.nih.gov/BLAST/ maintained by the National Center for Biotechnology Information). Table 2.1 lists the molecular identification of bacterial strains isolated from diesel-contaminated soil and coal slurry.

Table 2.1 Confirmation of molecular identification of bacteria with coal-degrading potential isolated previously from coal slurry and diesel-contaminated soil (Edeki, 2015)

Accession number (MCC)	Genbank accession numbers (KC)	Taxonomic designation (identity %)	Source
MCC0016	KC620476	*Exiguobacterium* strain ECCN 21b (99%)	Diesel-contaminated soil
MCC0021	KC620474	*Serratia* strain ECCN 24b (99%)	Diesel-contaminated soil
MCC0022	KC620478	*Proteus* strain ECCN 23b (91%)	Diesel-contaminated soil
MCC0027	KC620475	*Proteus* strain ECCN 20b (94%)	Diesel-contaminated soil
MCC0033	KC700328	*Citrobacter* strain ECCN 19b (99%)	Coal slurry
MCC0034	KC620473	*Bacillus* strain ECCN 18b (98%)	Diesel-contaminated soil
MCC0039	KC758162	*Bacillus* strain ECCN 41b (99%)	Coal slurry
MCC0041	KC700329	*Escherichia* strain ECCN 25b (99%)	Coal slurry
MCC0042	KC620477	*Microbacterium* strain ECCN 22b (99%)	Diesel-contaminated soil
MCC0062	KC700330	*Bacillus* strain ECCN 26b (99%)	Coal slurry

2.2.2 Origin and characterisation of coal discard and geologically weathered coal

Geologically weathered coal and coal discard samples used were obtained from coal mines in eMalahleni (Witbank), Mpumalanga Province, South Africa. Coal discard comprised low-grade coal with calorific value of 8 −10 $MJkg^{-1}$ obtained from the void following extraction of high-grade coal. Geologically weathered coal was sourced from the No.2 Seam, Kromdraai Section, Landau Colliery (Cowan *et al.*, 2016). Samples were air-dried and ground using a HP-M 100 Pulverizer (HERZOG Maschinenfabrik GmbH & Co., Osnabruck, Germany) and sieved to yield particles of approximately 0.2 ─0.5mm in diameter.

2.2.2.1 Proximate analysis of coal discard and geologically weathered coal

Proximate analysis is the determination of the amount of fixed carbon, moisture, volatile matter, and ash in a coal sample. These variables were measured as the weight percent (%) and calculated on several different bases in accordance with the American Society for Testing and Materials (ASTM) standards. All measurements were in triplicate and results are reported as the mean with standard errors (mean ±SE). Moisture content was determined after 1 g aliquots of coal were heated at 110 °C for 1 h. The relative loss of mass was determined and reported as percentage moisture content. Ash content was determined by heating 1 g aliquots of coal in a muffle furnace (Model JK-222M, Jingke Scientific Instrument Co., Ltd., Shanghai) at 815 °C.

First the temperature of the sample was heated to 400 °C over 30 min., then to 815 ^{0}C for further 30 min., and finally isothermally at 815 °C for 2 h. After heating, the difference in mass was measured, and the residue was reported as percentage ash content. To determine the volatile matter, 1 g aliquots of coal were placed in a platinum crucible of 10 —20 mL capacity. This was inserted into a muffle furnace (Model JK-222M, Jingke Scientific Instrument Co., Ltd., Shanghai) at 910 °C for 7 min on a mineral wood pad. The loss in mass was calculated and reported as the percentage of volatile matter.

The fixed carbon content is the carbon found in the material, which is left after volatile materials are driven off. Fixed carbon content was derived by subtracting the sum of % moisture, plus % ash plus % volatile matter from 100 as follows.

fixed carbon (%) = 100 − (% moisture + % volatile matter + % ash).

Physicochemical properties analysis (Table 2.3) shows that coal discard consisted of 8% moisture, 35% ash, 39% volatile matter, 18% fixed carbon, and geologically weathered coal consisted of 4% moisture, 40% ash, 49% volatile matter and 7% fixed carbon.

2.2.2.2 Ultimate analysis of coal discard and geologically weathered coal

The ultimate analysis determines the amount of carbon (C), hydrogen (H), oxygen (O), sulphur (S), and nitrogen (N) within a coal sample. Elemental analysis of coal samples was carried out using an elemental analyser (PE 2400 CHNS/O, PerkinElmer, Waltham, MA). Percentages of C, H, N, and S were determined experimentally while O percentage was determined by difference method.

2.2.2.3 Electrical conductivity and pH of coal sample

Electrical conductivity (EC) and pH were determined as described by Rayment and Higginson (1992). Aliquots (10 g) of coal were placed into a 100 mL beaker, to which MilliQ water (10 mL) was added and stirred vigorously for 5 s. The stirrer was removed from the beaker and the coal-water suspension was allowed to stand for 10 min. The pH of the suspension was measured at 25 °C without disturbing the settled solids using a pH meter (HANNA Instruments HI 8314) which had been calibrated using standard buffer solutions.

Electrical conductivity (EC) was determined by suspending 10 g of coal sample in 50 mL MilliQ water and the content thoroughly mixed. The mixture was allowed to settle prior to measurement of EC using a waterproof EC Testr11Dual Range EUTECH conductivity meter (EUTECH Instruments/Oakton Instruments). The pH and electrical conductivity of coal discard were 3.43 and 120 µs/cm respectively, and that of geologically weathered coal 3.40 and 210 µs/cm, respectively (Table 2.3).

2.2.3 Screening for biodegradation potential using coal discard and geologically weathered coal

2.2.3.1 Solid culture screening

Powdered coal samples were sterilised using liquid nitrogen (three cycles). Confirmation of the sterilisation of coal samples was achieved by monitoring bacterial growth following serial dilution of aliquots of coal discard and geologically weathered coal samples suspended in sterile Milli-Q water and subsequent plating on solid nutrient agar, which was incubated at 37 °C for 48 h.

Screening of the bacterial strains for biodegradation potential was carried out using a mineral salt medium agar plate assay. A mineral salt medium (MSM) (K_2HPO_4 1.71 g, KH_2PO_4 1.32 g, NH_4Cl 1.26 g, $MgCl_2.6H_2O$ 0.011 g, $CaCl_2$ 0.02 g, 15 g nutrient agar per litre of MilliQ water) was used to assess the biodegradation capability of the bacterial strains on geologically weathered and coal discard. The media was sterilised by autoclaving at 121 °C for 15 min. Bacteria cultivated at 37 °C for 24 h in Luria broth were harvested in log phase by centrifugation at 10000 rpm for 5 min. The supernatants were discarded, and the cell pellets washed in phosphate-buffered saline (PBS) twice. Tenfold serial dilutions of bacteria culture were performed and about 200 µL aliquots of 10^{-6} to 10^{-7} of bacteria culture were inoculated at the centre of 90mm diameter Petri plates containing sterilised solidified mineral salt medium agar

and incubated at 37 °C for 24 h. Particles of coal discard and geologically weathered coal sterilised using liquid nitrogen were dispersed using a sterised forcep directly onto the bacterial colonies and the plates incubated at 37 °C for a further 7 –14 d. The appearance of a brown halo around the bacteria colonies was taken to indicate the formation of 'soluble' breakdown products. The controls for this experiment were the mineral salt medium agar with coal but without bacteria inoculation. This experiment was carried out in a sterile laminar flow cabinet.

2.2.3.2 Liquid culture screening

Bacterial strains were further screened in a liquid culture in 150 mL volumes of mineral salt medium (MSM) containing 4 mL of trace element solution (TES) ($MnSO_4.H_2O$ 5 g, NaCl 10 g, $FeSO_4.H_2O$ 5 g, $COCl_2$ 1 g, $CaCl_2$ 1 g, $ZnSO_4.7H_2O$ 1 g, $CUSO_4.5H_2O$ 0.1 g, ALK $(SO_4).12H_2O$ 0.8 g, H_3BO_3 0.1 g, $Na_2MoO_4.2H_2O$ 0.9 g) and 1 g of either ground geologically weathered coal or coal discard that had been sterilised as described above.

Following inoculation with bacterial strains cultures were incubated without shaking at 37 °C for periods up to 21 d and a change in colour of the incubation medium to yellow/brown liquid was considered to indicate biodegradation potential. This was done in a sterile laminar flow cabinet.

2.2.3.3 Evaluation of coal biodegradation potential

Parameters such as the diameter of the brown halo on the bacteria solid culture plates, and colour intensity of supernatants of the bacterial strains culture (liquid) in the presence of either coal discard or geologically weathered coal incubated at 37 °C for 14d –21d were studied. Indicators such as pH, the intensity of brown/yellow colour, absorbance at 280nm and 450nm were subjectively related to coal biodegradation ability and used to evaluate the degree of potential bacterial degradation of coal discard and geologically weathered coal.

2.3 Results

2.3.1 Bacterial strain colony characteristics

Colonies on nutrient agar plates after incubation were circular, smooth with orange-yellow and yellow pigments observed in *Exiguobacterium* strain ECCN 21b and *Microbacterium* strain

ECCN 22b. Undulating margins around the colonies were found in *Bacillus* strains (data not shown).

2.3.2 Molecular characterisations of bacterial strains by 16S rDNA gene sequencing

Blast results corresponded to the sequences queried and were similar to the original sequences submitted and contained in the NCIB data base. Thus, sequences submitted to GenBank using BankIT, wild-type strains deposited with Microbial Culture Collection (MCC), Maharashtra, India, and accession numbers and nearest taxonomic designation assigned to each bacterial isolate were confirmed.

Based on this investigation, these bacterial strains are: ECCN 18b belonging to genus *Bacillus*, revealing 100% identity to *Bacillus* strain; ECCN 19b belonging to genus *Citrobacter*, revealing 99% identity to *Citrobacter* strain; ECCN 20b belonging to the genus *Proteus*, revealing 99% *Proteus* strain; ECCN 21b belonging to genus *Exiguobacterium*, revealing 99% *Exiguobacteriun* strain; ECCN 22b belonging to genus *Microbacterium*, revealing 99% *Microbacterium* strain; ECCN 23b belonging to genus *Proteus*, revealing 99% *Proteus* strain; ECCN 24b belonging to the genus *Serratia*, revealing 100% *Serratia* strain; ECCN 25b belonging to genus *Escherichia*, revealing 99% *Escherichia* strain; ECCN 26b belonging to genus *Bacillus*, revealing 100% *Bacillus* strain, and ECCN 41b belonging to genus *Bacillus*, also revealing 100% *Bacillus strain* (Table 2. 2). In this present study, the bacteria will be referred to as; *Bacillus* strain ECCN 18b, *Citrobacter* strain ECCN 19b, *Proteus* strain ECCN 20b, *Exiguobacterium* strain ECCN 21b, *Microbacterium* strain ECCN 22b, *Proteus* strain ECCN 23b, *Serratia* strain ECCN 24b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b and *Bacillus* strain ECCN 41b

Table 2.2 Confirmation of molecular identity of bacterial strains isolated from diesel-contaminated soil and coal slurry

Accession Number (MCC)	Genbank Number (KC)	Taxonomic Designation (identity %)	Source
MCC0016	KJ722475.1	*Exiguobacterium* strain ECCN 21b (99%)	Diesel-contaminated soil
MCC0021	KC178028.1	*Serratia* strain ECCN 24b (99%)	Diesel-contaminated soil
MCC0022	KX058415.1	*Proteus* strain ECCN 23b (99%)	Diesel-contaminated soil

MCC0027	JF775423.1	*Proteus* strain ECCN 20b (99%)	Diesel-contaminated soil
MCC0033	CP016952.1	*Citrobacter* strain ECCN 19b (99%)	Coal slurry
MCC0034	KM114617.1	*Bacillus* strain ECCN 18b (100%)	Diesel-contaminated soil
MCC0039	KJ127191.1	*Bacillus* strain ECCN 41b (100)	Coal slurry
MCC0041	KC700329	*Escherichia* strain ECCN 25b (99%)	Coal slurry
MCC0042	KC620477	*Microbacterium* strain ECCN 22b (99%)	Diesel-contaminated soil
MCC0062	KX242272.1	*Bacillus* strain ECCN 26b (100%)	Coal slurry

Table 2.3 Physicochemical properties of coal discard and geologically weathered coal (mean ± SE).

Physicochemical analysis	**Unit**	**Coal discard**	**Geologically weathered coal**
Moisture	**(mass%)**	**8 ± 0.70**	**4 ± 0.00**
Ash	**(mass%)**	**35 ± 0.70**	**40 ± 0.69**
Volatile Matter	**(mass%)**	**39 ± 1.70**	**49 ± 2.29**
Fixed Carbon	**(mass%)**	**18 ± 1.90**	**7 ± 2.25**
pH	**-**	**3.43**	**3.40**
Electrical Conductivity (EC)	**(µs/cm)**	**120**	**210**

Table 2.4 Elemental analysis of coal discard and geologically weathered coal (mean ± SE)

Ultimate Analysis	Unit	Coal discard	Geologically weathered coal
Carbon (C)	**(mass %)**	**43.5 ± 1.07**	**25 ± 1.74**
Hydrogen (H)	**(mass %)**	**2.7 ± 0.55**	**1.4 ± 0.39**
Nitrogen (N)	**(mass %)**	**1.0 ± 0.19**	**0.7 ± 0.34**
Sulphur (S)	**(mass %)**	**0.2 ± 0.28**	**0.2 ± 0.18**
Oxygen (O)	**(mass %)**	**52.7 ± 1.08**	**72 ± 1.81**

Coal discard consists of 43.5% C, 2.75 % H, 1% N, 0.2% S, 52.7% O and geologically weathered coal 25% C, hydrogen 1.4% H, nitrogen 0.7 % N, sulphur 0.2% S and oxygen 72% O (Table 2.4).

2.3.3 Screening for coal biodegradation potential

2.3.3.1 Plate screening

Brown halos were observed on MSM agar after addition of either geologically weathered coal or coal discard within 14 d of incubation for all of the bacterial strains. The appearance of halos was considered to indicate diffusion of substances released from the coal particles as a result of bacterial activity. Zones of colour formation for each bacterial strain ranged from 5 to 25 mm. Halos became wider and the colour more intense when plates were incubated for longer periods. As might be expected, no coloured halos were observed in the controls, indicating that spontaneous leaching of any latent or residual material in the coal substrates had not occurred (Figures 2.1, 2.2).

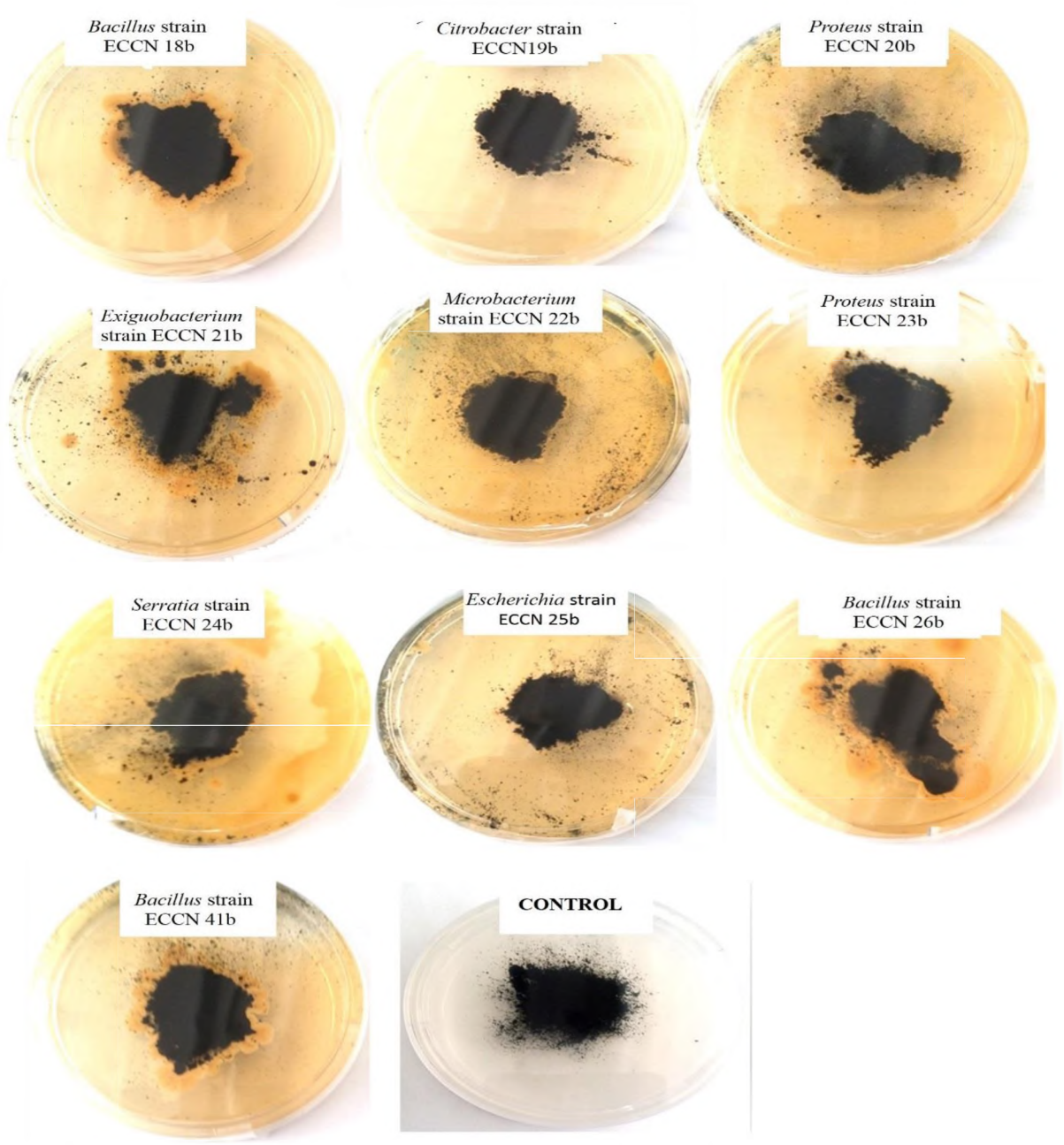


Figure 2.1 Coloured halos indicating bacterial biodegradation of coal discard in solid culture. No coloured halo was observed in control.

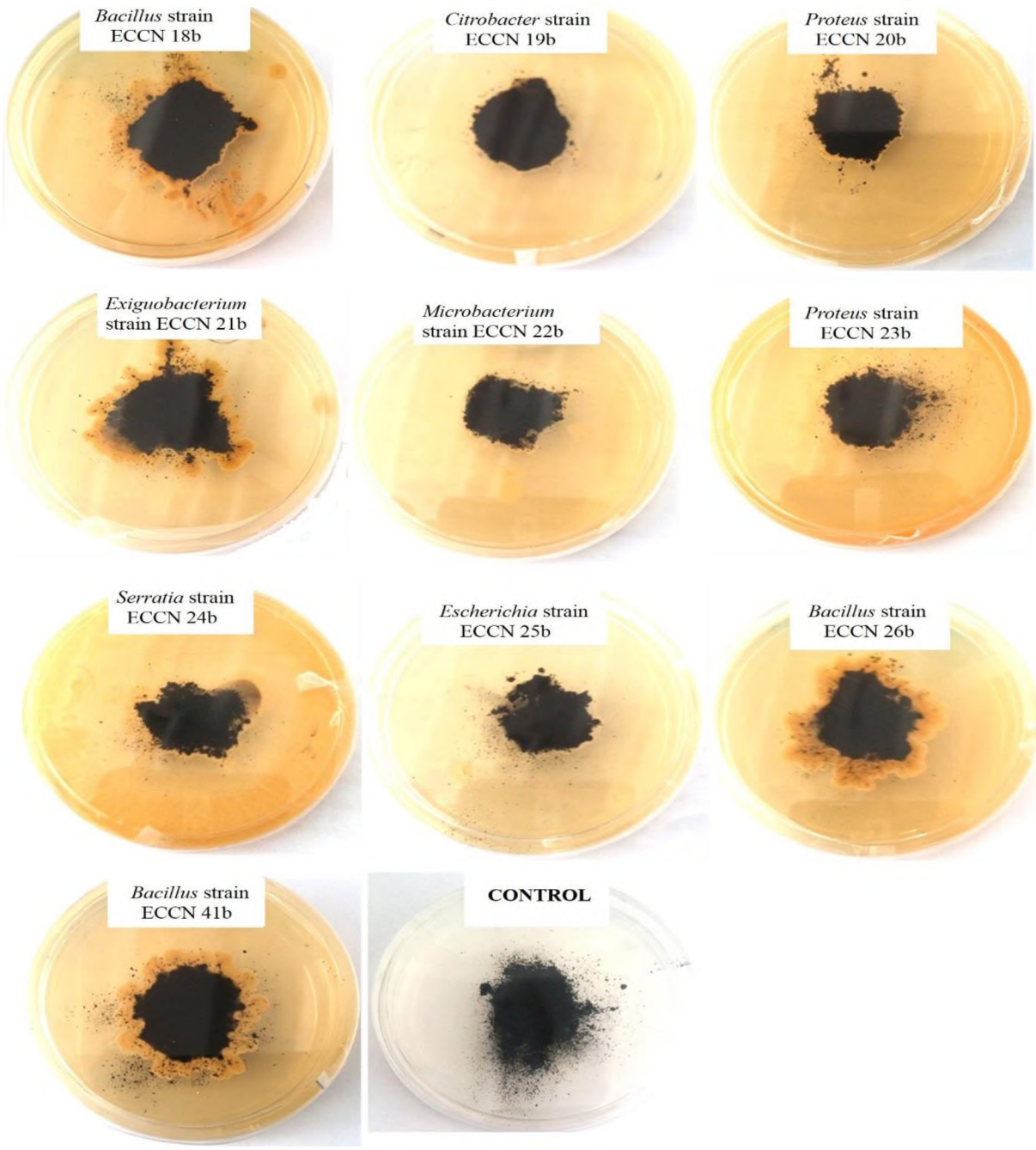


Figure 2.2 Coloured halos indicating bacterial biodegradation of geologically weathered coal in solid culture. No coloured halo was observed in control.

2.3.3.2 Liquid culture screening

Following degradation capability of these bacterial strains on coal discard and geologically weathered coal substrates on MSM agar plates (plate screening), further screening was performed in liquid culture using MSM. Erlenmeyer flasks containing 150 mL MSM and 1 g

coal substrate was inoculated with bacterial strains and incubated at 37 °C without shaking for 21 d. The pH of the culture medium after adding coal substrates (coal discard and weathered coal) declined from 6.82 to 6.52 –6.70 in all cultures in the first 3 d (Figure 2.3). The pH started to rise after 3 d to pH 7.00 at 18 d. The pH of un-inoculated controls declined from 6.82 to 6.50 –6.37. These results showed that there was no appreciable increase in pH of the culture medium. The decrease in pH of the control might be as a result of the acidic nature of coal substrates (Yuan *et al.*, 2013; Romanowska *et al.,* 2015). The brown/yellowish colour of the culture medium (Figures 2.4 and 2.5) was used as an indicator of bacterial ability to degrade coal discard and geologically weathered coal. The results of the coal discard and geologically weathered coal degradation in liquid culture medium (MSM) showed that all bacterial strains individually developing a brown colour after 21 d incubation (Figures 2.4 and 2.5). Colour change was not observed in the uninoculated control.

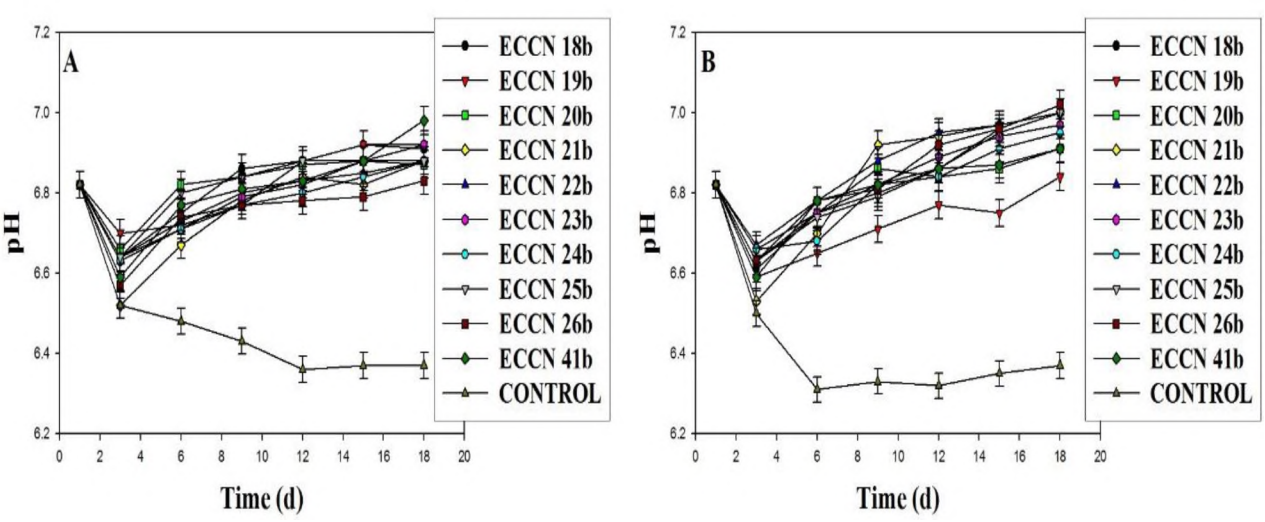


Figure 2.3 pH change in MSM containing coal discard (A) and geologically weathered coal (B) after inoculation with *Bacillus* strain ECCN 18b, *Citrobacter* strain ECCN 19b, *Proteus* strain ECCN 20b, *Exiguobacterium* strain ECCN 21b, *Microbacterium* strain ECCN 22b, *Proteus* strain ECCN 23b, *Serratia* strain ECCN 24b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b and *Bacillus* strain ECCN 41b and incubated at 37 °C without shaking for 21 d. Error bars represent the standard error of at least three replicates

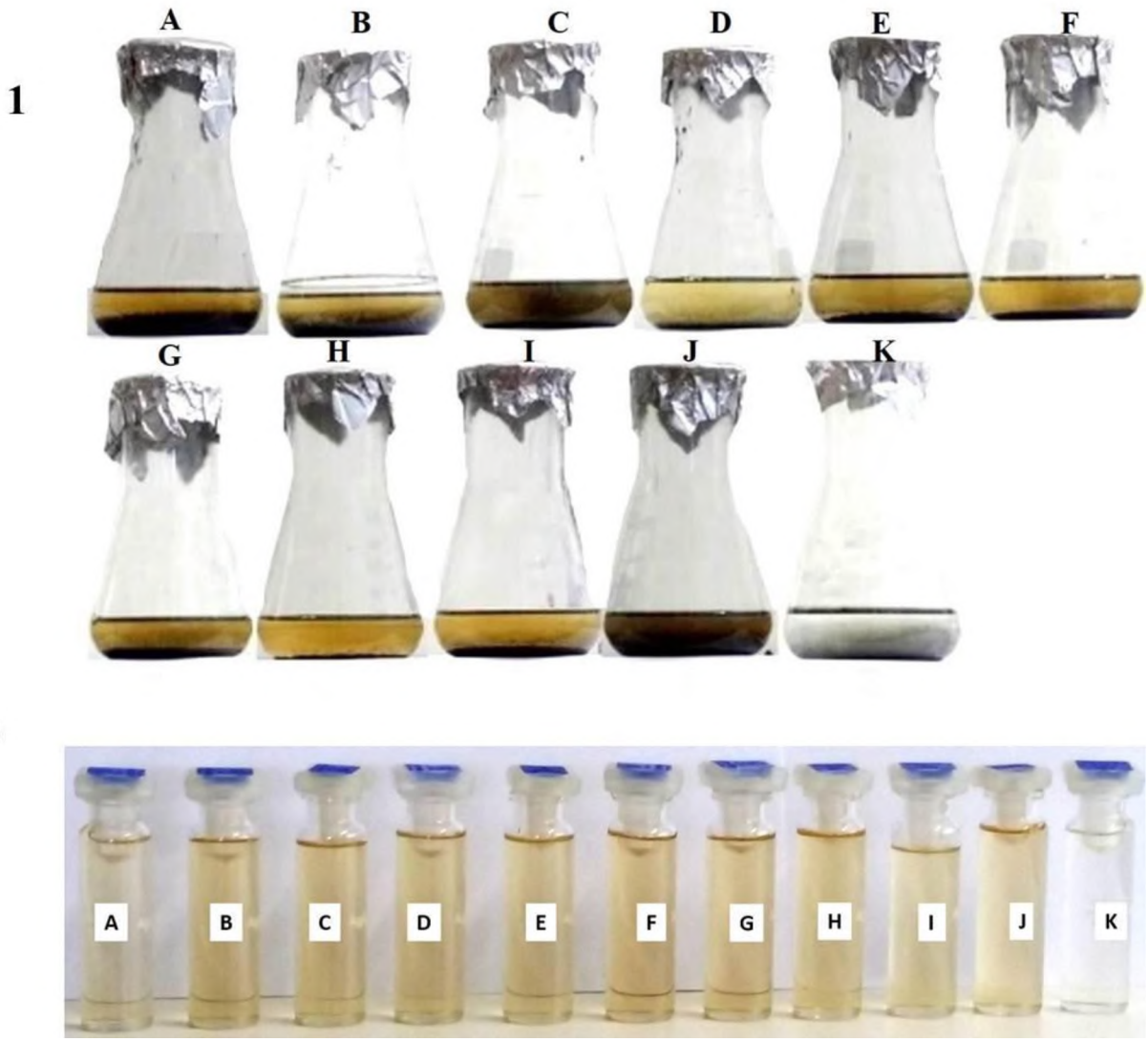


Figure 2.4 Flasks containing MSM medium with coal discard (1) and supernatants (2) inoculated with *Bacillus* strain ECCN 18b (A), *Citrobacter* strain ECCN 19b (B), *Proteus* strain ECCN 20b (C), *Exiguobacterium* strain ECCN 21b (D), *Microbacterium* strain ECCN 22b (E), *Proteus* strain ECCN 23b (F), *Serratia* strain ECCN 24b (G), *Escherichia* strain ECCN 25b (H), *Bacillus* strain ECCN 26b (I), *Bacillus* strain ECCN 41b (J) and uninoculated Control (K) incubated at 37 ^{o}C without shaking for 21d. The supernatants were centrifuged and filtered to obtain cells and particles free filtrate.

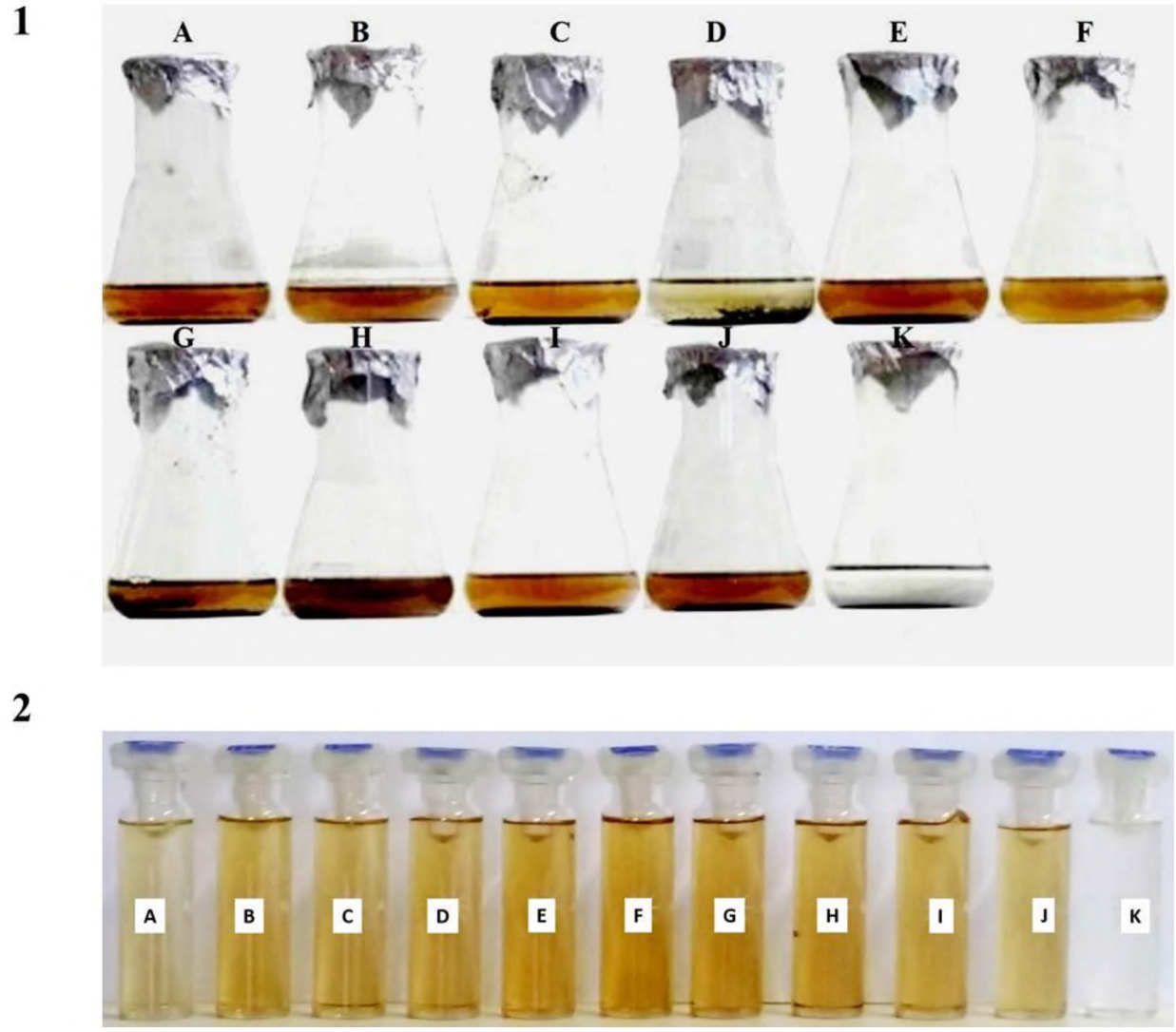


Figure 2.5 Flasks containing MSM medium with geologically weathered coal (1) and supernatants (2) inoculated with *Bacillus* strain ECCN 18b (A), *Citrobacter* strain ECCN 19b (B), *Proteus* strain ECCN 20b (C), *Exiguobacterium* strain ECCN 21b (D), *Microbacterium* strain ECCN 22b (E), *Proteus* strain ECCN 23b (F), *Serratia* strain ECCN 24b (G), *Escherichia* strain ECCN 25b (H), *Bacillus* strain ECCN 26b (I), *Bacillus* strain ECCN 41b (J) and uninoculated Control (K) incubated at 37 °C without shaking for 21d. The supernatants were centrifuged and filtered to obtain cells and particles free filtrate.

2.3.4 Evaluation of coal biodegradation potential

Table 2.5 shows the ranking of the bacterial strains for the potential degradation for coal discard and geologically weathered coal. Brown halo formation around the bacterial strains colony was considered wider in diameter for *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN

21b and *Serratia* strain ECCN 24b on MSM agar plates containing either coal discard or geologically weathered coal, when the period of incubation was extended beyond 14d .

Furthermore, UV/Vis spectroscopy analysis revealed an increase in the absorbance at 450 nm and 280 nm of biosolubilisation product of both coal discard and geologically weathered coal treated with the following bacterial strains: *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in MSM. In addition, there was a minor change in the pH of culture media during the biodegradation process by these bacterial strains of both coal discard or geologically weathered coal. Based on these parameters, *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b showed similar results based on the ranking (Table 2.5), and were therefore considered more efficient than other bacterial strains in the degradation of coal discard and geologically weathered coal in both MSM agar plates or MSM liquid medium containing coal substrates.

Table 2.5 Ranking of bacterial coal degradation potential

	Coal discard					Geologically weathered coal				
Bacterial strains	Zone of brown halo	pH	Colour of supernatant	A_{450}	A_{280}	Zone of brown halo	pH	Colour of supernatant	A_{450}	A_{280}
Citrobacter strain ECCN 19b	+++	6.92	+++	+++	+++	+++	6.92	+++	+++	+++
Exiguobacterium strain ECCN 21b	+++	6.89	+++	+++	+++	+++	6.98	+++	+++	+++
Serratia strain ECCN 24b	+++	6.89	+++	+++	+++	+++	6.95	+++	+++	+++
Bacillus strain ECC 18b	+++	6.83	+++	+++	++	+++	6.91	+++	++	++
Microbacterium strain ECCN 22b	+++	6.87	++	++	++	++	6.92	++	++	++
Escherichia strain ECCN 25b	++	6.85	++	++	++	++	6.84	++	++	++
Bacillus strain ECCN 26b	+++	6.88	+++	+++	++	+++	6.94	+++	+++	++
Bacillus strain ECCN 41b	+++	6.88	+++	+++	++	+++	6.91	++	++	++
Proteus strain ECCN 23b	++	6.40	++	++	++	++	6.90	++	++	++
Proteus strain ECCN 20b	++	6.84	++	++	++	++	6.89	++	++	++
Control	-	6.37	-	-	-	-	6.35	-	-	-

Zone of brown halo; ++, 5 -15 mm; +++, 15 - 25 mm

Colour of supernatant; ++, light brown; +++, deep brown

pH; ++, <6.9; +++, >6.9

$A_{280;}$ ++, 0.00 – 1.00; +++, 1.00 – 2.00

$A_{450;}$ ++, 0.00 – 0.15; +++, 0.15 – 0.30

2.4 Summary

The results of screening bacterial strains for coal discard and geologically weathered coal degradation potential using solid and liquid screening methods showed that all the bacteria strains: *Bacillus* strain ECCN 18b, *Citrobacter* strain ECCN 19b, *Proteus* strain ECCN 20b, *Exiguobacterium* strain ECCN 21b, *Microbacterium* strain ECCN 22b, *Proteus* strain ECCN 23b, *Serratia* strain ECCN 24b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b and *Bacillus* strain ECCN 41b, have the ability to degrade coal discard and geologically weathered coal individually, but the extent of degradation was greatest in *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b when the period of incubation was prolonged. The extent of these bacteria degradation potential is based on the evaluation by ranking, using parameters such as the diameter of the brown halo on the bacteria solid culture plates, and the colour intensity of supernatants of the bacterial strains culture (liquid) in the presence of either coal discard or geologically weathered coal. In addition, indicators such as pH, the intensity of brown/yellow colour, absorbance at 280 nm and 450 nm related to coal biodegradation ability were also used. Before the screening exercise, bacterial strains DNA analysed using pyrosequencing of 16S rDNA gene confirmed the identity sequences indicating pure bacterial strains was used for the study. This work highlights the importance of conducting a detailed screening study of bacteria degradation potential on coal before characterisation of both soluble and insoluble biodegradation products, or any coal biotechnology can be implemented.

CHAPTER 3

Growth and colonisation of different bacterial strains on discard and geologically weathered coal

3.1 Introduction

Adherence of the bacterial strains to coal substrate is an important step in the biotechnological study of coal biodegradation. Many studies have examined bacteria attachment to coal as a first step in coal biodegradation (Chen and Skidmore, 1988; Vitaya and Toda, 1991). Results from these experiments have successfully shown bacterial modification of the physical and chemical structure of coal due, presumably, to bacterial attachment. During the process of attachment, organisms are brought into close proximity with the surface (Prakash *et al.*, 2003). The main components of the cell surface that may contribute to the adhesion properties of the microorganisms are the extra polymeric substance (EPS), lipopolysaccharide (LPS) and outer membrane protein (Lugtemberg *et al.*, 1983).

Exopolymeric substances (EPS) are organic molecules formed by polymerisation of organic fractions, carbohydrates, proteins, and humic substances (Bramhachari and Dubey, 2006). They are known to be produced during the process of microbial adherence to a particular substrate (Geesey and White, 1990), otherwise responsible for the interaction of bacteria with each other as well as with interfaces (Flemming 2002; Neu *et al.*, 2001). Nonetheless, they are secreted by microorganisms and are mostly composed of polysaccharides, protein, lipids and humic substances. Percentage composition of these substances are: exopolysaccharide (40 – 95%), protein (1 –60%), nucleic acids (1 –10%), and lipids (1 –40%) (Davey and O'Toole, 2000; Flemming and Wingende, 2001). Lipopolysaccharide plays a role in the interaction of some microbial cells with the solid surface (Labishchinski *et al.*, 1985). In general, the attachment process to a surface is predominantly mediated by the production of EPS. Initial studies of bacterial cell-coal surface interactions focus on the presence of EPS observed on SEM images i.e. *Pseudomonas fluoroscens* (Hazrin-Chong and Manefield, 2012).

Scanning electron microscopy provides a means of observing bacteria cell adhesion to various substrates, including coal. The technique has been used to observe at high resolution the morphology of bacteria adherences to the material surface, the morphology of the material surface, the relationship between them (Peters *et al.*, 1982), and to analyse initial cell attachment and colonisation (Manefield and Hazrin-Chong, 2012). The ability of microorganisms to grow

depends on the availability of nutrients within the medium (e.g. sulphur and carbon as a source of energy), and the diversity of environments that support growth is the fundamental corpus of scientific knowledge (Makie *et al.*, 2014). Although, growth conditions for microorganisms differ from one environment to the other, it is imperative that bacterial growth is assessed under conditions that are similar to the natural environment (Novak *et al.*, 2009).

The ability of bacteria to grow on coal can indicate that they use coal as the carbon source. Various methods have been explored in order to quantify bacterial growth on coal substrates. Previous studies demonstrated and confirmed the use of protein concentration (Tripathi and Singh, 2011) and turbidity (Baylon *et al.*, 2017) as indicators of bacterial growth. However, the latter may not be appropriate because of the interference of insoluble substances released from biodegraded coal. To facilitate the ease and speed of growth measurement in the present study, a molecular technique based on the indirect-quantification of bacterial deoxyribonucleic acid (DNA) estimation using diphenylamine (DPA) was developed (Zhao *et al.*, 2013).
Of the 10 bacterial strains screened in Chapter 2, *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were considered to possess the highest potential for coal biodegradation. These three strains, individually and in the consortium, were used to further study bacterial colonisation and degradation of coal. In this chapter, studies were carried out to determine bacterial growth using coal as the sole carbon source, and whether these bacteria produce EPS in the presence of coal substrates. Electron microscope analysis was used to examine and confirm colonisation of substrate coal by these bacteria.

3.2 Materials and methods

3.2.1 Determination of bacterial growth on coal substrates

The cell density of growth rate and biomass productivity of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b on either coal discard or geologically weathered coal were investigated in a mineral salt medium (MSM). The growth was determined by quantification of deoxyribonucleic acid (DNA) using diphenylamine reagent (colorimetric method). Diphenylamine reagent was prepared by dissolving 1.5 g of reagent grade diphenylamine in 100 mL glacial acetic acid followed by the addition of 1.5 mL concentrated H_2SO_4 (Zhao *et al.*, 2013). On the day of use, a fresh solution of 1 mL acetaldehyde

in 50 mL distilled water was prepared and 0.5 mL of this solution was added to 100 mL of the diphenylamine solution.

Bacteria colonies from the plates were inoculated into 10 mL of mineral salt medium (MSM) and incubated overnight at 37 ^{o}C (primary culture) with constant shaking at 120 rpm. The seed cultures were harvested by gentle centrifugation at 5000 rpm for 5 min and rinsed in sterile PBS to remove the waste products produced during growth and also to change the carbon/nutrient source. The supernatant was discarded and appropriate dilutions prepared using MSM, enriched with 4 mL of trace element solution (TES) per litre of distilled water. Two (2 mL) millilitres of bacteria culture was inoculated into 250 mL Erlenmeyer flasks containing 150 mL MSM plus 1 g of sterile coal discard or geologically weathered coal.

The controls used for this experiment were (1) un-inoculated coal in MSM and (2) inoculated MSM only. All the experiments were performed in triplicate. Cultures were incubated on a rotatory shaker at 37 ^{0}C at 120 rpm for 7 d. Aliquots of 1.5 mL were transferred aseptically to a sterile Eppendorf tube and centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the bacterial cell pellets washed in PBS, resuspended in 3 mL diphenylamine reagent, and incubated at 60 ^{o}C for 1 h (Figure 3.1). This was centrifuged again, and the supernatant pipetted into 2 mL cuvettes; the absorbance of the blue colour formed at 600 nm was measured using a UV-VIS Spectrophotometer, Shimadzu (UV-1201, Japan). The triplicate values were averaged and A_{600} was plotted against time (d).

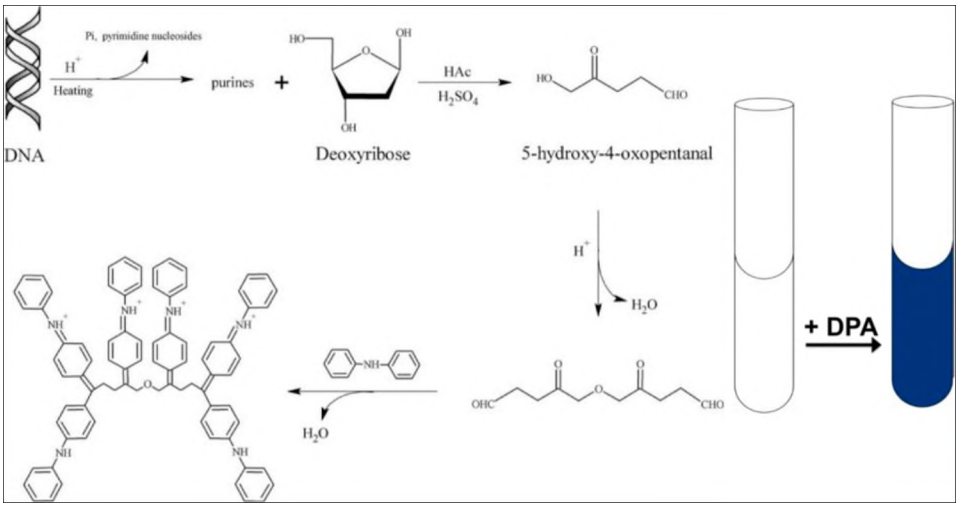


Figure 3.1 Mechanism of diphenylamine colorimetric reaction of bacterial strains DNA quantification (modified after Zhao *et al.*, 2013)

3.2.2 Scanning electron microscopy (SEM) of bacteria on coal

Residual weathered coal and coal discard that had been incubated with bacterial strains for 14 d were taken out of the culture medium by centrifugation at 5000 rpm for 5 min. The pellet was washed with MSM and distilled water to remove unattached bacterial cells. The coal residue containing adhered bacteria was fixed in 2 mL 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer for 24 h at 4 °C. After removal of glutaraldehyde solution, the samples were washed twice with PBS and then dehydrated using a graded series of ethanol solution with concentrations; 30, 50, 70, 80, 90, and 100% first for 5 min, and then for 10 min. Dehydrated samples were immersed in 1 mL 100% hexamethyldisilazane (HMDS) for 10 min. After 10 min, HMDS was decanted and the samples left to air-dry at room temperature in the fume cupboard overnight. After drying, the samples were mounted on specimen stubs with double-sided conductive tape, gold-coated, and then viewed using a Vega 3 LMU (TESCAN, Brno, Czech Republic) analytical scanning electron microscope at 30 kV. The number of adhered bacteria was determined by averaging the counts per observation field. At least 20 images of each sample were analysed.

3.2.3 Transmission electron microscopy (TEM) of bacteria on coal

Following incubation of coal discard and geologically weathered coal in MSM inoculated with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, *Serratia* strain ECCN 24b respectively, 15 mL of the culture medium was harvested, centrifuged and the cell pellet suspended in phosphate buffer. The sample for analysis was prepared by placing 100 µL of a very dilute solution of bacteria pellet in phosphate buffer on a standard carbon-coated copper TEM grid. The sample was allowed to evaporate slowly at room temperature and the excess removed using blotting paper. Micrographs were obtained using a ZEISS LIBRA 120 °C TEM at a 90 kV accelerating voltage.

3.2.4 Extraction of extracellular polymeric substance (EPS) from liquid culture of bacterial

Following incubation of the culture medium, the whole content of the flask's medium was centrifuged at 10000 rpm for 20 min. The supernatant was decanted and mixed with an equal volume of chilled ethanol. The mixture was agitated and kept overnight at 4 °C for precipitation of EPS. This was followed by centrifuging at 10000 rpm for 20 min to precipitate EPS. After centrifugation, the precipitate was collected and suspended in 10 mL distilled water followed

by lyophilization at -80 °C. The weight of the freeze dried EPS was determined and then analysed, using Fourier Transform Infrared (FTIR) spectroscopy.

3.3 Results

3.3.1 Growth studies of bacterial strains on coal substrates

The bacterial strains, *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, *Serratia* strain ECCN 24b grew successfully on nutrient agar plates, developing colonies with variations in morphology such as size, shape and colour (see appendix II). The growth was also evaluated in LB to obtain the conventional growth curve profiles. The growth curves obtained from nutrient broth for the incubation period for all cultures consisted of a characteristic lag phase and exponential phase (Figure 3.2).

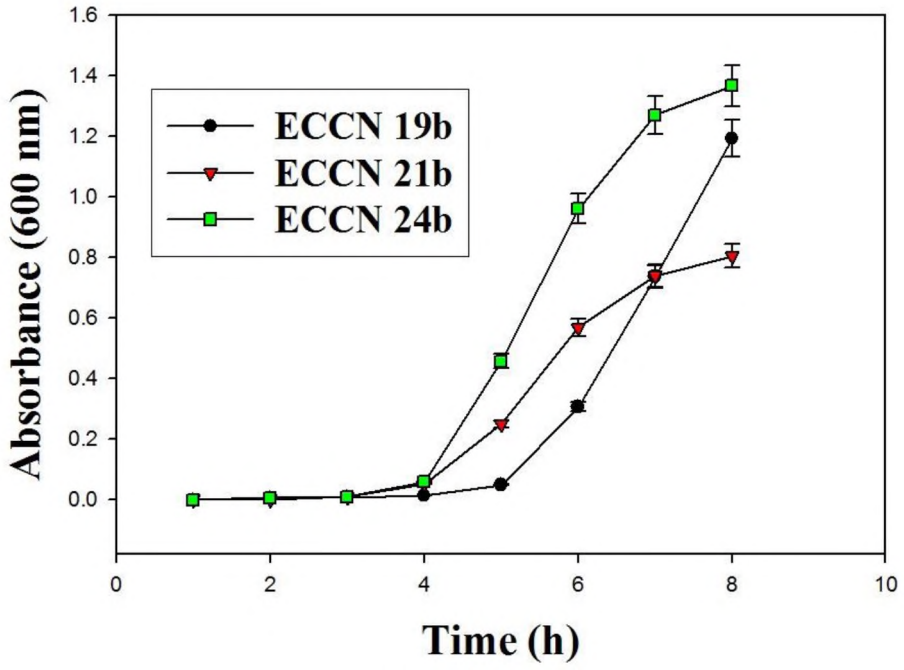


Figure 3.2 Growth curves of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, and *Serratia* strain ECCN 24b in Luria broth (LB). A colony of bacteria was inoculated into Luria broth and incubated on a rotatory shaker at 120 rpm at 37 °C for 12 h. At 1 h intervals, biomass accumulation was determined based on turbidity by measuring the absorbance at 600 nm. Error bars represent standard error of at least three determinations

Figure 3.3 shows the accumulation of DNA in an exponential culture with time (d). Biomass productivity of the three organisms using diphenylamine reagent (for DNA quantification) in coal discard and geologically weathered coal increased rapidly for first three days and maximum values 1.7, 1.9 and 1.7, for ECCN 19b, ECCN 21b and ECCN 24b, respectively (Figure 3.3 A), for all bacterial strains on coal discard of an absorbance at 600nm were obtained. Maximum values on geologically weathered coal were 2.0, 2.0 and 1.9, for ECCN 19b, ECCN 21b and ECCN 24b, respectively (Figure 3.3 B), suggesting higher growth on this coal substrate. By the time maximum productivity occurred, the value remained more or less stable (Figure 3.3).

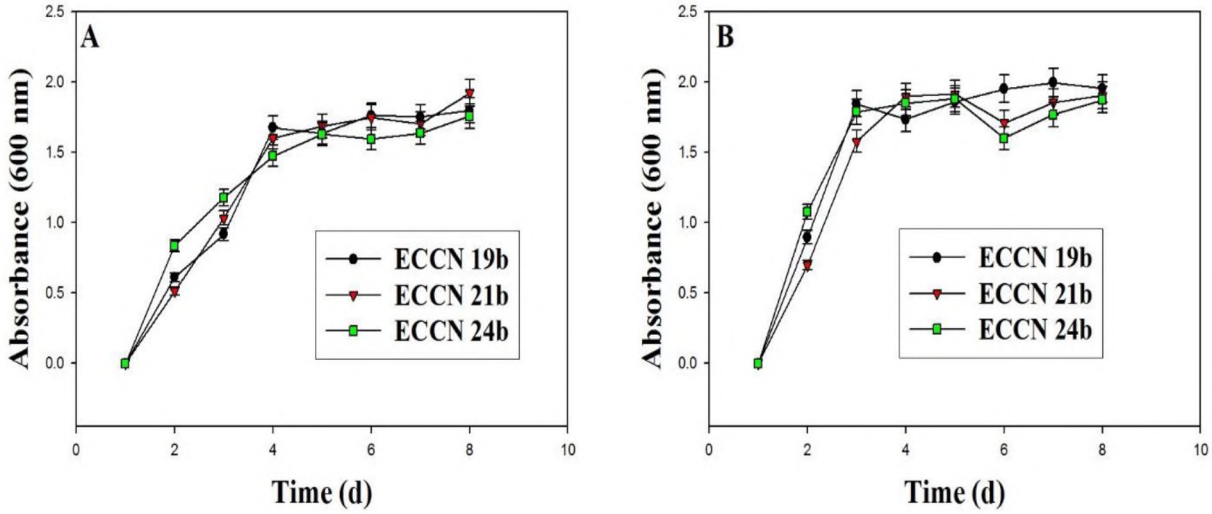


Figure 3.3 Growth curves of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in MSM containing either coal discard (A) or, geologically weathered coal (B). Bacterial strains were inoculated and incubated for at 37 °C for 7 d. At 1 d intervals, growth was determined using the diphenylamine reagent at 600nm. Error bars represent standard error of at least mean of three measurement

The growth curve of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b obtained using LB and MSM in either coal discard or geologically weathered coal were quite similar but maximum growth differed in the tested media. Since bacteria organisms are in the process of replication, they are said to contain more than one genome equivalent of DNA detected as a blue colour, and measure at an absorbance of 600 nm. The genome equivalent in bacteria strains increased exponentially with the days.

The liquid medium containing coal substrate as a carbon source showed a higher rate of growth compared to the control as measured at an absorbance of 600nm. Little or slow growth was observed in the mineral salt medium inoculated with organisms because carbon, sulphur, and other nitrogenous sources were absent or at low levels or concentrations (Figure 3.3).

However, using diphenylamine reagent, the exponential phase was observed immediately after the lag phase, followed by a stationary phase after 4d of incubation (Figure 3.3). Varying concentrations of DNA, as shown by the optical density of the blue colour based on the accumulation of DNA, is assumed to be a function of bacterial replication. Thus, DNA quantification from the bacterial strains is a reference for biomass on coal discard and geologically weathered coal. The difference in the growth pattern of microorganisms is a function of the nutritional content (carbon and nitrogen) of the substrate i.e. coal, as the source of energy.

The source of energy requirement differed from one organism to another and this depends on the substrate on which they were grown. The growth of the bacterial strains was slower in the coal discard medium, with the stationary phase reached at day 4 (Figure 3.3 A). In Figure 3.3 B, growth is more rapid and the stationary phase is reached at day 3. This difference may be attributed to the more oxidised nature/ status of geologically weathered coal as confirmed in the previous Chapter by a greater concentration of oxygen. As shown in the results, the growth of these bacterial strains was increased in the presence of coal discard and geologically weathered coal (Figure 3.3). Results indicate that a medium with coal as the substrate promotes the growth of the three bacterial strains.

3.3.2 Bacterial colonisation of coal discard and geologically weathered coal

3.3.2.1 Scanning electron microscopy (SEM) analysis of bacterial strains on coal discard and geologically weathered coal

Scanning electron microscopy (SEM) was used to monitor surface colonisation of coal discard and geologically weathered coal by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. Figures 3.4, 3.5 show the scanning electron micrographs of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b attachment to coal discard and geologically weathered coal particles. Bacteria cells ranging from short to long rod morphology up to 2 µm —10 µm in length were found to be attached to coal substrates. Using the extracellular polymeric substance (EPS), some

bacterial strains were attached to the coal substrate individually, while others were assemblages (clusters) on surfaces and crevices.

Micro colonies of *Citrobacter* strain ECCN 19b up to 10 μm in length were observed on the surface of geologically weathered coal particles. Attached bacterial strains on the coal substrates were estimated by averaging the number of cells on at least 15 different fields on each sample at a different magnification and in different locations on the sample surfaces. An average of about 1.8×10^8 —6×10^7 cells/mm^2 bacterial strains were found attached to coal discard and geologically weathered coal. Geologically weathered coal had higher number of bacterial cells attached than coal discard (Table 3.1). The average length of bacterial long rod cells was approximately 1257.40 nm and short rod cells were 119.16 nm; observations that are similar in size and shape of the organisms on coal observed with TEM.

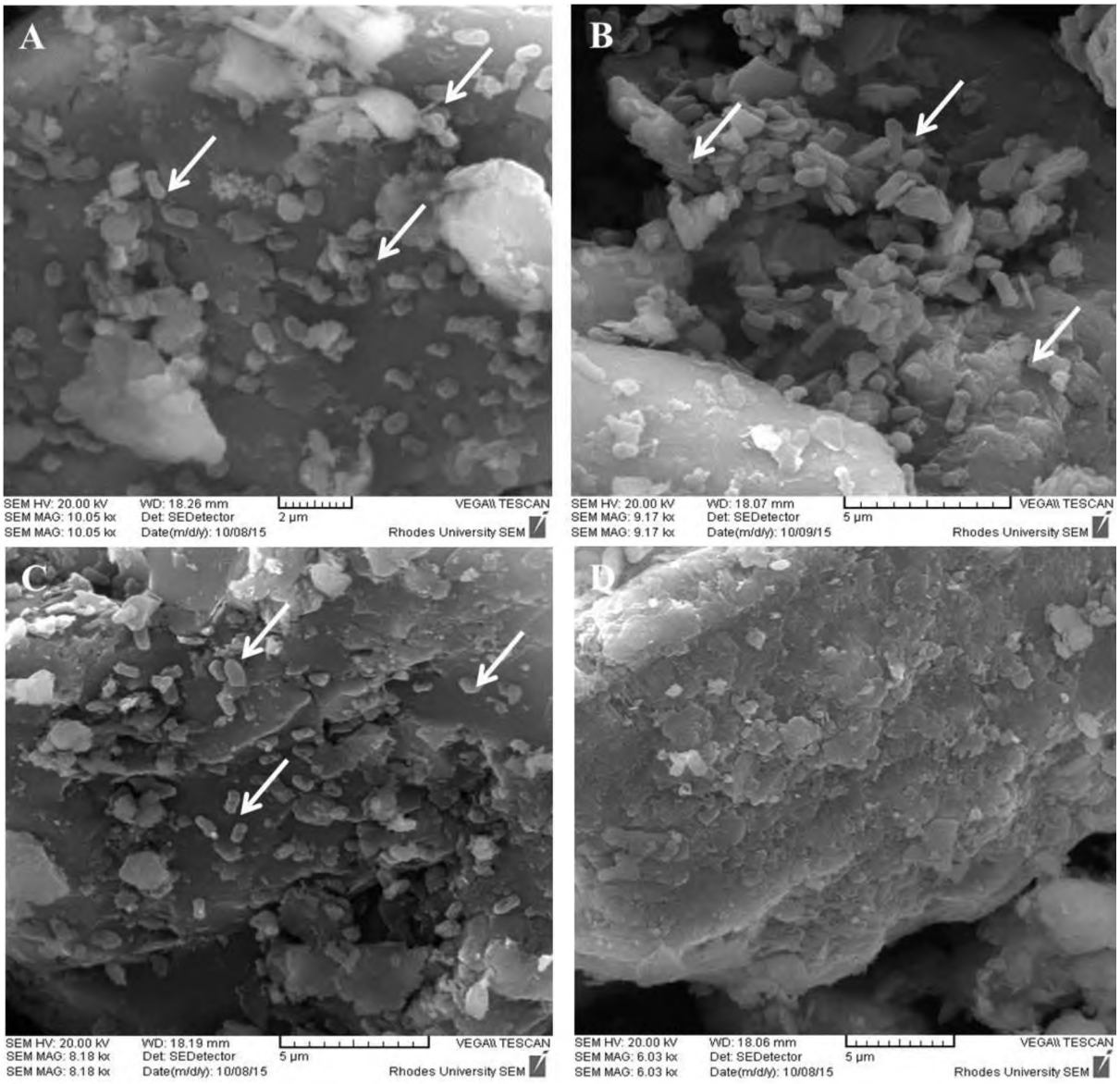


Figure 3.4 Scanning electron micrographs showing attachment (arrows) of *Citrobacter* strains ECCN 19b (A), *Exiguobacterium* strain ECCN 21b (B), and *Serratia* strain ECCN 24b (C) to the surface of coal discard particles after 14d of incubation at 37 °C. D, uninoculated coal discard. Arrows indicate bacterial colonisation and attachment to coal.

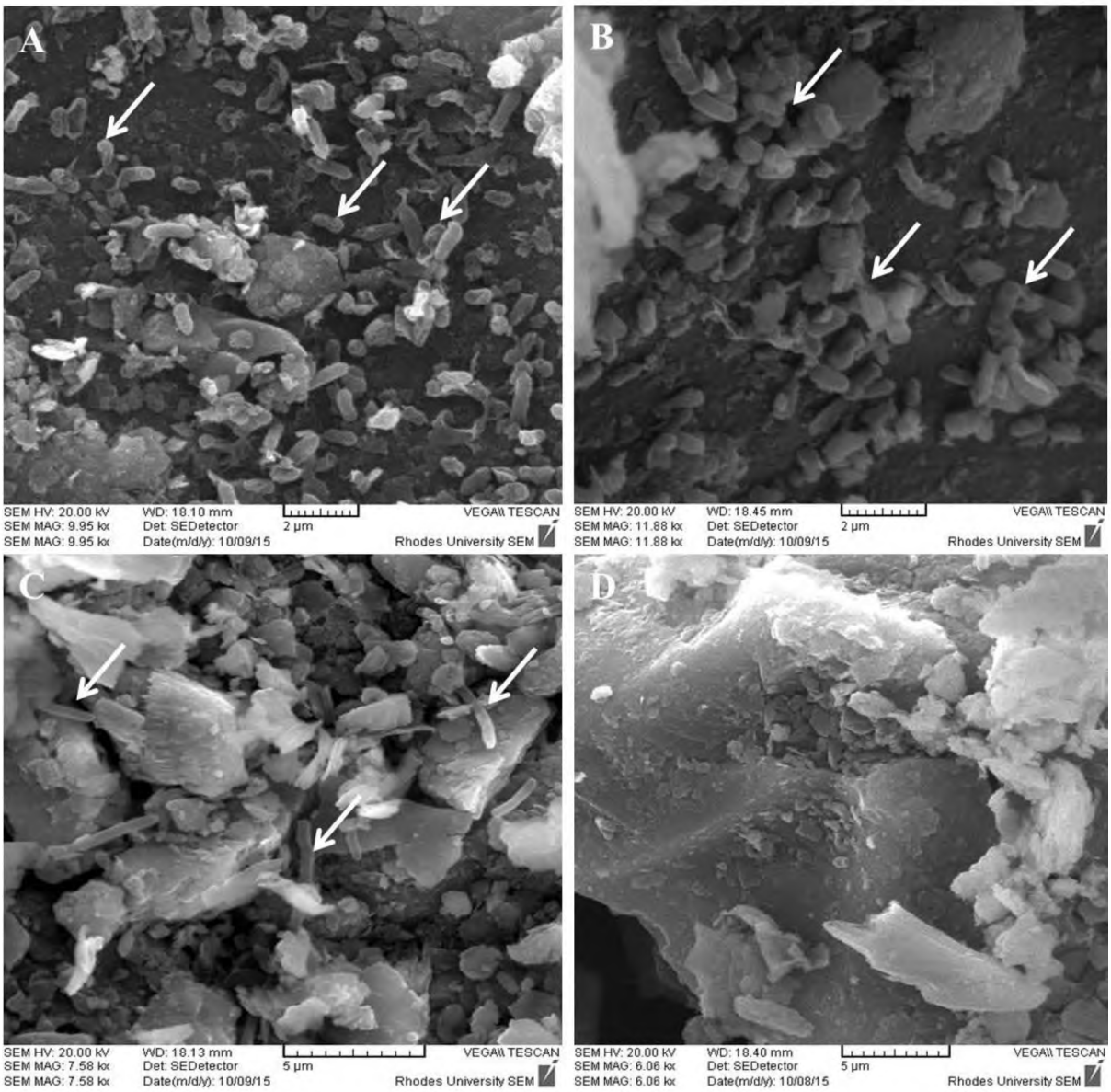


Figure 3.5 Scanning electron micrographs showing attachment (arrows) of *Citrobacter* strains ECCN 19b (A), *Exiguobacterium* strain ECCN 21b (B) and *Serratia* strain ECCN 24b (C) to the surface of geologically weathered coal particles after 14d of incubation at 37 °C. D, uninoculated geologically weathered coal. Arrows indicate bacterial colonisation and attachment to coal.

Table 3.1 Bacteria cell density on coal, based on SEM images

Bacteria	Unit	Coal discard	Geologically weathered coal
Citrobacter strain ECCN 19b	cells/mm^2	2.1×10^8	3.9×10^8
Exiguobacterium strain ECCN 21b	cells/mm^2	2.8×10^8	3×10^8
Serratia strain ECCN 24b	cells/mm^2	1.8×10^8	6×10^7

3.3.2.2 Transmission electron microscopy (TEM) analysis of bacteria on coal substrates

Transmission electron microscopy (TEM) images reveal distinct phenotypic structure of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b such as shape, size, flagellum and production of EPS on coal substrates (Figure 3.6). These bacterial strains in the presence of coal were surrounded by what appears to be extracellular polymeric substances (EPS) as electron-dense materials around the bacteria cells (see arrows). From the micrograph, *Citrobacter* strain ECCN 19b and *Serratia* strain ECCN 24b are shown to have flagella. This different phenotypic variation (EPS/or flagellum) observed among these bacterial strains on coal are evidence of their colonisation and growth on coal discard and geologically weathered coal substrates.

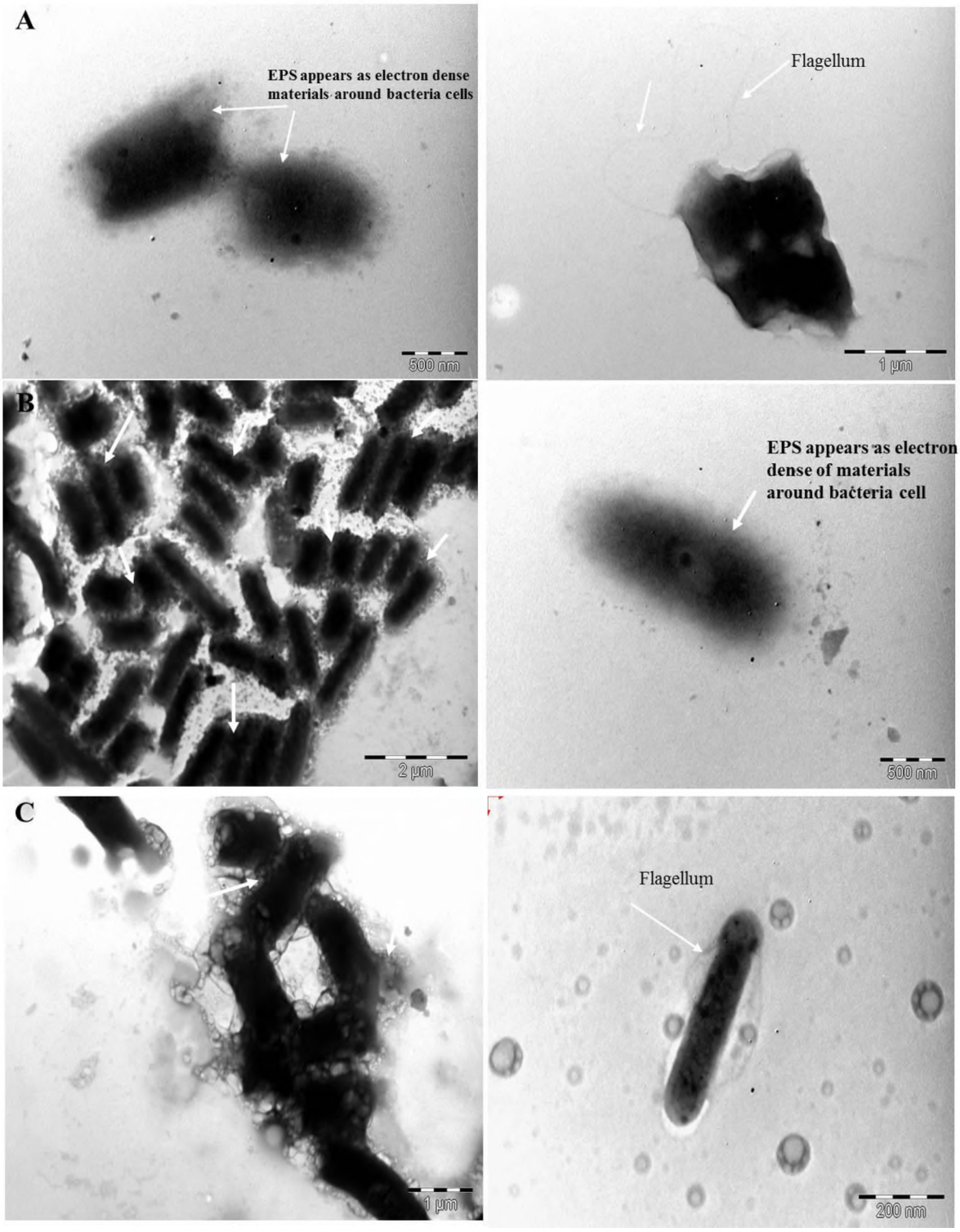


Figure 3.6 TEM images of *Citrobacter* strain ECCN 19b (A), *Exiguobacterium* strain ECCN 21b (B) and *Serratia strain* ECCN 24b (C) grown on coal discard after 14d of incubation at 37 ^{o}C. Arrows indicate electron dense material possibly, EPS and/or flagella

3.3.2.3 Extracellular polymeric substance (EPS) of bacterial strains on coal-induced liquid culture after a period of incubation

Extracellular polymeric substances (EPS) produced by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, *Serratia* strain ECCN 24b and bacteria consortium in MSM containing either coal discard or geologically weathered coal incubated at 37 °C for 21d was extracted using chilled ethanol to precipitate the culture filtrates which were kept overnight at 4 °C and lyophilized at -80 °C followed by its characterisation using fourier transform infrared (FTIR). Figure 3.7 represents the IR spectrum of EPS produced by the bacterial strains. The spectrum revealed different functional groups such as broad and intense stretching characteristics of a hydroxyl group (O-H), asymmetrical C-H stretching of aliphatic C-H groups, N-H bonding (Amide I & II) corresponding to the functional groups in protein, stretching C-O-C corresponding to the presence of carbohydrates, and O-H bonding of carboxylic acid.

EPS of different bacterial strains accumulated in MSM containing either coal discard or geologically weathered coal revealed the characteristic of absorption bands of EPS (crude) in the medium without coal substrate. However, differences were observed in the absorption intensity mostly in the protein and carbohydrate peak (Figure 3.8). This EPS demonstrated the adherence effects of bacteria during colonisation on the coal discard and geologically weathered coal. However, this result clearly showed that the enhancement of cell adherence on coal substrates due to the production of EPS by the organisms was relevant to all the bacteria strains examined.

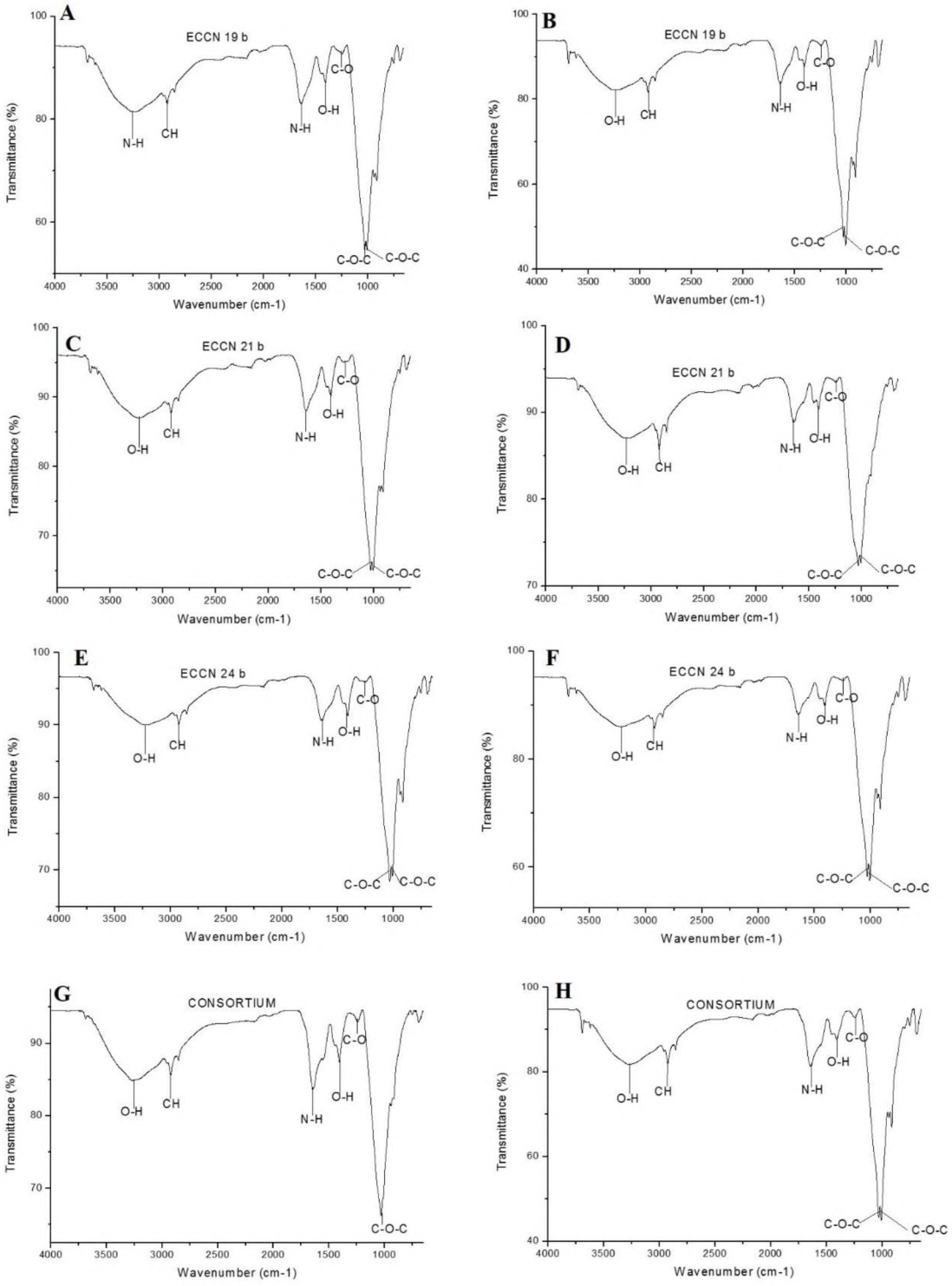


Figure 3.7 FTIR spectra of EPS produced on coal discard (A, C, E, G) and geologically weathered coal (B, D, F, H) by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, *Serratia* strain ECCN 24b and consortium of bacterial strains mix inoculated in MSM containing coal discard (A) and geologically weathered coal (B) incubated at 37 ^{o}C for 21 d.

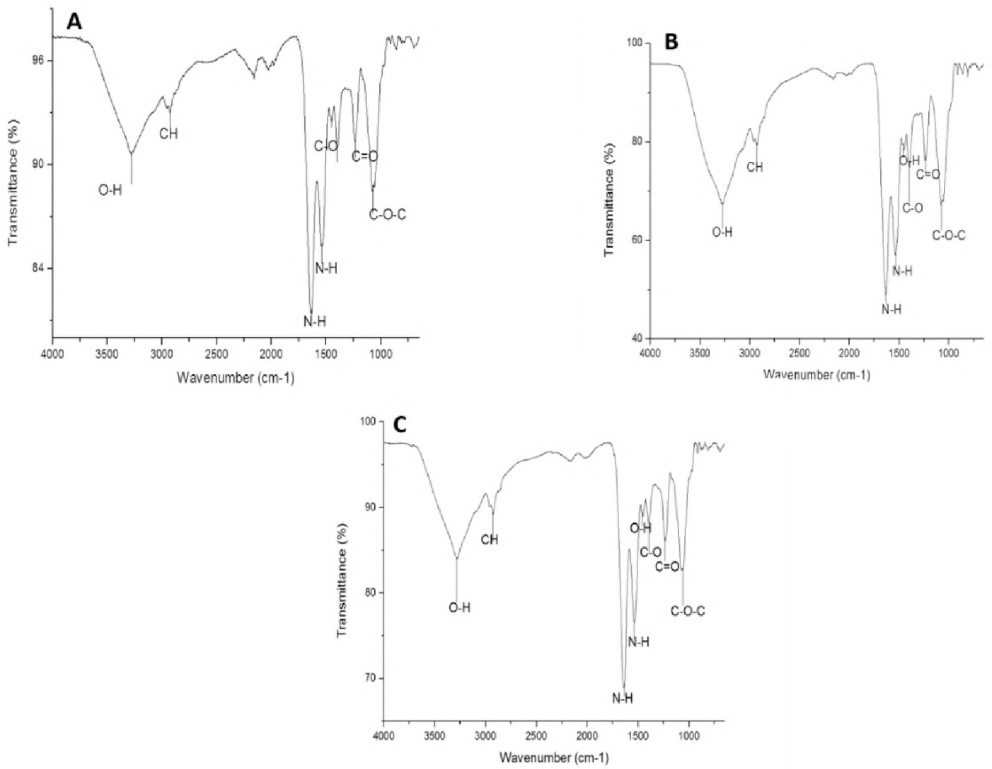


Figure 3.8 FTIR spectra of crude EPS produced by *Citrobacter* strain ECCN 19b (A), *Exiguobacterium* strain ECCN 21b (B), and *Serratia* strain ECCN 24b (C) in LB incubated at 37 ^{o}C for 24 h.

3.4 Summary

The results of the studies confirmed the growth and colonisation of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b on coal discard and geologically weathered coal substrates. Results showed that the bacterial strains' genomic DNA increased exponentially within days in culture mediums containing either coal discard or geologically weathered coal. Growth curves depicted from the molecular technique indicated that bacterial strains utilise components of coal discard and geologically weathered coal as the sole source of carbon. SEM and TEM analysis showed the cells of bacterial strains strongly attached to coal substrates. The micrographs show flagella and EPS produced by the bacterial strains when growing in the presence of coal discard and geologically weathered coal, which could be used for the attachment and uptake of nutrient from coal substrate. The growth of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN

24b on coal discard and geologically weathered coal resulted in the production of EPS, and an increase in the number of bacterial strain cells attached to the coal particle surface confirmed the capability to utilise coal as the energy source.

CHAPTER 4

Bacterial biodegradation of coal discard and geologically weathered coal

4.1 Introduction

Citrobacter strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were selected as promising candidate strains for further study. They were shown to colonise coal discard and geologically weathered coal and use these substrates for growth, as demonstrated in Chapter 3. The ability of an organism to utilise a substrate as the sole carbon source for growth implies that biodegradation of the substrate must have occurred (Romanowska *et al.*, 2015).

Many bacteria are able to degrade recalcitrant materials such as coal, e.g. *Rhodococcus ruber*, *Rhodococcus erythropolis, Flavobacterium* sp. *Corynebacterium* sp. *Paenibacillus* sp. (Mishra *et al.*, 2017). It is perhaps therefore not surprising that *Citrobacter freundii* has been shown to degrade lignin (Chandra and Abhisek, 2011; Chandra and Bharagava, 2013), that *Exiguobacterium aurantiacum* degrades diesel (Mohanty and Mukherji, 2008) and that *Serratia* sp. degrades hydrocarbons (Benedek *et al.*, 2011; Wi *et al.*, 2012). However, the potential for coal biodegradation by these bacterial species has not been reported yet. Degradation studies of *Exiguobacterium* sp. on diesel were accomplished through the exhibition of high cell surface hydrophobicity when grown on diesel, and established on the basis of contact angle measurement on cells deposited on 0.45µm cellulose acetate buffer (Mohanty and Mukherji, 2008). In the present study, the attractive features of biotechnological importance are the production of peroxidases (MnP, LiP) (Chandra and Bharagava, 2013) and biosurfactant (Ibrahim, 2016) by *Citrobacter freundii*, laccases by *Exiguobacterium* sp. (Hosseini *et al.*, 2012), and *Serratia* sp. *(Kaira et al., 2015)* for degradation of toluene and hydrocarbon, respectively. In addition, *Citrobacter freundii, Exiguobacterium* sp. and *Serratia* sp. produce biosurfactant to degrade used engine oil, petroleum and hydrocarbons (Wongsa *et al.*, 2004; Shende, 2013; Ibrahim, 2016).

The ability of these bacterial strains to colonise and grow on coal discard and geologically weathered coal provided an ideal opportunity to investigate in some detail the bacterial biodegradation of these substrates and to explore the underlying mechanisms involved.

Traditionally, the so-called ABCDE (Alkaline, Biocatalyst, Chelation, Detergent and, Esterase), model of coal biodegradation has been proposed (Fakoussa and Hofrichter, 1999). In this model, microorganisms degrade coal either by producing alkaline substances, oxidative enzymes (biocatalysts), chelators, biosurfactant secretion (detergents), or esterases. The first mechanism (alkaline production) consists of non-enzymatic degradation of coal which occurs at alkaline pH. A rise in pH of the culture medium during coal degradation is an indication of the secretion of alkaline substances by microorganisms (Romanowska *et al.*, 2015). Consequently, alkaline compounds solubilise the acidic group in coal by deprotonation, and increase the hydrophilicity (Ghani *et al.*, 2015). Bacteria species such as *Streptomyces setonii* have been documented for the production of the extracellular compound for solubilising coal substrate (Strandberg and Lewis, 1987), and are thus implicated in this mechanism. The second mechanism, biocatalysts, involves the secretion of enzymes such as lignin-degrading oxidoreductases (peroxidases and laccases) on coal substrates. Bacteria organisms involved in this case, *Pseudomonas cepacia* (Gupta and Crawford, 1991), *Pseudomonas fluoroscens* (Hazrin-Chong *et al.*, 2014), *Gordonia alkanivorans* and *Bacillus mycoides* (Romanowska *et al.*, 2015) incorporate extracellular enzyme in the degradation of coal. The third mechanism, chelation, involves utilizing components of the mobile part of coal with the aid of chelating agents such as oxalate ions (Cohen *et al.*, 1990). The oxalate ions remove Fe^{3+}, Mg^{2+} and Ca^{2+} involved in ionic linkages in coal. *Chelatococcus* sp. utilise and biosolubilise coal by producing metal ion chelators (Catcheside and Ralph, 1999). Fourthly, detergent, which involve the secretion of surfactants by microbes, are another means of coal biodegradation and is regarded as the simplest method for coal solubilisation (Ghani *et al.*, 2015). Finally, esterases, such as hydrolases, have been found to contribute to coal degradation and lead to the release of humic acid-like substances from coal (Crawford and Gupta, 1991). All these mechanisms biologically convert coal to clean, economical energy and a source of aromatic compounds for biocatalytic conversion to valuable industrial products (Ghani *et al.*, 2015).

This chapter describes experiments carried out to evaluate the soluble and insoluble products of bacterial biodegradation of coal discard and geologically weathered coal in order to understand the underlying mechanisms involved. This is the first report on the degradation of coal discard and geologically weathered coal using *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b.

4.2 Materials and methods

4.2.1 Coal biodegradation

Coal degradation experiments were carried out using *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b on coal discard and geologically weathered coal to release functional organic compounds from coal matrix; compounds which could be of biotechnological importance for the sustainable development of coal utilisation. Bacterial strains were grown in Luria broth and incubated at 37 °C overnight. Aliquots of the bacteria culture were harvested in log phase and centrifuged at 10000 rpm for 5 min. The supernatants were discarded and the cell pellets washed in phosphate buffer saline (PBS) twice to remove waste products of the bacteria during growth and to enhance enough nutrients for the survival of the bacteria or change the carbon/nutrient source. The washed cells were re-suspended in PBS, after which 10-fold serial dilutions were made to obtain a working medium (CFU/mL) for the three organisms. To assay the capacity of these bacteria to degrade coal discard and geologically weathered coal, mineral salt medium (MSM), enriched with 4 mL trace element solution (TES) per litre of MilliQ water was used.

The pH of the solution was adjusted to 6.8. Erlenmeyer flasks (250 mL) containing (150 mL) MSM plus 1 g of sterile coal sample (coal discard and geologically weathered coal) inoculated with each one of the organisms individually and in the consortium. The inoculum concentration was approximately 2.6×10^8, 5.3×10^6, and 2.2×10^9 cells/ mL, respectively. A consortium was prepared by inoculating equal proportions (2 mL) of all bacterial strains in a flask in the same condition as the individual test. Inoculated MSM without coal and un-inoculated MSM with coal were used as controls. All the glassware used was sterilised using an autoclave at 121 °C for 15 min to avoid any contaminants. The experimental cultures were incubated at 37 °C for 21 d without shaking. A whole culture medium from each flask at 7 d intervals was centrifuged and the supernatant and coal residues analysed.

4.2.1.1 Ultra-violet and visible (UV-VIS) spectrophotometric analysis of residual supernatant of biodegraded coal

Ultraviolet and visible spectroscopy was used to analyse the product of coal solubilisation. The brown/yellow supernatant from each flask was filtered and then centrifuged using a Beckman Coulter Centrifuge (Beckman Coulter Inc, U.S.A) to remove the organic substrates and pellet

the bacterial cells. The supernatant was analysed using a Perkin Elmer, Lambda 25 and UV/VIS Spectrophotometer with a wavelength from 200 to 700nm using 1 cm path quartz cuvettes.

4.2.1.2 Gravimetric analysis of biodegraded (residual) coal substrates

Gravimetric analysis of residual coal was carried out by determining the loss in mass of substrate discard and geologically weathered coal. The mass of residual coal after solubilisation was determined using an analytical balance and was calculated by the formula;

$$P = (1 - W_1/W_0) \times 100 .$$

Where P= percentage solubilisation (%); W_0= initial weight of coal (g); W_1= residual weight

4.2.1.3 Elemental analysis of residual coal

Elemental analysis of the residual (in soluble) degradation products of coal discard and geologically weathered coal was performed to measure the mass percent of carbon, hydrogen, nitrogen, sulphur and oxygen using an elemental analyser (PE 2400 CHNS/O, PerkinElmer, Waltham, MA).

4.2.1.4 FT-IR analysis of residual (insoluble) coal discard and geologically weathered coal

Residual coal discard and geologically weathered coal remaining after bacterial treatment was collected by centrifuging at 10000 rpm for 10 min, washed in MSM followed by MilliQ water three times and dried at 60 °C. A spectrum 400 FT-IR/FT-FIR Spectrophotometer (Perkin Elmer–Fourier), with measuring regions of 4000 −650 cm^{-1} was used for analysis . Samples were placed on a translucent disc and pressure was applied until an adequate consistency was obtained. In order to generate the spectra, background scan ranging from 4000 −650 cm^{-1} was first obtained before the sample scan to overlay the background scan. The sample spectrum was processed by deleting the background scan, followed by carrying out automatic baseline correction and automatic smoothing. The most recent spectrum was selected for examination by deleting all other generated spectra.

4.2.2 Humic acid extraction from coal discard and geologically weathered coal using alkali solubilisation

Humic acid was extracted from raw coal discard and geologically weathered coal in 10 mL of 0.5 M NaOH at 70 °C for 2 h. The extract was filtered and coal residue washed with distilled water until the supernatant was clear. The supernatant was acidified using H_2SO_4 to pH 2 and

allowed to stand for 24 h. Precipitated humic acid was separated by centrifugation and gel-like humic acids were oven dried at 60 °C. Fourier transform infrared (FTIR) spectra of the extracted humic acids were recorded using a Perkin-Elmer Spectrum instrument in a spectral range of 4000 to 650 cm^{-1}.

4.3 Results

4.3.1 Bacterial degradation of coal discard and geologically weathered coal

4.3.1.1 pH changes of bacterial cultures

After *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were incubated with either coal discard or geologically weathered coal as the sole carbon source, the effect of pH for coal degradation was evaluated. Figure 4.1 shows the pH change of the culture medium containing coal discard and geologically weathered coal treated with bacterial strains. Interestingly, some differences were observed in the pH of the bacterial strains' culture medium containing coal discard and geologically weathered coal, irrespective of the starting pH. The initial pH of the culture medium was 6.82 which declined to a minimum (6.52) in the absence of bacterial strains. This decline was less pronounced for coal discard (Figure 4.1 A) than for geologically weathered coal (Figure 4.1 B). On the third day, there was a steep rise in the culture containing either coal discard or geologically weathered coal. Figure 4.1A showed that the pH of the culture containing *Citrobacter* strain ECCN 19b returned to 6.8 (the starting pH) and then remained constant after 12d of incubation. Hence, the medium pH was more alkaline after 12d (6.92).

In B, the medium pH also recovered, but less so, for *Citrobacter* strain ECCN 19b whereas *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24 b generated greater alkalinity.

Thus, there is a possibility that, for the pH to recover, an alkaline substance must have been produced by all bacterial strains. The difference in the recovery of the pH of the culture medium containing coal discard and that containing geologically weathered coal seems to have been more pronounced in Figure 4.1 B (geologically weathered coal), and this ought to have been attributed to the more acidic state of coal discard than geologically weathered coal.

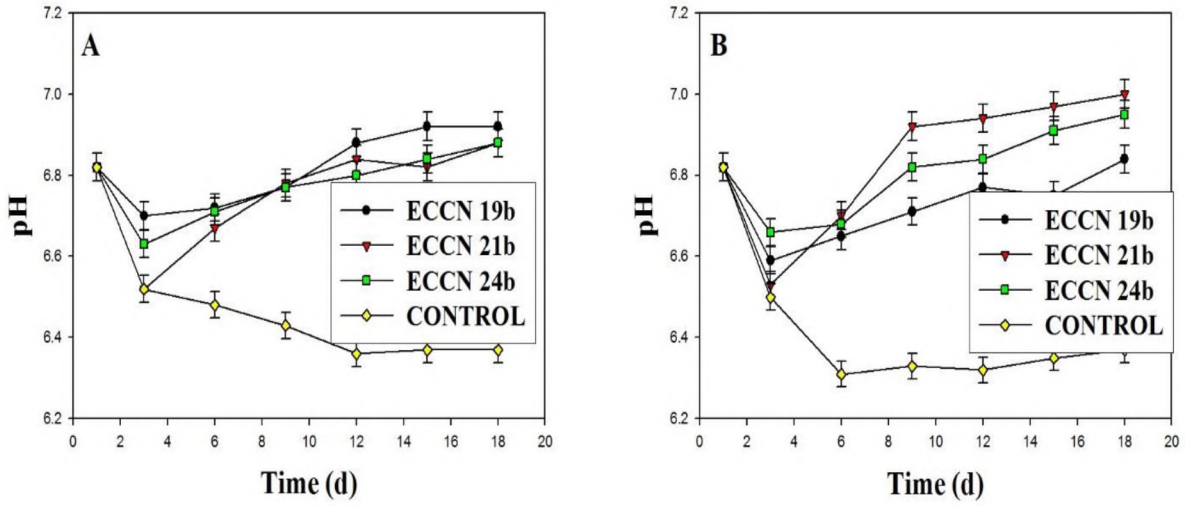


Figure 4.1 Change in pH of the culture medium containing coal discard (A) and geologically weathered coal (B) after inoculation with either *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, or *Serratia* strain ECCN 24b, and control, and incubated statically at 37 °C for 21 d. The error bars represent standard error for a mean value of at least three replicates.

4.3.1.2 Spectrophotometric analyses of soluble (liquid) products of biodegraded coal discard and geologically weathered coal

Coal discard and geologically weathered coal released brown/yellow liquid products into MSM when inoculated with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, *Serratia* strain ECCN 24b and incubated for 21d. This brown/yellow colour mediums at 7 d interval, filtered and centrifuged were measured at the absorbance of 450 nm (Figure 4.2) and, 280 nm (Figure 4.3). This colour supernatant increased in absorbance at 450 nm in all bacterial strain culture mediums containing coal discard (Figure 4.2 A) up to 7 d, after which it remained fairly stable. In Figure 4.2 B, absorbance continues to increase up to 21 d of incubation.

The absorbance value observed at 280 nm did not change much after 7 d for *Citrobacter* strain ECCN 19b compared to *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b (Figure 4.3 A), but, as shown in Figure 4.3 B, continued to increase for all the bacterial strains. The observed increase in the absorbance value at 450 nm and 280 nm of the brown/yellow colour indicates the release of soluble substances, typically humic acid-like substances from coal discard and geologically weathered coal upon treatment with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. This result indicated

that absorbance at these wavelengths increased up to 21 d in the medium of all bacterial cultures, suggesting that these bacterial strains are effective in coal degradation. The coal medium without bacterial strains (control) did not show any increase in the absorbance at 280 nm and 450 nm throughout the period of incubation (data not shown).

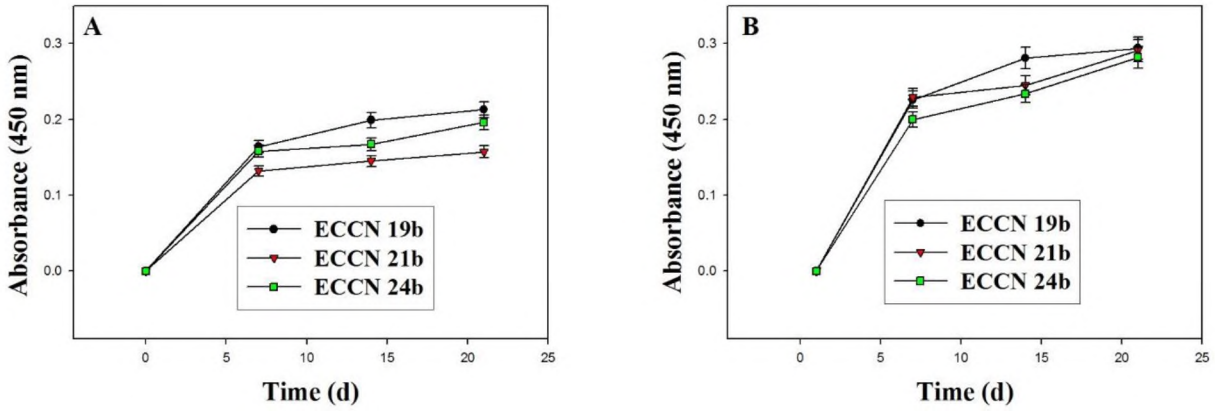


Figure 4.2 Optical density of soluble products of coal discard (A) and geologically weathered coal (B) of MSM culture of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b incubated at 37 °C for 21 d. Colour intensity of the culture medium was measured at 450 nm. The error bars represent standard error for a mean value of at least three replicates.

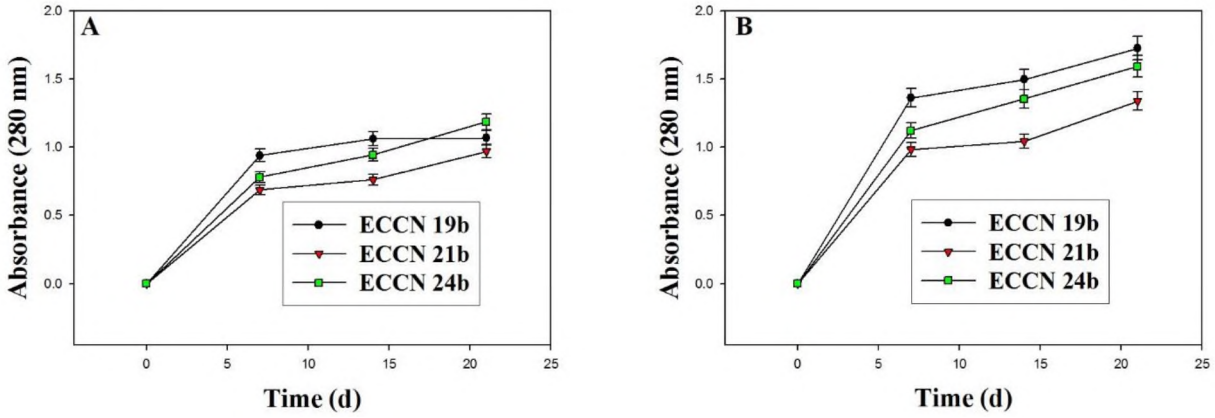


Figure 4.3 Optical density of soluble products of coal discard (A) and geologically weathered coal (B) of MSM culture of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b incubated at 37 °C for 21 d. Colour intensity of the culture medium was measured at 280 nm. The error bars represent standard error for a mean value of at least three replicates.

4.3.1.3 UV-Vis scanning spectra of soluble products of biodegraded coal discard and geologically weathered coal

During incubation of bacterial strains in the presence of geologically weathered /or coal discard, coloured products were released into the culture medium. To gain insight into these potential biodegradation products, samples of the coloured (brown) materials were recovered by centrifugation of the culture at 7d intervals. The supernatants thus collected were analysed using a UV-Vis spectrophotometer by scanning in the range 200 –700 nm and the results are shown in Figure 4.4, and 4.5. The UV-Vis results showed an increase in absorbance in the UV region of the spectrum for soluble products derived from coal discard (Figure 4.4) and geologically weathered coal (Figure 4.5) treated with the bacterial strains, either individually or in the consortium. Furthermore, the UV-Vis spectra obtained displayed characteristics similar to published spectra for authentic humic acid and soil-extracted humics with maxima between 250 and 300 nm (Peuravuori *et al.*, 2002; Gao *et al.*, 2016). Absorbance in this region strongly suggests that humic acid-like substances were released from the coal substrates and that these products show characteristics of Type B humic acid (Kumada, 1987; Cunha *et al.*, 2009). This result was in line with the results of Cunha *et al.*, (2009) on the spectroscopic characterisation of humic acids isolated from Amazonian dark earth soil. An increase in the absorbance of the degradation products was observed up to 21 d of incubation.

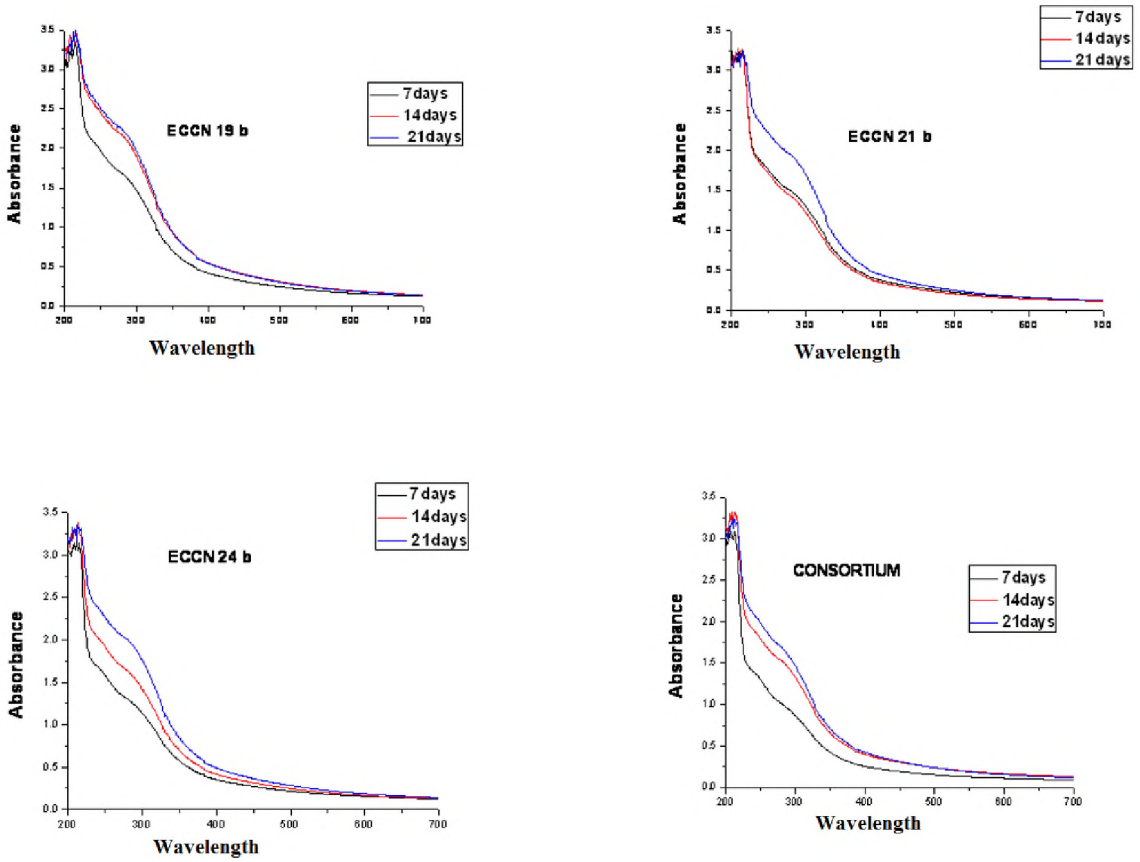


Figure 4.4 UV-Vis scanning spectra of biodegradation products of coal discard by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b incubated at 37 ºC for 21 d . The supernatants from bacteria strains MSM culture supplemented with coal discard were centrifuged to obtain bacteria cells and coal particles free and scanned between 200 −700 nm.

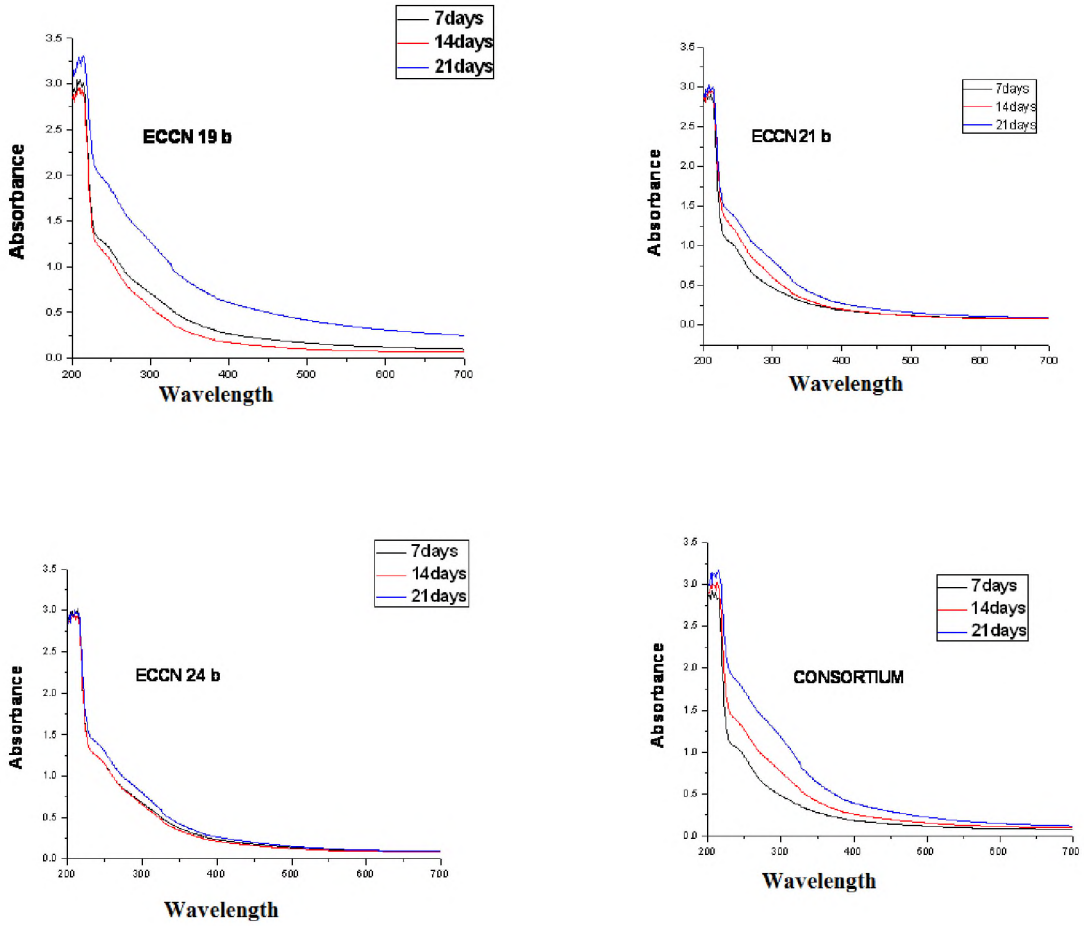


Figure 4.5 UV-VIS scanning spectra of biodegradation products of geologically weathered coal by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b incubated at 37 °C for 21 d. The supernatants from bacteria strains MSM culture supplemented with geologically weathered coal were centrifuged to obtain bacteria cells and coal particles free and scanned between 200 −700 nm.

4.3.1.4 Analysis of insoluble (residual) biodegradation products of coal discard and geologically weathered coal

4.3.1.4.1 Gravimetric analysis of residual coal substrate

To confirm bacterial biodegradation of the coal substrates, the residual coal was quantified gravimetrically. Residual coal substrate was recovered from the incubation medium, washed with MilliQ water, dried at 60 °C, the mass determined using an analytical balance, and percentage biodegradation calculated. The mass of residual coal discard and geologically weathered coal degraded by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b

and *Serratia* strain ECCN 24b are shown in Figure 4.6. The degree of coal discard degradation by *Citrobacter* strain ECCN 19b was 11% on 21 d of the incubation. The extent of degradation was 10% and 9% for *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b (Figure 4.6 A). For geologically weathered coal, the rate of degradation was considerably higher (22%) when the coal was treated with *Exiguobacterium* strain ECCN 21b. The maximum degradation of 19% was obtained for *Citrobacter* strain ECCN 19b on 21d of incubation. Biodegradation of geologically weathered coal with *Serratia* strain ECCN 24b decreased by 16% (Figure 4.6 B).

The degrading efficiency when *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b are present in the consortium was 28% for geologically weathered coal and 12% for coal discard (Figure 4.6). Results showed that geologically weathered coal was more effectively degraded by the bacterial strains during the period of incubation (Figure 4.6) and this could be associated with the high oxidation status of geologically weathered coal compared to coal discard.

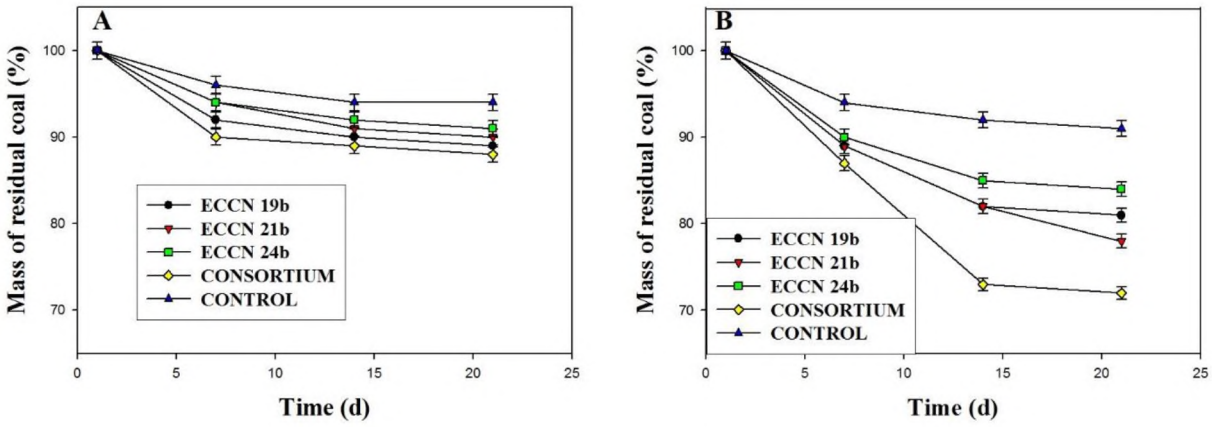


Figure 4.6 Mass of residual coal discard (A) and geologically weathered coal (B) biodegraded by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in MSM incubated without shaking at 37 °C for 21 d . The error bars represent standard error for a mean value of at least three replicates.

4.3.1.4.2 Elemental analysis of residual coal discard and geologically weathered coal

Coal discard and geologically weathered coal remaining after treatment with bacterial strains either individually or in the consortium were analysed to determine the elemental composition.

The coal residue samples were dried for elemental analysis. Tables 4.1 & 4.2 shows C, H, N, S and O content in residual coal discard and geologically weathered coal. The elemental compositions of the insoluble (residual) product of coal discard are characterised by higher N and O content with an appreciably lower C content after treatment with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, and *Serratia* strain ECCN 24b. Mass of carbon content was reduced by *Citrobacter* strain ECCN 19b to 36.33% followed by *Exiguobacterium* strain ECCN 21b with 36.02%, and 36.01% by *Serratia* strain ECCN 24b from initial 43.5%. The nitrogen content increased to 1.60%, 1.66% and 1.54%, respectively, from the initial 1.0%. In addition, the oxygen content increased to 59.73%, 60.02%, and 59.58%, respectively, from 52.7%. Hydrogen content of the insoluble product (residual) of coal discard was 2.13% when the coal substrate was treated with *Citrobacter* strain ECCN 19b and 2.01% in the presence of *Exiguobacterium* strain ECCN 24b from initial mass of 2.7% (Table 4.1).

For geologically weathered coal treated with bacterial strains, the increase in N, H and C with an appreciable lower O content was observed in the insoluble biodegradation product (Table 4.2). Elemental nitrogen was increased to 1.16%, 1.19%, and 1.15%, from initial 0.7% when the coal substrate was treated with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b, respectively. Oxygen content was observed to be reduced by *Citrobacter* strain ECCN 19b by 59.73%, 60.02% by *Exiguobacterium* strain ECCN 21b and 59.58% by *Serratia* strain ECCN 24b from the initial 72% mass. This was followed by the increase in carbon content to 30.47%, 29.11%, and 28.09%, respectively, from the initial 25%. Hydrogen content was increased to 1.99%, 1.93%, and 2.04% from initial 1.4% for all the bacterial strains. The insoluble (residual) product of coal discard and geologically weathered coal after treatment with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24 b in the consortium were characterised by an increase in the nitrogen content (1.71% and 1.40% from initial 1.0% and 0.7%). The bacterial strain consortium increased the carbon content of geologically weathered coal to 31.34% from 25 % and reduced oxygen content from 72% to 65.3%.

Differences observed in the elemental composition of residual coal discard and geologically weathered coal are presumably due to the oxidised nature of the latter. In fact, increase in N and H content of the residual coal may be resulted from the consumed C of coal structure. In addition, increases in nitrogen content could also be attributed to the bacterial strain's ammonia. Clearly, the observed mass % differences in the insoluble (residual) product of coal discard and

geologically weathered coal compared to the starting coal showed that the elemental composition of the coal substrates was modified by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24 b.

Table 4.1 Ultimate analysis of residual biodegraded coal discard by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b incubated in MSM under a static condition at 37 °C for 21 d. Results are presented as the mean of three analyses (Mean ± SE)

S/N	SAMPLE	% C	% H	% N	% S	% O
1	Coal +ECCN 19b	36.33±0.55	2.13±0.63	1.60±0.22	0.21±0.18	59.73±0.12
2	Coal +ECCN 21b	36.02±0.74	2.01±0.72	1.66±0.17	0.29±0.20	60.02±1.00
3	Coal +ECCN 24b	36.01±0.16	2.74±0.17	1.54±0.12	0.21±0.21	59.58±0.15
4	Coal +Consortium	36.32±0.30	2.19±0.66	1.71±0.07	0.31±0.28	59.47±0.67

Table 4.2 Ultimate analysis of residual biodegraded geologically weathered coal by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b incubated in MSM under a static condition at 37 °C for 21 d. Results are presented as the mean of three analyses (Mean ± SE)

S/N	SAMPLE	% C	% H	% N	% S	% O
1	Coal +ECCN 19b	30.47±0.72	1.99±0.24	1.16±0.20	0.29±0.29	66.09±0.79
2	Coal +ECCN 21b	29.11±0.62	1.93±0.46	1.19±0.14	0.26±0.18	67.51±0.47
3	Coal +ECCN 24b	28.09±0.49	2.04±0.24	1.15±0.16	0.21±0.14	68.51±0.43
4	Coal +Consortium	31.34±0.60	1.58±0.54	1.40±0.17	0.32±0.28	65.36±0.42

4.3.1.4.3 FT-IR spectra analysis of residual coal discard and geologically weathered coal

To gain insight into the mechanisms used by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in the biodegradation of coal, residue substrate was recovered, analysed by FTIR and the spectra shown in Figure 4.7. The relative intensity of the various bands reflects the functional groups of the substrate after bacterial biodegradation. Results show that there was little or no difference in the spectra generated from untreated (i.e without bacteria) and control (raw substrate) coal discard and geologically weathered coal samples (Figure 4.7). Thus, any change in the FTIR spectrum for a given substrate must arise as a consequence of bacterial action. The spectra for the residual substrate after treatment of

coal discard with either *Citrobacter* strain ECCN 19b or *Serratia* strain ECCN 24b are similar (Figure 4.7 A and E).

Coal discard treated with *Citrobacter* strain ECCN 19b showed an obvious band at 2970 cm^{-1} which is characteristic of aliphatic C-H groups. This peak was shifted to 3026 cm^{-1} (aromatics, C-H stretch) after treatment with *Serratia* strain ECCN 24b. The peak for coal discard at 1737 cm^{-1} (aldehyde, ketones, carboxylic acids, esters, C=O stretch) treated with *Citrobacter* strain ECCN 19b is stronger than that of the sample treated with *Serratia* strain ECCN 24b. The bands at 1571 cm^{-1}, 1366 cm^{-1}, 1228 cm^{-1} and 1216 cm^{-1} are almost the same after treatment of coal discard with *Citrobacter* strain ECCN 19b and *Serratia* strain ECCN 24b. However, spectral bands at 1228 cm^{-1} and 1216 cm^{-1} indicate an enhanced proportion of aliphatic amine group, while the band at 1571 cm^{-1} and 1366 cm^{-1} correspond to the amine (N-H), and alkane (C-H) group respectively. These bands were absent in untreated coal and control samples, indicating that coal discard has been biodegraded by bacterial strains. There was no discernable difference in the FTIR spectra of residual geologically weathered coal after treatment with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, or *Serratia* strain ECCN 24 b. One possible difference was in the broad spectral region of 3500 — 3200 cm^{-1} which is attributed to O-H groups (alcohol, and phenol). This might due to the highly oxidised nature of geologically weathered coal.

Citrobacter strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b when present in the consortium, these effects are masked (Figure 4.7, G, H). As it stands, the bacterial strain consortium showed little or no effect on weathered coal. On coal discard, the bacterial strain consortium had some effect, but it is not additive, suggesting that one strain may suppress the activity of others, or that humic acids inhibit bacterial strain activity. Nonetheless, the O-H spectrum or absorption band between 3200 and 3600 cm^{-1} indicates either an alcohol or phenol in the band at 1369 cm^{-1} (alkane, C-H) and is conspicuous on the coal treated with the consortium (Figure 4.7, G). This finding confirms that, in the presence of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b, coal discard and geologically weathered coal release functional organic compounds from coal.

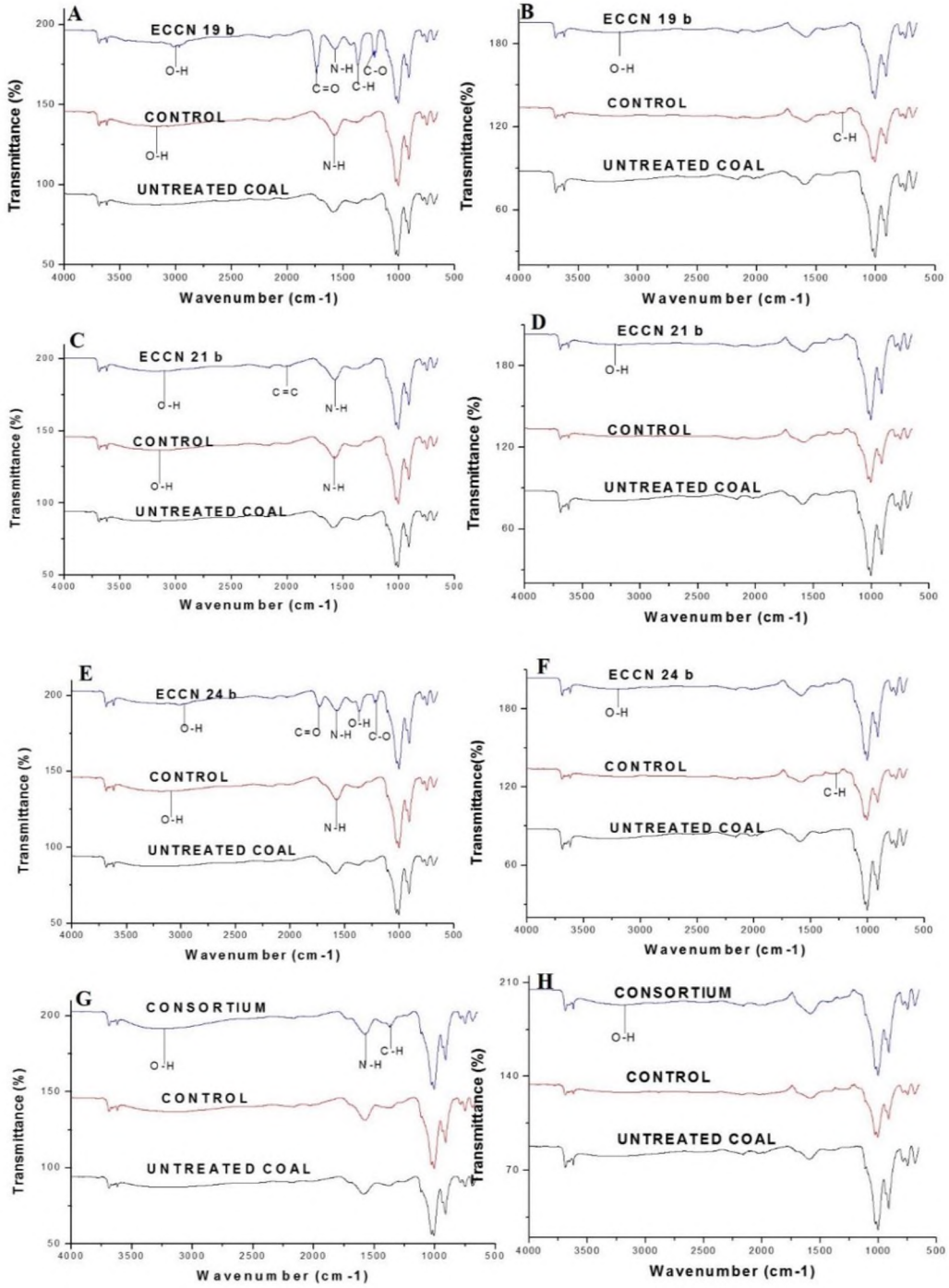


Figure 4.7 FTIR spectra of residual biodegraded coal discard (A, C, E, G) and geologically weathered coal (B, D, F, H) in MSM inoculated with *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b, *Serratia* strain ECCN 24b and consortium of bacterial strains incubated under static condition at 37 ^{o}C after 21 d .

4.3.1.5 FTIR spectra of extracted humic acid from coal discard and geologically weathered coal

A representative FTIR spectrum of the humic acid-like substances extracted from coal discard and geologically weathered coal exhibit the same absorbance as commercial humic acid (Sigma-Aldrich, Switzerland), as shown in Figure 4.8. The extracted humic acid-like substances were characterised by a broad absorption band in the region between 3400 –3200 cm^{-1} corresponding to OH groups of alcohol, phenols and organic acid. Also, C=C conjugated with C=O noticed around 1500 –1650 cm^{-1} was associated with aromatic hydrocarbon and carboxylic compounds. Similarly, the sharp band was observed in the region of 1300 $cm^{-1}$and 1050 cm^{-1} attributing to C-O of a polysaccharide. An absorption band in the region between 1000 –1300 cm^{-1}, observed in the humic acid-like substances extracted from geologically weathered coal suggested that the coal was highly oxidised (A). The result indicated that the FTIR spectra of humic acid-like substances extracted from coal discard and geologically weathered coal showed features typically attributed to humic acid that were closely similar to reference commercial humic acid. The differences were only observed in the relative intensities of the spectra generated.

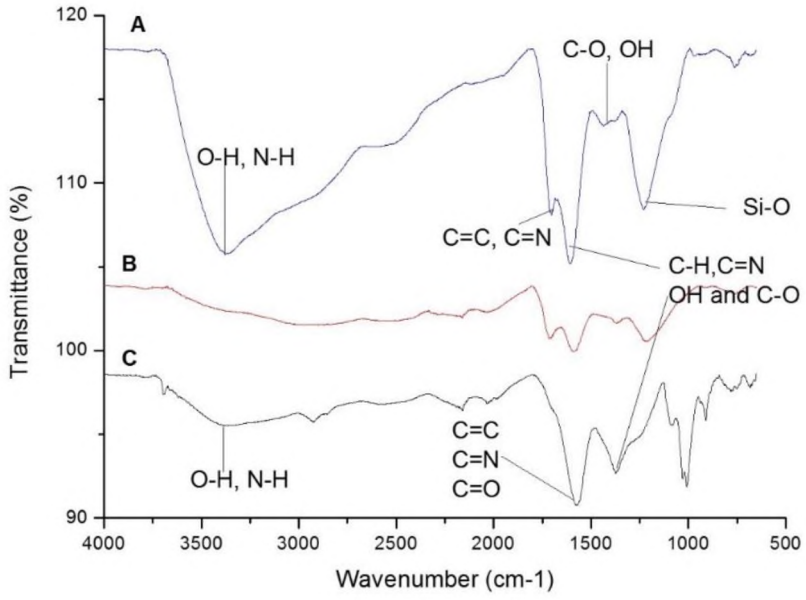


Figure 4.8 FTIR spectrum of extracted humic acid-like substances from geologically weathered coal (A), coal discard (B) and commercial humic acid (C).

4.4 Summary

Results of the analyses show that *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b released alkaline substances which significantly raised the pH of the culture medium containing coal during the period of incubation. The pH was lowered at the beginning, then rose and remained almost constant throughout the period of incubation. Also, bacterial strains exhibited the ability of coal discard and geologically weathered coal degradation to release soluble (brown/yellow colour) and insoluble (residual solid) products into the culture medium. Coal discard and geologically weathered coal degraded by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b exhibited increased absorbance at 450 nm and 280 nm in the brown/ yellow colour released into the culture medium; this was related to the rise in pH of the culture medium for all the bacterial strains.

The released soluble product (brown/yellow colour substance) spectra observed at the UV-Visible region of 200 –700 nm increased in absorption in the ultraviolet region and decreased towards the visible region for all the bacterial strains. Based on the analysis, this soluble product was assumed to be humic acid-like substances, which were confirmed through UV-Vis spectra maxima at 200 –300 nm, characteristic of humic acid. Gravimetric analysis of the residual (insoluble) product of coal degradation showed that geologically weathered coal was more effectively degraded than coal discard. FTIR spectra of the residual biodegraded coal discard and geologically weathered coal depicted significant modification in the coal functional groups. The appearance of new peaks at 1737 cm^{-1}, 1366 cm^{-1}, 1228 cm^{-1} and 1216 cm^{-1} were observed after coal discard degradation by bacterial strains. These new peaks were attributed to aldehyde, ketones, carboxylic acids, esters, amine and alkane. A broad spectral region of 3500 –3200 cm^{-1} attributed to O-H groups (alcohol, and phenol) was noticed for geologically weathered which might be due to the highly oxidised nature of geologically weathered coal. FTIR spectra of humic acid-like substances extracted from coal discard and geologically weathered coal showed features typically attributed to humic acid that were very similar to reference commercial humic acid, thus indicating that they are a potential source of humic acid and are capable of being metabolised by bacteria to release this product. Elemental composition of coal discard and geologically weathered coal was modified after treatment with bacterial strains. However, evaluation of both soluble and insoluble products of coal discard and geologically weathered coal degradation showed that *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b

and *Serratia* strain ECCN 24b, efficiently degraded coal discard and geologically weathered coal to release humic acid-like substances.

CHAPTER 5

Involvement of laccase and cytochrome P450 in the bacterial degradation of coal

5.1 Introduction

The principal mechanisms of microbial coal conversion, solubilisation, which involves the secretion of alkaline products (non-enzymatic dissolution); depolymerisation (enzymatic action), and chelation are well documented (Sekhohola *et al.*, 2013; Romanowska *et al.*, 2015; Ghani *et al.*, 2015). Alkaline materials produced by bacteria solubilise coal by deprotonation, thereby increasing coal hydrophilicity (Baylon *et al.*, 2017). Although, much of the information on microbial degradation of coal has been derived from studies using fungi, bacteria are known to degrade lignin, and since lignin has been considered one of the most important substances in the plant constituent of coal, it is possible that bacteria also produce enzymes that degrade lignin and coal. Various studies have shown that oxidative enzymes such as peroxidases, laccases (LAC, E.C.1.10.3.2) and hydrolytic enzymes (esterases) are responsible for microbial coal degradation (Fakoussa and Hofrichter, 1999). Bacterial strains found in coal environment are apparently more efficient coal degraders not only because of adaptations to local environmental conditions such as temperature, pH, salinity (Chang *et al.*, 2011; Kota *et al.*, 2004), but also of their ability to produce laccase (Kaira *et al.*, 2015).

The use of microorganisms and enzymes such as laccase and peroxidase to degrade and even convert coal to useful products has been growing rapidly. The enzymatic biodegradation ability of bacteria is associated with both intracellular and extracellular oxidoreductive enzyme systems, which includes laccase (LAC, E.C.1.10.3.2), azoreductase, and NADH-DCIP-reductase (Telke *et al.*, 2009). Ihssen *et al* (2015) recently described the cloning and expression of five novel bacterial LAC-like multicopper oxidases (LMCOs) of diverse origin that were identified by homology searches in online databases. Laccases have many possible applications in bioremediation because of an ability to degrade various substances such as undesirable contaminants, by-products, or discarded materials (Xu, 1999). Detoxification of phenolic pollutants such as o-chlorophenol, 2, 4-dichlorophenol, 2, 4, 5-TCP, 4-chloro-2-methylphenol (Bollag *et al.,* 1988) and xenobiotics present in polycyclic aromatic hydrocarbon (PAH) in oil deposits and fossil fuels has been achieved (Pointing, 2001; Anastasi *et al.,* 2009 and Murugan *et al.*, 2014).

Cytochrome P450 (CYT450) is another candidate enzyme that might play a role in biodegradation of coal. This enzyme has been detected in fungi and bacteria and appears to be involved in the catabolic/enzymatic conversion of PAH (Masaphy *et al.*, 1996), lignin (Doddapaneni *et al.*, 2005; Matsuzaki and Wariishi, 2005) and petrol additives, e.g. methyl tert-butyl ether, ethyl tert-butyl ether and tert-amyl butyl ether (Malandain *et al.*, 2010). There is adequate evidence to suggest that both enzymatic and non-enzymatic mechanisms are involved in coal biodegradation. However, the production of LAC and CYT450 enzymes in coal biodegradation has not apparently been explored. The ability of the bacterial strains *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b to degrade coal discard and geologically weathered coal (see Chapter 4) provides an ideal opportunity therefore, to attempt isolation and identification of candidate enzymes that might play a key role in coal degradation. This chapter describes the studies that were carried out to detect and quantify LAC and CYT450 activity in bacteria capable of degrading coal discard and geologically weathered coal.

5.2 Materials and methods

5.2.1 Extracellular protein determination of bacterial strains on coal discard and geologically weathered coal

The protein content of the culture medium from *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b was determined according to Bradford (1976). The reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol. One hundred millilitres 85% (w/v) phosphoric acid was added to this solution and diluted to a final volume of 1 L. The prepared reagent was filtered and kept at 4 ^{o}C. Bovine serum albumin (BSA; 2 mg/mL) was used as the standard at concentration ranges from 0 -100 µg/mL. A protein assay was prepared by centrifuging the whole culture medium at 10000 rpm for 5 min. Aliquots of the supernatant (100 µL) were added to 5 mL protein reagent (Bradford) which was thoroughly mixed. Absorbance was measured using a UV-1201, UV-Vis spectrophotometer (Shimadzu, Japan) at 595 nm in 2 mL cuvettes after 5 min of incubation at room temperature. Total protein content was calculated based on the standard calibration curve for BSA (Appendix V).

5.2.1.1 Purification of cellular bacterial proteins

Ammonium sulphate ($(NH_4)_2SO_4$) was used for salting out protein from the bacterial culture. This precipitates most proteins and protects bacteria proteins in solution from denaturation (Kapoor, 2015). To achieve this, bacteria strains were incubated in 100 mL MSM with and without coal substrates (coal discard and geologically weathered coal) as the sole carbon sources. Two millilitres (2 mL), containing 2.3×10^8 CFU/mL of bacteria were used as inoculum and the flasks incubated for 7 d prior to filtration and centrifugation at 10000 rpm at 4 ^oC for 20 min. Bacterial pellets were washed in PBS and lysed by sonication using a 550 Sonic Dismembrator (Fisher Scientific, 2000 Park Lane, Pittsburgh) at 6 cycles of pulses for 5 s at 20% amplitude with 5 s intervals. Sonicated samples were then centrifuged at 10000 rpm for 20min, the pellet discarded and solid ammonium sulphate added to achieve 30% saturation. Precipitated protein was removed by centrifugation at 10000 rpm for 20 min and more ammonium sulphate was added to the supernatant to yield 60% saturation. Precipitates were resuspended in 1 mL buffer (PBS) and subjected to gel column filtration .

5.2.1.2 Size exclusion chromatography of bacterial protein

In size exclusion chromatography, sephadex G-75 column resin was used. About 5 g of sephadex G-75 was suspended in 100 mL of 0.1 M Tris-HCl buffer (pH 7.0) and swelled by keeping it for 3-4 h at room temperature with intermittent stirring. Excess buffer was decanted along with suspended fines in order to obtain a slurry of reasonable consistency. Next, resin material was packed into 6 mL plastic columns fixed on a retort stand and allowed to settle completely without air bubbles. Column chromatography was carried out using 5 mL of 0.1 M Tris-HCl buffer, pH 7.0. Aliquots (1 mL) of ammonium sulphate precipitated bacterial strains protein was loaded onto the sephadex G-75 column pre-equilibrated with 0.1 M Tris-buffer (pH 7.0) and eluted with the same buffer. Size exclusion chromatography of the protein was carried out at room temperature, and fractions of 500 µL were collected manually at a flow rate of 10 −12 drops per tube. Elution of proteins was measured at 280 nm using a UV-Vis spectrophotometer and, the molecular weight of the purified proteins was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses using 12% polyacrylamide gel and Coomassie Brilliant Blue R-250 staining. Protein markers (10 −250 kDa, obtained from Bio-rad), were used to determine the approximate molecular mass of purified proteins.

5.2.1.3 SDS-PAGE analysis of bacterial protein

SDS-PAGE analysis of the sample was performed according to Simpson (2004), on a Bio-Rad Mini-PTROTEAN 3 system (Hemel Hempstead, Hertfordshire, U. K). Glass plates were assembled on a casting rack, which was followed by pouring of 12% gel mixture into the space between the glass plates and covered with sterilised distilled water. When the resolving gel polymerised, water was discarded, and a 5% stacking gel mixture was added and a 10-well comb inserted. After polymerisation, glass plates containing gels were placed in the electrophoretic chamber containing the running buffer. Protein samples for SDS-PAGE were prepared by washing the pellets in PBS, centrifuging and resuspending in SDS sample solubilisation buffer, and vortexing thoroughly.

Protein solution collected after bacteria lysis (sonication) was incubated at 95 ^{o}C for 5 min. Samples were cooled to room temperature and mixed with an equal volume of SDS-PAGE sample buffer (ratio 1:1). This was kept in the water bath for 2 min at 95 ^{o}C to denature the protein and to ensure the maximum amount of SDS binding to the protein. Samples were loaded onto the polymerised gel immersed in the electrophoretic chamber containing the running buffer. Electrophoresis was at 150 V for about 1.5 h using a power Bio-Rad Pac device operated at room temperature. After electrophoresis was finished, the gel was removed from the glass plates, washed with Milli Q water and stored in 5% acetic acid solution at 4 ^{o}C. Staining solution for protein involved using (Fairbanks) Coomassie Brilliant Blue G-250. After staining and destaining, the gel was photograph using a Canon camera. Positions of the band for molecular weight were determined by comparison with standard molecular weight marker from Bio-Rad.

Preparation of running buffer: 3 g of Tris base 14.4 g of glycine and 1 g of sodium dodecyl sulphate were weighed and dissolved in 1000 mL of distilled water. The pH was adjusted to 8.3 and kept at room temperature.

Acrylamide stock (30%): Acrylamide 30 g and bisacrylamide 0.8 g were weighed and dissolved in 70 mL of distilled water and made up to 100 mL. This was stored at 4 ^{o}C.

Resolving gel buffer: 18.7 g Tris base was suspended in 70 mL of distilled water. The pH was adjusted to 8.8 with Hcl and volume made up to 100 mL.

Stacking gel buffer: 6.05 g Tri-base was dissolved in 70 mL of distilled water followed by adjustment of pH to 6.8 with Hcl and volume made up to 100 mL.

Ammonium persulphate (10%): 1 g of ammonium persulphate was suspended in 10 mL of distilled water.

SDS-sample solubilization buffer (50 mL): 0.50 g of SDS and 0.60 g Tris base was dissolved in 50 mL of distilled water.

SDS-sample buffer (10 mL): 2 mL of stacking gel buffer, 1.6 mL of glycerol, 3.2 mL of 10% SDS, 0.8 mL of 2-mercaptoethanol and 0.4 mL of 1% bromophenol blue were mixed together in dark blue container.

5.2.2 Laccase assay

Bacterial laccase enzyme activity was monitored using guaiacol and catechol as substrates in the liquid medium and solid medium, respectively. Screening of bacterial strains for laccase secretion was performed by pipetting 0.1 mL aliquots of bacteria culture onto sterilised nutrient agar plates supplemented with 0.4 mM $CuSO_4.5H_2O$. Plates were incubated at 37 ^oC for 48 h. After incubation, individual colonies formed on plates were flooded with guaiacol and catechol substrate. The appearance of a brown/purple colour around bacteria colonies indicates the formation of laccase. For analysis of laccase from liquid cultures, 2 mL aliquots containing $10^{-6}-10^{-9}$ CFU of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were inoculated into the liquid medium containing either coal discard or geologically weathered coal and incubated without shaking for 7 d at 37 ^oC. To determine laccase production 2mL aliquots of culture were sampled daily and centrifuged at 10000 rpm for 10 min. Supernatant collected was used as the source of laccase enzyme.

Laccase activity from the culture media of the bacterial strains was measured using guaiacol as a substrate. One millilitre of culture media was mixed with 1mL of 10 mM guaiacol in 3 mL of 0.1 M sodium acetate buffer (pH 5) and incubated for 15 min. Oxidation of guaiacol was detected spectrophotometrically at 450 nm (UV-1201, UV-Vis spectrophotometer, Shimadzu, Japan). One unit of laccase activity was defined as the amount of enzyme required to oxidise one mole of guaiacol per min at 35 ^oC. The laccase activity in U/mL was calculated using the extinction coefficient of guaiacol (12,100 $M^{-1}cm^{-1}$) at 450 nm using the formula:

E.A= (A*V)/t*e*v), where E.A =Enzyme Activity (U/mL), A= absorbance at 450 nm, V= total volume of reaction mixture (mL), t= incubation time (min) and e= extinction coefficient ($M^{-1}cm^{-1}$) (Desai *et al.*, 2011).

5.2.3 Cytochrome P450 enzyme assay

Citrobacter strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were screened for production of cytochrome P450 in the presence of either coal discard and geologically weathered coal using a cytochrome P450 reductase activity assay kit (Colorimetric) (Abcam biotech, U.K.). Bacterial culture broth from each flask was sampled and centrifuged at 10000 rpm for 5 min to pellet bacteria cells.

Cell pellets were frozen at -20 °C for later use. On the day of analysis, pellets were thawed and resuspended in 100 µL of 2 mg/mL lysozyme and 500 µL cytochrome P450 reductase assay buffer followed by sonication for 2 min. The sonicated sample was centrifuged and the supernatant used as the source of cytochrome P450 enzyme. Thirty microliters (30 µL) of the supernatant was mixed with an equal volume of CPR assay buffer, reaction mix (NADPH substrate+ CPR assay buffer) and 10 µL of 20 mM glucose-6-phosphate (G6P) solutions in the 96-well micro plate. Relative CPR activity was detected spectrophotometrically using a micro plate reader at 460 nm in kinetic mode for 25 min at 25 °C. Protein concentrations were measured according to Bradford (1976) using BSA as the standard. The concentration of CPR was calculated using the formula:

$$\text{CPR activity} = (B/\Delta T \times P),$$

where CPR activity (nmol/min/mg), B=amount of G6P consumed calculated from the standard curve (nmol), ΔT=Reaction time (min), P= Original amount of protein sample added into the well (in mg)

5.3 Results

5.3.1 Extracellular protein production by bacteria cultivated in coal discard and geologically weathered coal-containing medium

To demonstrate extracellular protein production/accumulation in the culture of bacteria growing in the presence of either coal discard or geologically weathered coal as the sole carbon source, aliquots of the culture medium were sampled and the total protein determined. Figure 5.1 shows the time course of increasing concentration of extracellular protein in the culture medium from bacteria supplied with either coal discard or geologically weathered coal as the sole carbon source. The protein content secreted by bacterial strains in the medium containing

coal discard continued to increase with the incubation time. The maximum protein (1.24 µg/mL) was produced from *Citrobacter* strain ECCN 19b, followed by *Exiguobacterium* strain ECCN 21b (1.26 µg/mL), and *Serratia* strain ECCN 24b (1.27 µg/mL). The maximum protein concentration was observed on 7d for *Citrobacter* strain ECCN 19b, 6d for *Exiguobacterium* strain ECCN 21b and 5d for *Serratia* strain ECCN 24b. Maximum protein secreted by the bacterial strains, appeared to be stable till 7d suggesting maximum bacterial metabolic activity on days 5 and 7 (Figure 5.1 A).

The maximum protein content recorded in the medium in the presence of geologically weathered coal was 1.47 µgmL, 1.37 µg/mL and 1.36 µg/mL for *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b, respectively (Figure 5.1 B). All these appeared to be on day 7. The reason for the secretion of much bacterial strain protein in the presence of geologically weathered coal was apparently due to its more oxidised nature compared to coal discard. The bacterial strain consortium in the medium containing geologically weathered coal secreted the maximum protein of 1.72 µg/mL compared to discard coal (1.20 µg/mL). The protein contents increased, indicating that bacterial cells were utilising coal substrates as the source of carbon which resulted in its degradation.

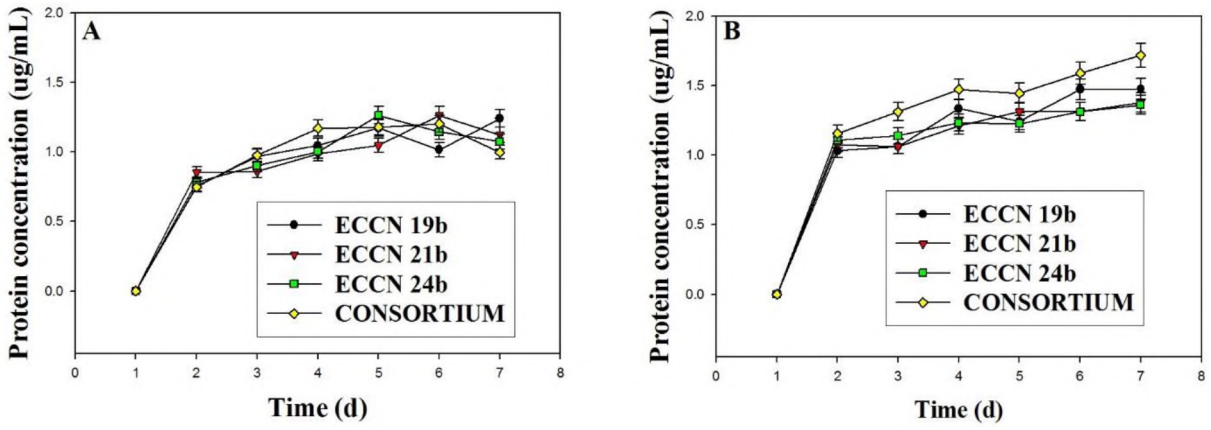


Figure 5.1 Concentration of extracellular protein in culture medium from *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b supplied with coal discard (A) and geologically weathered coal (B) as sole carbon source and incubated statically at 37 °C for 21 d. The error bars represent standard error for a mean value of at least three replicates.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to investigate the spectrum of extracellular proteins in the culture medium. Many proteins of

different molecular mass were detected for the three bacterial strains and these are shown in Figure 5.2. The spectrum of extracellular proteins was more diverse and intense for the *Citrobacter* strain ECCN 19b than the *Serratia* strain ECCN 24b and the *Exiguobacterium* strain ECCN 21b in the presence of coal discard (Figure 5.2 A). Of particular interest were the three intensely stained protein bands with an approximate molecular mass of 53 kDa, 72 kDa and 82 kDa, respectively (arrows; Figure 5.2). These were detected in mediums from all bacterial strains containing coal discard. A similar spectrum of extracellular proteins was observed after incubation of *Citrobacter* strain ECCN 19 b, *Serratia* strain ECCN 24b and *Exiguobacterium* strain ECCN 21b in MSM containing geologically weathered coal (Figure 5.2 B). In these incubates, a prominent protein band corresponding to the molecular mass of 53 kDa was also present. Proteins of 72 kDa and 82 kDa, while visible were diffuse in fractions from both *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b .

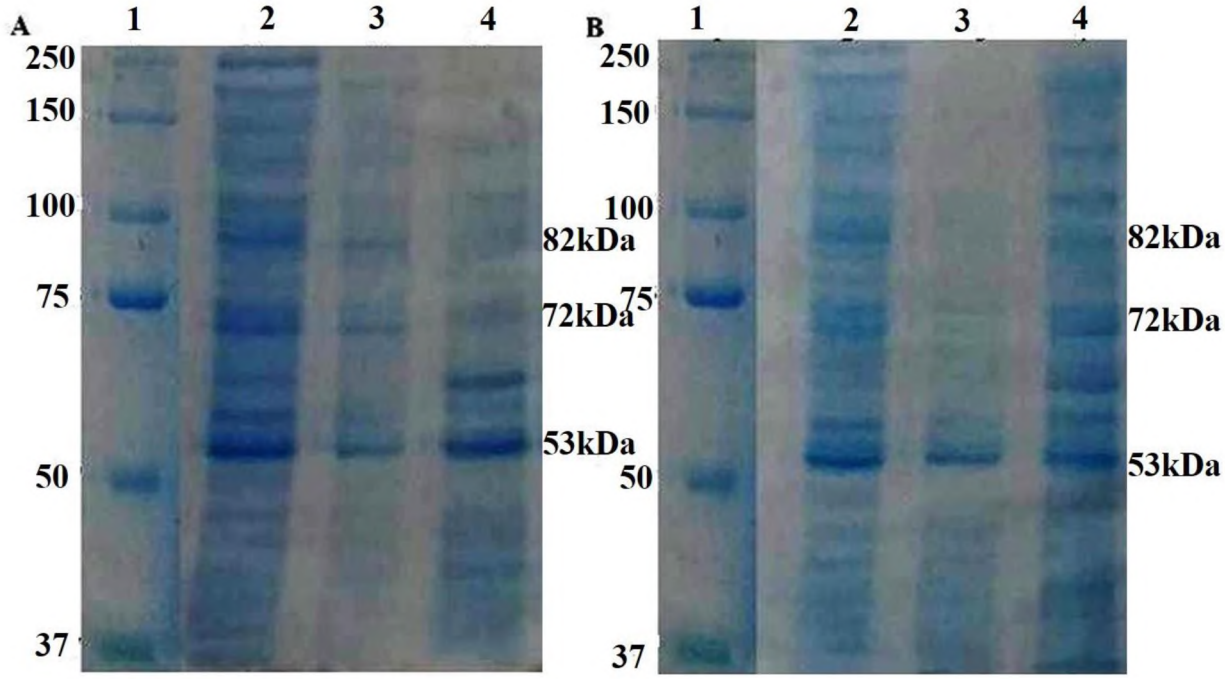


Figure 5.2 SDS-PAGE separation of extracellular protein from the medium of bacterial cultures supplied with coal discard (A) or geologically weathered coal (B) as the sole carbon source. Lane 1: protein molecular weight markers; Lane 2: *Citrobacter* strain ECCN 19b; Lane 3: *Exiguobacterium* strain ECCN 21b; Lane 4: *Serratia* strain ECCN 24b

The extracellular proteins from *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24 b from MSM was concentrated using 30 −60% ammonium

sulphate, desalted by gel filtration using Sephadex G-75 and fractions with high absorbance at 280 nm (Figure 5.3) were pooled together and re-analysed on SDS-PAGE.

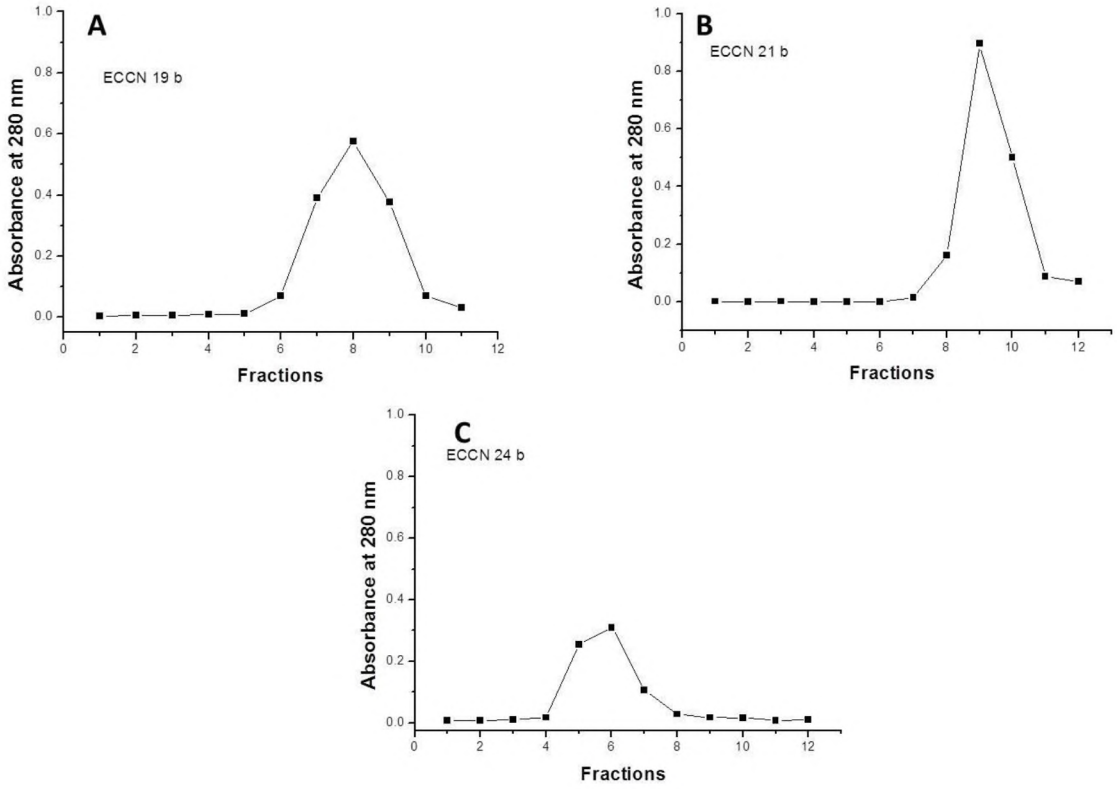


Figure 5.3 Profiles of *Citrobacter* strain ECCN 19b (A), *Exiguobacterium* strain ECCN 21b (B) and *Serratia* strain ECCN 24b (C) protein from the sephadex column.

Figure 5.4 A, B, and C shows the SDS-PAGE analysis of bacterial strains protein after ammonium sulphate precipitation and gel filtration chromatography purification. The purified protein from *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b *and Serratia* strain ECCN 24b upon SDS-PAGE analyses showed it to be composed of several protein subunits, as shown in Figure 5.4. These proteins had different apparent subunit molecular masses. Two protein fractions with approximatly 53 kDa and 82 kDa were precipitated against coal degradation activity of the protein, and had a molecular mass of 72 kDa on deletion. Three protein fractions were identified through gel-filtration chromatography for *Citrobacter* strain ECCN 19b, having the molecular mass of 16 kDa, 33 kDa and 43 kDa. The molecular mass of the band of purified protein from *Exiguobacterium* strain ECCN 21b was estimated to be 37 kDa, 53 kDa and 82 kDa. In the same way, two subunits of molecular mass of 37 kDa and 43

kDa were purified from *Serratia* strain ECCN 24b. As indicated in Figure 5.6 A, B, more proteins were purified from *Citrobacter* strain ECCN 19b and *Exiguobacterium* strain ECCN 21b than from *Serratia* strain ECCN 24b. These proteins could therefore be cooperatively responsible for coal discard and geologically weathered coal degradation.

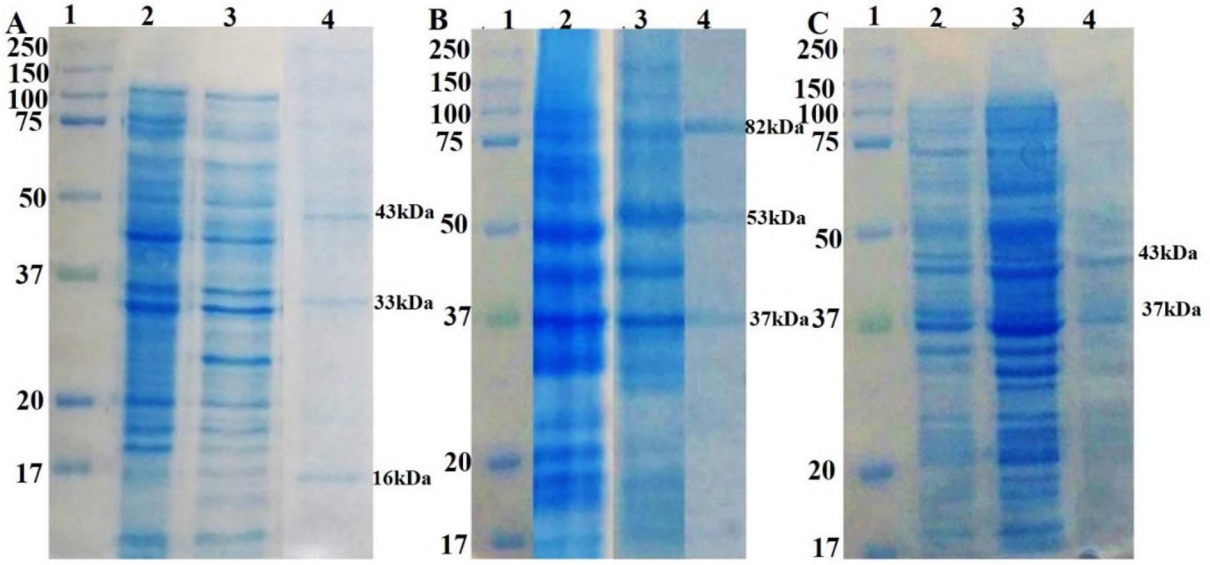
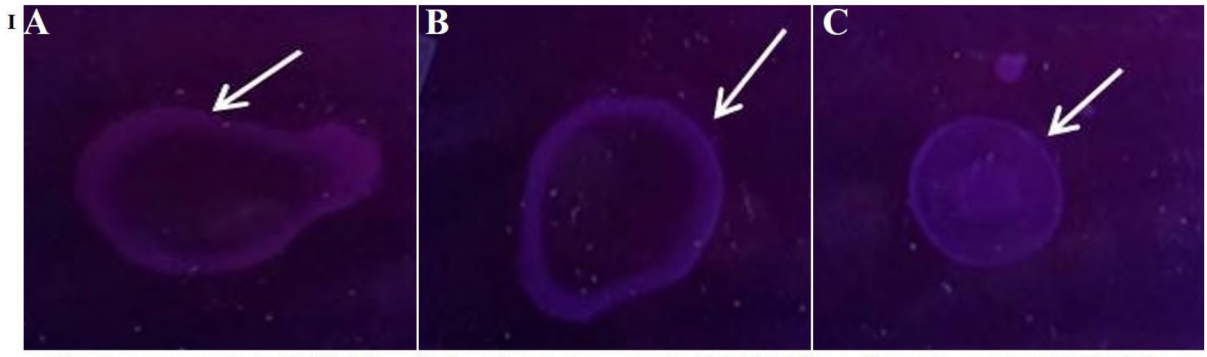


Figure 5.4 SDS-PAGE of crude and purified protein produced by *Citrobacter* strain ECCN 19b (A), *Exiguobacterium* strain ECCN 21b (B) *and Serratia strain* ECCN 24b (C). Aliquots of the extract, equivalent to 5, 4 and 5 µg protein, were analysed by polyacrylamide gel electrophoresis. Lane 1, molecular weight markers; lane 2, crude protein; lane 3, protein after ammonium sulphate purification; lane 4, after gel filtration.

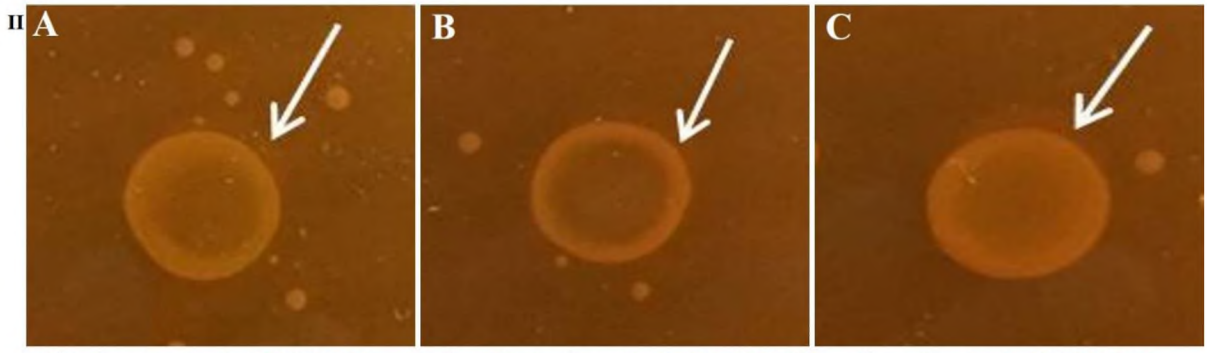
5.3.2 Detection of LAC activity in bacterial strain culture induced by coal substrate

Laccase and CYTP450 enzymes have been implicated in lignin and hydrocarbon degradation, and the bacterial strains under study were screened to determine whether these enzymes are involved in coal degradation. In order to test for the presence of LAC, *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were initially screened using nutrient agar supplemented with bromophenol blue, eosin dyes and guaiacol and catechol on Petri plates, and were incubated at 37 ^{o}C for 7d. The ability of the bacterial strains to decolourise bromophenol blue and eosin was taken to indicate the production of ligninolytic enzymes including LAC. The results in Figure 5.5. I and II show that *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b produced ligninolytic

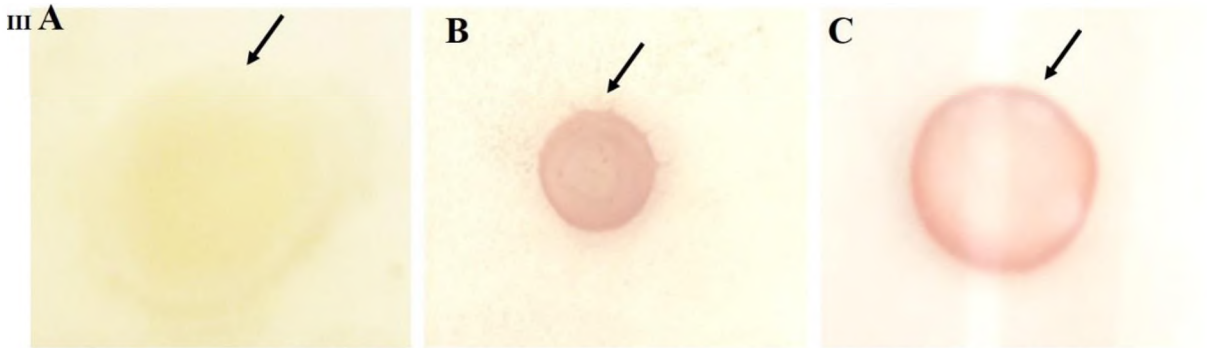
enzymes which enabled them to grow on and decolourise bromophenol blue and eosin (arrow pointing to the zone of growth; Figure 5.5).



***Citrobacter* strain ECCN 19b** ***Exiguobacterium* strain ECCN 21b** ***Serratia* strain ECCN 24b**



***Citrobacter* strain ECCN 19b** ***Exiguobacterium* strain ECCN 21b** ***Serratia* strain ECCN 24b**



***Citrobacter* strain ECCN 19b** ***Exiguobacterium* strain ECCN 21b** ***Serratia* strain ECCN 24b**

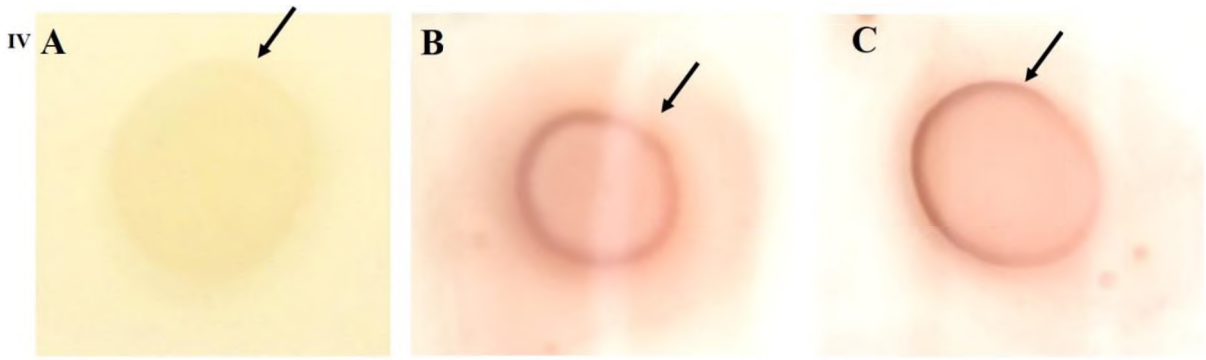


Figure 5.5 Oxidoreductive producing assays. 100ul of a fresh broth culture of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b was placed on nutrient agar plates to assay for the presence of laccase enzyme; I (bromophenol agar plates), II (eosin agar plates), III (quaiacol) and IV (catechol) agar plate grown at 37 ^{o}C for 24 h. Arrows pointing to the zone of growth (white) and production of laccase enzyme (black).

Ability to decolourise lignin (dye) was confirmed by the appearance of halo formation around the bacterial colonies. Following this oxidative screening, the bacterial strains, *Citrobacter* strain ECCN 19 b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were investigated for their ability to produce laccase on coal discard and geologically weathered coal. The presence of laccase in these bacteria was identified by flooding 10 mM guaiacol and 75 mM catechol onto bacterial strain colonies on nutrient agar plates (plate screening). Extracellular laccase activity was monitored by observing the colour change on the bacteria inoculated plate, which indicated the oxidation of the substrates, guaiacol, and catechol. Guaiacol and catechol were oxidised by laccase with the formation of reddish brown and pink colour zones, respectively (Figure 5.5, III & IV).

Of the three organisms screened, *Exiguobacterium* strain ECCN 21b *and Serratia* strain ECCN 24b developed these colours after flooding with catechol and guaiacol (Figure 5.5, III & IV). However, *Citrobacter* strain ECCN 19b was unable to oxidise guaiacol and catechol to develop any colour, suggesting that it did not produce any extracellular laccase. Following the screening of these organisms for LAC production, an assay was performed in MSM supplemented with and without coal (coal discard or geologically weathered coal) substrates as the sole source of

carbon. LAC activity for the bacterial strains was measured at daily intervals for a period of 7d. Guaiacol was used as a substrate to monitor the LAC activity, with the oxidation being monitored using spectrophotometer by measuring the absorbance at 450 nm. The enzyme units were calculated using molar extinction of 12100 M^{-1} cm^{-1}. It was observed that bacterial strain LAC activity in the extract of culture medium containing geologically weathered coal was higher than the extract of culture containing coal discard. LAC enzyme production on coal discard by *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were recorded maximum (4.78 U/mL and 6.24 U/mL) on day 7 (Figure 5.6 A). For geologically weathered coal, a maximum value of 5.76 U/mL was quantified from *Exiguobacterium* strain ECCN 21b, and 6.38 U/mL from *Serratia* strain ECCN 24b (Figure 5.6 B). Evidence of LAC was not detected in the culture supernatant of *Citrobacter* strain ECCN 19b during coal discard and geologically weathered coal biodegradation.

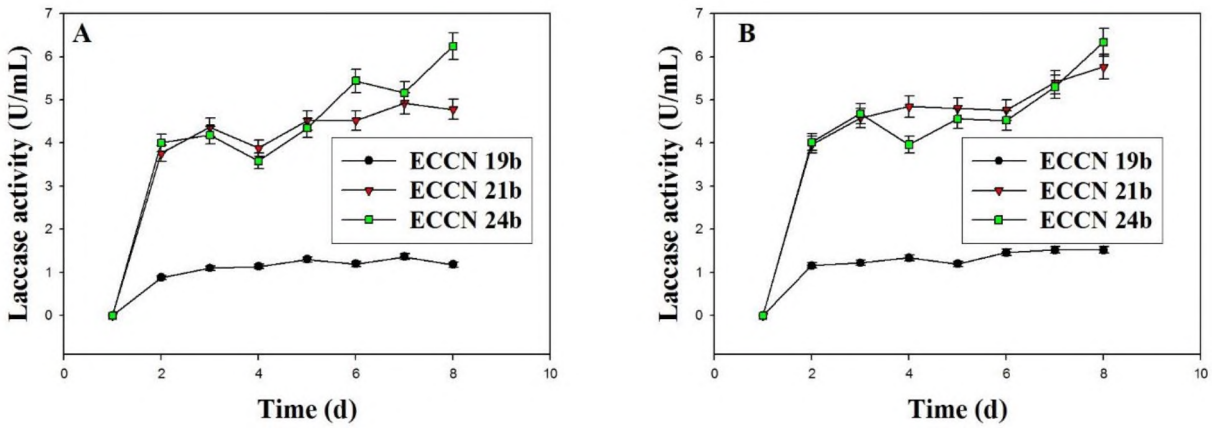


Figure 5.6 Coal discard (A) and geologically weathered coal (B) induced changes in extracellular LAC activity in culture filtrates for *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. Filtrates were assayed for extracellular LAC activity by monitoring the oxidation of guaicol at 450nm. The error bars represent standard error for a mean value of at least three replicates.

5.3.3 Detection of CYTP450 activity in bacterial strains

Following the determination of a standard curve for CYTP450 enzyme assay (see appendix VI), the enzyme in *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in medium supplied with either coal discard or geologically weathered coal, assay variability was verified. Samples were prepared according to the standard and transferred

to a 96-well microplate. The bacterial microsome prepared showed a bright yellow colour, indicating the production of the CYTP450 enzyme by the organisms under investigation. Figure 5.7 shows the relative activity of CYTP450 enzyme detected in *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. The result was based on the detection of bright yellow-coloured products which were measured kinetically at an absorbance of 460 nm for 25 min at 25 °C. For coal discard, the concentration of CYTP450 produced by *Citrobacter* strain ECCN 19b was 53 nmol/min/mg. The value increased (i.e.139 nmol/min/mg) when coal discard was treated with *Exiguobacterium* strain ECCN 21b. In *Serratia* strain ECCN 24b, 72 nmol/min/mg of CTYP450 activity was produced (Figure 5.7 A). For geologically weathered coal, the relative CYTP450 activity produced by *Citrobacter* strain ECCN 19b was 56 nmol/min/mg. In *Exiguobacterium* strain ECCN 21b, the concentration was somewhat higher, as 139 nmol/min/mg was produced. It was noticed that lower concentration (14 nmol/min/mg) of CYTP450 activity was yielded by *Serratia* strain ECCN 24b on coal after 14 d of incubation (Figure 5.7 B). The control did not show any absorbance peak (data not shown).

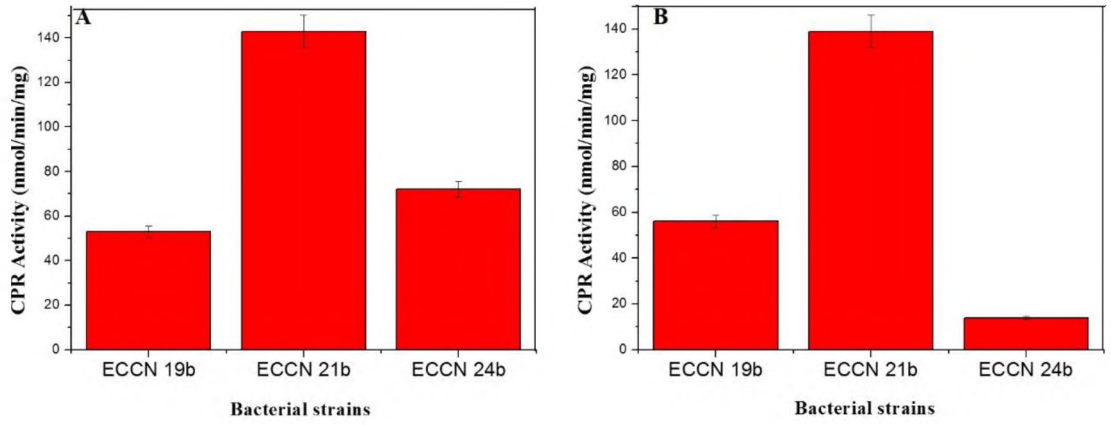


Figure 5.7 Relative CPR activity detected in *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b on coal discard (A) and geologically weathered coal (B) incubated at 37 °C for 14 d. Error bars represent standard errors for a mean value of at least three replicates.

5.4 Summary

Citrobacter strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b produced the protein responsible for degradation of coal discard and geologically weathered coal. Three protein fractions of molecular weights 53 kDa, 72 kDa and 82 kDa were detected using SDS-PAGE from *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in the presence of either coal discard or geologically weathered coal, suggesting their cooperation in coal degradation. Upon purification, protein fractions of molecular weight 16 kDa, 33 kDa and 43 kDa detected from *Citrobacter strain* ECCN 19b, three subunits of molecular weights of 37 kDa, 53 kDa and 82 kDa from *Exiguobacterium* strain ECCN 21b *Serratia* strain ECCN 24b produced 37 kDa and 43 kDa molecular weights of protein, respectively.

The appearance of halo zone around the colonies indicated that the bacterial strains had decolourised bromophenol blue and eosin dye, which seemed to confirm the presence of oxidative enzymes. The screening of oxidative enzymes using guaiacol and catechol, produced by *Exiguobacterium* strain ECCN 21b *and Serratia* strain ECCN 24b, revealed the production of laccase in the formation of pink and reddish-brown colour on the plates. No evidence of laccase production was detected in *Citrobacter* strain ECCN 19b during biodegradation of coal discard and geologically weathered coal. This result suggests a potentially different mechanism for coal degradation for *Citrobacter* strain ECCN 19b.

The laccase detected were quantified using quaiacol assay. For coal discard, a maximum value of 4.78 U/mL laccase activity was quantified for *Exiguobacterium* strain ECCN 21b and 6.24 U/mL for *Serratia* strain ECCN 24b on 7 d of incubation. For geologically weathered coal, a maximum value of 5.76 U/mL was quantified for *Exiguobacterium* strain ECCN 21b and 6.38 U/mL for *Serratia* strain ECCN 24b. Similarly, CYTP450 enzyme was detected in all the bacterial strains in the culture medium containing coal discard and geologically weathered coal. The CYTP450 quantified for *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were 53 nmol/min/mg, 143 nmol/min/mg and 72 nmol/min/mg, respectively, on coal discard. On geologically weathered coal, the result were: 56 nmol/min/mg, 139 nmol/min/mg and 14 nmol/min/mg respectively. This is the first study of bacterial cytochrome P450 enzyme involvement in coal degradation. In other words, the mechanism of degradation of coal discard and geologically weathered coal by *Citrobacter* strain ECCN 19b,

Exiguobacterium strain ECCN 21b and *Serratia* strain ECCN 24b can be attributed to LAC and CYTP450 enzyme action.

CHAPTER 6

General discussion and conclusion

6.1 General discussion

Biological conversion of coal to useful products is an environmentally friendly alternative to conventional chemical and thermal processing (Kang, 2014). Likewise, biological beneficiation of low-grade coal and coal discard through its microbiological conversion to products of value can provide an environmentally sustainable method to mitigate the adverse effects of stockpiled waste. Fungi have historically been the microorganisms of choice in coal biodegradation studies, but recently, bacteria-based biosolubilisation has also been described. Indeed, a previous study at the Institute for Environmental Biotechnology, Rhodes University (EBRU) resulted in the isolation and identification of a suite of bacteria with coal biodegradation potential (Edeki, 2015). Bacteria were bio-prospected from diesel-contaminated soil and coal slurry and studies revealed effective coal-degrading activity when assembled as consortia. Unfortunately, no scientific basis was provided for the choice of organism for each consortium; colonisation of the coal substrate by the various consortia was only partially demonstrated, and the mode of biodegradation was not investigated. This study therefore aimed to investigate the bacterial colonisation and degradation of waste coal using highly oxidised coal discard and geologically weathered coal as substrates in an effort to gain insight into the mechanisms involved in the process.

Coal discard is low calorific residual material generated as a by-product of coal mining and processing. The coal mining industry considers coal discard not marketable because it is rich in pollutants, usually oxidised and of low calorific value (Chassapis and Roulia, 2008; Baldwin, 2004). By comparison, geologically weathered coal resembles Leonardite and is soft, brown mineraloid that is soluble in alkaline solutions and a rich source of humic acid (Asing *et al.*, 2009). It is an oxidation product of lignite and is typically associated with near-surface mining and has high O content and low calorific value making it unsuitable for power generation. Most previous work on coal biosolubilisation using bacteria was conducted after pre-treating coal using nitric acid, sodium hydroxide or hydrogen peroxide, and in some cases, permanganate (Hung *et al.*, 2013). The rationale for pre-treatment is that the oxidised form of coal enhances the susceptibility of coal substrates to bacteria degradation (Maka *et al.*, 1989). By contrast, this study employed naturally oxidised coal that did not necessitate treatment with any acid.

Microorganisms isolated from coal environments have been shown to have the potential to biodegrade coal (Malik *et al.*, 2015; David *et al.*, 2017). A previous study by Edeki (2015) described ten bacterial strains isolated from diesel-contaminated soil and coal slurry that were shown to have coal biodegradation potential as consortia. These bacteria strains were designated as *Bacillus* strain ECCN 18b, *Citrobacter* strain ECCN 19b, *Proteus* strain ECCN 20b, *Exiguobacterium* strain ECCN 21b, *Microbacterium* strain ECCN 22b, *Proteus* strain ECCN 23b, *Serratia* strain ECCN 24b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b, and *Bacillus* strain ECCN 41b. Unfortunately, whether these strains were able to degrade coal individually was never investigated, and there was no apparent scientific basis for the choice of organism adopted for each bacterial consortium. Furthermore, the mechanism of biodegradation used by these consortia was not investigated.

Screening methods for coal degradation using bacterial consortia are usually not suitable for confirming the biodegradation potential capability of the individual organisms to degrade coal substrate (Maka *et al.*, 1989). However, a single organism is considered able to produce substances useful for biodegradation (David *et al.*, 2017). Consequently, in the present study, screening methods which aimed more directly at coal degradation by the individual bacteria organism were used.

Several studies have reported the isolation, characterisation and screening of microorganism for coal biodegradation (Malik *et al.*, 2015), that is, numerous bacteria are known to degrade coal and most of them have been isolated from soil polluted with crude oil (Yperman *et al.*, 2013), cow dung, paddy field soil, termites, mine water (Guptal and Guptal, 2014), and coal slurry (Edeki, 2014; David *et al.*, 2017). Among these bacteria are: *Pseudomonas putida* (Machnikowska *et al.*, 2002), *Pseudomonas cepacia* (Guptal *et al.*, 990; Crawford and Guptal, 1991), *Pseudomonas fluoroscens* (Hazrin-Chong *et al.*, 2014; Fakoussa and Troper, 1983), *Escherichia freundi* and *Pseudomonas rathonia* (Korburger, 1964), *Escherichia coli* and *Streptococci* sp., (Sharma *et al.*, 1992), *Pseudomonas stutzeri* (Singh and Tripathi, 2011), *Bacillus cereus* (Maka *et al.*, 1989), *Streptomyces viridosporous* (Strandberg and Lewis, 1987), *Alcaligenes* sp., *Cupriavidus* sp., *Pseudomonas* sp. (David *et al.*, 2017).

Screening of bacteria for coal degradation using solid (plate) and suspended (liquid) culture techniques (Silva-Stenico *et al.*, 2007; Malik *et al.*, 2015) is a major breakthrough in the biotechnology of coal, and was thus the screening method adopted in this study. Based on the results of screening experiments, all the bacterial strains were confirmed as possessing some capability to degrade coal discard and geologically weathered coal. However, a ranking system, albeit subjective, indicated that *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b were the best candidate strains and these were therefore selected for more detailed study. Furthermore, a bacterial consortium was established using a combination of the three bacterial strains and the biodegradation capability of this consortium determined. This is the first report of the coal-degrading activity of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b using coal discard and geologically weathered coal substrates. It has to be noted that the other bacterial strains had different degradation potential; they were also good, but it was likely that they used the product of coal degradation as a carbon source at the expense of coal (David *et al.*, 2017). The capability of all the bacterial strains for degrading coal discard and geologically weathered coal was based on the evaluation of the formation of brown halos/zones and a brown/yellow colour on the MSM agar plates and liquid medium (supernatant) (Silva-Stenico *et al.*, 2007; Tripathi *et al.*, 2011; Yuan *et al.*, 2013; Valero *et al.*, 2014; Romanowska *et al.*, 2015; Malik *et al.*, 2016), released from the coal in the presence of bacteria. The formation of brown halos/zones and a brown/yellow colour on MSM agar plate and MSM containing either coal discard and geologically weathered coal in the presence of the bacterial strains is in accordance with the results of earlier studies on coal solubilisation by *Bacillus* sp. (Yuan *et al.*, 2013), *Gordonia alkanivorans* and *Bacillus mycoides* (Romanowka *et al.*, 2015). However, the results of studies by Xiu-xiang *et al,* (2009) and Hofrichter *et al,* (1997) on coal solubilisation using fungal species demonstrated the formation of black droplets on the culture plates. The black droplets is due to the coal pretreatment using chemicals as well as rich nitrogen content of the medium that resulted in higher pH values (Hofrichter *et al.*, 1997).

A critical requirement for the bacterial biodegradation of coal is that the bacteria should be able to grow on and colonise the coal substrates. To monitor the growth and colonisation of bacteria on coal substrates, it is important that appropriate methods be used. Although, previous studies such as David *et al* (2017) used the turbidity of the culture medium to estimate bacterial growth, in the present study, a molecular technique based on the indirect-quantification of bacterial

deoxyribonucleic acid (DNA) using diphenylamine (DPA) was developed following Zhao *et al* (2013). The technique is based on the replication of bacterial strains resulting in accumulation of DNA in the growth medium. Since the amount of DNA in microbial cells represents a fairly constant proportion of the total biomass, its measurement can be used to estimate microbial growth; a technique which is a more accurate than the turbidity measure (Doelle *et al.*, 2009). Thus, the growth of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b on coal discard and geologically weathered coal were monitored using the quantification of the DNA. Results of the growth study showed that the bacterial strain's DNA increased exponentially in the presence of both coal discard and geologically weathered coal, suggesting that certain components of coal discard and geologically weathered coal are used as a source of carbon for growth. To the best of my knowledge, this is the first reported attempt to determine bacteria growth on coal using a diphenylamine reagent to quantify DNA accumulation. However, in colorimetric method using a diphenylamine reagent, it may be expected that DNA of dead and living bacteria will be quantified and this may possibly not have portrayed the desired result, but at present, it is expected that the number of dead cells is negligible as coal is a good source of carbon and energy for bacteria replication. Clearly, this method has been used successfully to determine the growth of bacteria species such as *Escherichia coli, Streptomysces clavuligerus, Saccharomyces cerevisiae* and *Trichoderma reesi* on solid particles, as reported in other cases (Zhao *et al.*, 2013).

It has been postulated that colonisation and utilisation of coal components for growth and as a source of energy is accompanied by bacteria degradation (Triphati and Narain, 2011). Many studies had been carried out on colonisation and growth of microorganism on substrates. In their studies, Hunt *et al* (2004), Jana *et al* (2000) and Santina *et al* (2015) concluded that colonisation of solid substrates for degradation purposes is a basic, natural strategy adopted by microorganisms in a wide variety of environments. Although, it is not easy to visualise microorganisms on coal because of its highly adsorptive surface (Mittal and Venkobachar, 1993; Vick *et al.*, 2016), scanning electron microscopy (SEM), being a well-established technique for determining the morphology of bacteria and adherence to material surfaces (Yuehuei and Friedman, 1997; Peters *et al.*, 1982), was used in the present study to confirm the colonisation and adherence of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b to the surface of coal discard and geologically weathered coal. The results of the SEM analysis showed that the cells of the bacterial strains strongly

attached to coal discard and geologically weathered coal surfaces. This finding is in accordance with the result of earlier studies on the adherence and degradation of coal by *Pseudoclavibacter* sp. (Handayani *et al.*, 2016), *Pseudomonas fluoroscens* (Hazrin-Chong and Manefield, 2012) and, *pseudomonas* sp. and *Bacillus* sp. (Malik *et al.*, 2016). The transmission electron microscopy (TEM) micrographs also indicated the presence of flagella and associated extracellular polymeric substance (EPS) on the bacterial strains in the presence of coal discard and geologically weathered coal, which were presumed to aid and facilitate cell attachment and uptake of nutrients from the coal substrate. The presence of flagella, production of microcolonies and associated EPS by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b is attributed to coal discard and geologically weathered coal degradation where in this process has been demonstrated and reported by several groups of researchers (Costerton *et al.,* 1995; Laborda *et al.*, 1997; Vick *et al.*, 2016).

A preliminary study of EPS from the bacterial strains was therefore performed. Extracellular polymeric substance was extracted from *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in a mineral salt medium containing either coal discard or geologically weathered coal, using chilled alcohol and analysed by FTIR. In the present study, the various functional groups of the EPS extracted from the bacterial strains as included: broad and intense stretching characteristic of hydroxyl groups (O-H), asymmetrical C-H stretching of aliphatic C-H groups, N-H bonding (Amide I & II) corresponding to functional groups in protein, stretching of C-O-C corresponding to the presence of carbohydrates, and O-H bonding of carboxylic acids (Hafsa and Asghari, 2014). These bacterial EPS's may therefore play an important role in adherence of cells to coal surfaces, and contribute to the biodegradation potential of bacteria (Handayani *et al.*, 2016).

Although bacteria adhere to coal surfaces using a variety of mechanisms, they naturally tend to adhere to surfaces as a survival strategy (Santina *et al.*, 2015). The surface roughness of coal is an important property that promotes bacterial adherence and colonisation (Characklis *et al.*, 1990; Raichur and Vijayalakshmi, 2003; Lakshmi *et al.*, 2012). This property may have promoted the bacterial growth and colonisation observed in the present study.

The ability of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b to grow on and colonise coal indicated that they use coal as the carbon source.

It is therefore distinctly possible that these bacterial strains have the capability to degrade coal discard and geologically weathered coal, and have to be described and characterised in relation to coal degradation. Several studies have shown that *Citrobacter freundi* degrades lignin (Chandra and Abhisek, 2011), dyes (An *et al.*, 2002), used engine oil (Ibrahim, 2016), and phenols (Dars and Kumar, 2015); *Exiguobacterium aurantiacum* degrades diesel and hydrocarbon (Mohanty and Mukherji, 2008; Shende, 2013; Chen *et al* 2017), and *Serratia* sp. degrades hydrocarbons, crude oil and lignin (Benedek *et al.*, 2011; Wi *et al.*, 2012; Wongsa *et al.*, 2004; Kassim *et al.*, 2016). Despite the good degrading potential of these bacterial species, they have not been used for the treatment of coal. According to a previous study, lignin and coal share similarities in structure (Hayatsu *et al.*, 1979; Olson and Brinckman, 1986), so it is not surprising that these bacterial strains possess the ability to degrade coal discard and geologically weathered coal effectively. In the same way, bacterial species such as *Pseudomonas fluoroscens* (Rahmanpour and Bugg, 2015) and *Streptomyces* sp. (Fernandes *et al.*, 2014), earlier reported for lignin degradation, have also been confirmed as good candidates for coal degradation.

During the process of growth on and colonisation of coal discard and geologically weathered coal by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b, culminating in coal degradation, there was a significant increase in the pH of the culture medium. Initially, pH declined on account of the acidic nature of these coal substrates; later it rose and remained almost stable throughout the period of incubation. Increased in pH during biodegradation of coal discard and geologically weathered coal were attributed to the release of alkaline substances by the bacterial strains. Thus, in this study, alkaline substances presumably released by the bacterial strains in the culture medium containing either coal discard or geologically weathered coal may not be responsible for coal substrates degradation; instead, it is possible that biodegradation is more likely to be the result of enzymatic action. Although, studies have confirmed that microbial alkaline alone could degrade coal substrate (Yuan *et al.*, 2006; Yuan *et al.*, 2013), cooperative activity of alkaline substances and enzymatic reactions have been implicated in coal degradation (David *et al.*, 2017). Thus, alkaline production by microorganisms is known to be the initial steps of enzymatic reactions during the process of coal degradation (Stranberg and Lewis, 1987).

Coal discard and geologically weathered coal released soluble and insoluble (residual) products after treatment with *Citrobacte*r strain ECCN 19b, *Exiguobacterium* strain ECCN 21b or *Serratia* strain ECCN 24b either individually or in consortium. The soluble products caused the medium to change from near colourless to brown/yellow (supernatant) and this colour change was measured spectrophotometrically at 450 nm and 280 nm. The results of the soluble product analysed showed increased absorbance at 450 nm, which surpported the biodegradation of coal discard and geologically weathered coal by bacteria. The finding is in line with the results of earlier studies on coal degradation using *Rhizobium* and *Chelatoccocus* (Triphati *et al.,* 2011), *Bacillus* sp. (Yuan *et al.*, 2012) and *Pseudomonas* sp. and *Bacillus* sp. (Malik *et al.*, 2016). Similarly, various studies have reported the use of UV-Vis spectrophotometric analysis of the soluble product of coal degradation in the range of 200 —700 nm to determine coal solubilisation (Yuan *et al.,* 2006; Yuan *et al.*, 2009; Tao *et al.*, 2009). In the present study, the released soluble product was scanned using spectroscopy in the region of 200 —700 nm. Scanning this soluble product in this region revealed a discrete 'shoulder' around 270 nm (Cunha *et al.*, 2009). Overall, the results of the spectrophotometric analysis of the soluble product strongly suggest that humic acid-like substances, characteristic of a Type B (Kumada, 1987; Cunha *et al.,* 2009) were released from the coal discard and geologically weathered coal. In recent case studies, UV-Vis analysis of humic acid and soil-extracted humics displayed characteristic spectra with maxima between 250 and 300 nm (Peuravuori *et al.*, 2002; Gao *et al.*, 2016), similar to these results on UV-Vis scanning of the soluble products of coal discard and geologically weathered coal biodegradation. In the same way, maximum absorption in the region 230nm to 300nm has been attributed to aromatic compounds containing double bonds in coal discard and geologically weathered coal (Shi *et al.*, 2013). It is therefore not surprising that coal is commonly used as a source of humic acids for production of soil supplements in agriculture (Rahayu *et al.*, 2017). To confirm this possibility, humic acid was extracted from coal discard and geologically weathered coal using alkali solubilisation, which provides a useful and interesting comparison. Using FTIR spectroscopy, the humic acid-like substances extracted was similar to commercial humic acid. Thus, humic acid from coal could be used to rehabilitate coal dumps as well as providing a potential organic resource for use in agriculture, medicine and environmental protection (Ghani *et al.*, 2015).

Fourier transform infrared (FTIR) spectroscopy, a widely used analytical technique for determining the different functional groups in a coal structure (Manoj and Narayan, 2013;

Manoj, 2014), was used to analyse the insoluble product. The spectroscopy spectra generated for the insoluble (residual) product of coal discard and geologically weathered coal degraded by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b showed coal functional groups had been modified (Malik *et al.*, 2016; Jinshui *et al.*, 2004). This study showed the formation of carbonyl absorption bands at 1737 cm^{-1} due to the formation of ketones, aldehyde and carboxylic acids groups. Also, the corresponding O-H spectrum generated in the range 3600 –3200 cm^{-1} associated with alcohol or phenol in the residual coal discard and geologically weathered coal was stronger than the untreated and the control. This finding was in line with the previous result of coal degradation by *Pseudomonas fluorescens* (Hazring-Chong *et al.*, 2014), and *Pseudomanas putida* (Machnikowska *et al.*, 2002). The spectrum generated on residual coal discard is more discernible and stronger than geologically weathered coal, which had been pre-oxidised. No doubt some organisms do not have the ability to degrade oxidised forms of coal as reported in some cases (Reed, 1990; Akhtar *et al.*, 2016). Nonetheless, weathered coal underwent partial degradation both chemically and biologically (Opara *et al.*, 2013), making it a good source of humic acid for agriculture (Li *et al.*, 2012). In fact, the appearance of new peaks observed is similar to the pattern of results reported during coal degradation by microorganisms (Malik *et al.*, 2016).

Using the CHNS analyser on the insoluble (residual) product revealed the capability of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b to degrade coal discard and geologically weathered coal. The elemental analysis of the insoluble (residual) product revealed that the chemical composition of the coal discard and geologically weathered coal were modified during bacterial degradation. The finding is in agreement with the result of coal degradation by *Bacillus mycoides* and *Gordonia alkanivorans* (Romanowska *et al.*, 2015) and *Bacillus* sp. (Yaun *et al.*, 2013). An increase in elemental nitrogen was observed, and this was related to the release of humic acid-like substances from coal discard and geologically weathered coal (Yuan *et al.*, 2013). It is also conceivable that increased elemental nitrogen may be attributed to the alkaline materials secreted into the medium by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b, as reported during microbial degradation of sub-bituminous coal (Manoj and Elcey, 2010). In addition, the insoluble product of geologically weathered coal is characterised by higher hydrogen and lower oxygen content, as is consistent with the result of elemental analysis of residual coal degraded by *Pseudomonas putida* (Machnikowska *et al.*, 2002).

The soluble and insoluble products of coal discard and geologically weathered coal degradation can be explained by different reactions or mechanisms used by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. The only way to establish this reaction or mechanism was to follow the protein composition and enzymatic action. The capability of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b to degrade coal discard and geologically weathered coal appeared to be the result of production of extracellular protein. The extracellular proteins which were detected had bands of approximate molecular mass, 53 kDa, 72 kDa and 82 kDa on SDS-PAGE gel. Upon purification using ammonium sulphate and ion exchange chromatography, the deletion of one protein (72 kDa) was observed and four new proteins were detected with approximate molecular masses of 16 kDa, 33 kDa, 37 kDa and 43 kDa, irrespective of the bacterial strains. This result suggested the possibility that bacterial degradation of coal discard and geologically weathered coal degradation involves the cooperation of extracellular protein or enzymes produced by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b. Similarly, microbial extracellular protein was also reported for degradation of lignite coal (Xiu-Xang *et al.*, 2009). Thus, the bacterial strains extracellular protein enhances coal discard and geologically weathered coal degradation (Odom *et al.*, 1999).

Previous reports on microbial coal degradation have stressed the variability of mechanisms of degradation and have pointed out that either enzymatic, alkaline substance production or chelation is responsible for coal degradation (Yuan *et al.*, 2006; Jiang *et al.*, 2013; Romanowska *et al.*, 2015). It has been established that dyes serve as an appropriate base material for screening microorganisms for oxidative ligninolytic (oxidative) enzymes (Malik *et al.*, 2015: Baylon *et al.,* 2017). Previous results have demonstrated the capability of *Citrobacter freundii*, *Exiguobacterium* sp. *and Serratia* sp. to grow on and degrade dyes effectively (Dhanve *et al.*, 2008; Wang *et al.*, 2009; An *et al.*, 2013; Gusmanizar *et al.*, 2016), thus confirming the ability of these bacterial species to produce ligninolytic enzymes. To test for the presence of oxidative ligninolytic activity, the appearance of halos around bacterial colonies on backgrounds of either blue or Eosin dye was used which confirmed that *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b exhibit ligninolytic enzyme activity.

Although, it has been reported that *Citrobacter freundii, Exiguobacterium* sp. and *Serratia* sp. produce biosurfactant to degrade used engine oil, petroleum, and hydrocarbons (Wongsa *et al.*, 2004; Shende, 2013; Ibrahim, 2016), enzymes responsible for coal degradation may be present in the bacterial strains. Previous results have confirmed that laccase was produced by *Serratia* sp. to degrade and detoxify paper mill pulp effluent (Chandra *et al.*, 2012) and *Exiguobacterium* sp. for toluene degradation (Hosseini *et al.*, 2012).

The involvement of laccase and cytochrome P450 in the degradation of coal discard and geologically weathered coal may have been present in the bacterial strains. Evidence of LAC activity was established in *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b by oxidation of catechol and guaiacol substrates (Wang *et al.*, 2010; Mishra and Srivactava, 2016). *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b produce LAC with the formation of pink and reddish-brown colour on the plate (Wang *et al.,* 2010; Rajeswari and Bhuvaneswari, 2016). Catechol and guaiacol have also been used successfully for detection of LAC production by *Halobacillus halophilus* (Bhuvaneshwari, *et al.*, 2015), and *Bacillus subtilis* (Sheikhi *et al.*, 2012). Therefore, this confirmation is a baseline for LAC production before its optimisation and application (Kumar and Raphael, 2010). As shown in the results, *Serratia* strain ECCN 24b had more LAC activity than *Exiguobacterium* strain ECCN 21b on coal discard and geologically weathered coal. An increase in LAC production by the bacterial strains may be attributed to the nutritional content, i.e. carbon present in the coal substrates. As reported in other cases, carbon is a source of energy for growth of bacteria and enhanced production of LAC (Galhaup *et al.*, 2002; Kaira *et al.*, 2015). However, the growth of bacteria on the substrate is not necessarily an indicator of bacteria's biodegradation capability, but of its ability to produce the relevant enzyme (Itah and Essien, 2005).

With regard to ligninolytic oxidative enzymes such as LAC, MnP, LiP and cytochrome P450 involvement in lignin decolourisation and degradation (Fakousa and Hofrichter, 1999; Doddapaneni *et al.*, 2005; Matsuzaki and Wariishi, 2005; Bugg *et al.*, 2011; Baylon *et al.*, 2017), it is necessary to note that MnP and LiP, have not been detected in this work.

Similar to the findings in the present study, other bacterial strains, which have been involved in coal degradation, and produce extracellular LAC include *Bacillus mycoides*, *Acinetobacter baumanni* and *Microbacterium* sp. (Cubillos-Hinojosa *et al.*, 2015).

The production of cytochrome P450 by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b in the presence of coal discard and geologically weathered coal was established using a cytochrome P450 reductase activity assay kit (colorimetric). Several approaches have been adopted in earlier studies that have attempted to elucidate the biotechnological application of bacterial cytochrome P450. Cytochrome P450 has been reported as an inducible enzyme implicated in the biodegradation of various carbohydrates such as alkanes, aliphatic and aromatic compounds, and ethyl-tert-butyl ether (ETBE) (Khmelevtsova *et al.*, 2017). It is not surprising that cytochrome P450 initiates the attack on aromatic compounds in coal, as reported in another case (Field *et al.*, 1995). Despite the fact that there is no precedent of bacterial cytochrome P450 involvement in coal degradation, it has been implicated in the degradation of polycyclic aromatic hydrocarbon (Yadav *et al.*, 2006), petroleum hydrocarbon (Das and Chandran, 2011) and lignin (Subramanian and Yadav, 2008). Indeed, cytochrome P450 of *Exiguobacterium* sp. have been reported to degrade malachite green (Wang *et al.*, 2012). Similarly, the metabolism of cineole by *Citrobacter* sp. is known to involve cytochrome P450 (Hawkes *et al.*, 2002). Based on this result, there is no doubt that cytochrome P450 and laccase were used by *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b to degrade coal discard and geologically weathered coal. In light of the above, the combined action of bacterial strains cytochrome P450 and laccase have been implicated in the degradation of coal discard and geologically weathered coal as reported in the degradation of polycyclic aromatic hydrocarbon (Boomanithan and Reddy, 1992). However, it is likely that the other coal degrading enzymes may be discovered in the future.

Microbial degradation of compounds is often investigated in the laboratory to measure biodegradation rates that can be used to predict potential biodegradation rates in the environment (Joseph, 1990). This study established a central idea about the role of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b, in the biodegradation of coal discard and geologically weathered coal, an idea that, with further modifications, will have large-scale applications in the biotechnology of coal for sustainable development. Meanwhile, with the increasing knowledge of microbial biodegradation of coal, it goes without saying that it has been demonstrated and proved that many microorganisms (fungi and bacteria) have the potential for coal degradation, thus yielding products of

biotechnological importance. In other words, biodegradation of coal by several species of fungi has been reported widely but very little is known about the role and contribution of bacteria in this process. Biodegradation of coal is not confined to fungi alone but also to a number of bacterial species. Although not all the organisms isolated from the coal environment have the potential to biodegrade coal, screening activities have shown many bacterial species are implicated in coal degradation.

The ability of some indigenous bacteria, especially those isolated from contaminated soil to degrade hydrocarbons, is well known. The fossil fuel environment can be an important reservoir of bacteria of biotechnological importance. The ability to isolate a number of hydrocarbon-degrading organisms from an environment is commonly taken as evidence that those organisms are the active degraders of hydrocarbon contaminants (Okerentugba and Ezenroye, 2003). The investigation of the potential of indigenous bacteria rather than exogenous strains for coal degradation has been largely successful, showing that they are efficient in the breakdown of coal. This is significant because many of these bacteria, isolated from the coal environment, promote plant growth; hence, their effectiveness in rehabilitating waste coal dumbs.

A growing interest in the biodegradation of coal, and thus the production of valuable products of biotechnological importance i.e. Fungcoal, has led to tremendous achievements in the biotechnology of coal in recent years at the Institute for Environmental Biotechnology, Rhodes University, Grahamstown, South Africa. The high demand for coal and its associated products during the last decades has made polluted/degraded coal-mine areas inevitable consequences of mining activities. Microbial degradation of coal appears to be the most environmentally friendly method for the generation of value-added products (Ralph and Catcheside, 1994; Yuan *et al.*, 2006) since the conventional methods such as chemical and physical methods result in the release of more recalcitrant compounds into the environment.

A wide variety of microorganisms are capable of biosolubilising coal to release the humic acid-like substance that can be applied to rehabilitate degraded land caused by mining activities (Igbinigie *et al.*, 2009). *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b are not the only bacteria able to degrade coal discard and geologically weathered coal to release humic acid-like substances; it has also been reported that bacteria species such *Bacillus* sp. (Yuan *et al.*, 2013), *Bacillus mycoides, Acinetibacter baumanni*,

Microbacterium sp. and *Enterobacter aerogenes* (Cubillos-Hinojosa *et al.*, 2015; Valero *et al* ., 2014) used in the study of coal degradation have the same capability.

Research into and application of coal degradation by fungi have provided much useful information that can be used to design effective bacterial coal degradation with emphasis on the improvement and innovation. Historically, most microbial organisms capable of coal biodegradation have been isolated from the coal environment and only in this research have *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b been the subject of examination and experiment. To the best of my mknowledge, *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b have not been reported in the biodegradation of coal substrate, this is the first report of these bacterial strains' capability to degrade coal discard and geologically weathered coal. The fact that these bacteria were isolated from the coal environment is an indication of the adaptive advantages of indigenous bacteria over the exogenous strains. The investigation of these bacteria on coal discard and geologically weathered coal has been largely successful with research findings that they leached humic acid-like substances (Valero *et al.*, 2014). Thus, humic acids are of value in the fields like agriculture, industry, medicine and environmental protection (Ghani *et al.*, 2015).

The ability of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b to degrade coal discard and geologically weathered coal, so releasing humic acid-like substances, is not surprising as several researchers have demonstrated the unique properties of these bacteria to promote plant growth (Nguyen *et al.*, 2003; DiSalvo *et al.*, 2014; Yami *et al.*, 2016). Thus, *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b are promising candidates for technology in the development of green processes, converting coal discard and geologically weathered coal into a green energy source.

6.2 Conclusion

Waste coal, which is largely regarded as low-energy value discard and of little or no commercial value is the predominant waste generated in coal mining. In South Africa, waste coal is mostly stockpiled in surface impoundments referred to as discard dumps. These waste dumps are aesthetically unpleasing and a major source of dust and pollution. For example, as water percolates through this stockpiled waste, toxic metals are leached out polluting ground water

and streams and, causing acid-mine drainage. Waste coal, therefore, requires careful disposal and management to prevent and minimize air, water and soil contamination, and to mitigate the negative effects on human health. The current strategy used by the coal mining industry in South Africa is to treat discard dumps and opencast spoil with high levels of lime to negate any acid generating potential of the substrate, cover the dumps in a layer of topsoil (usually 50−100 cm), and re-vegetate using selected annual and perennial species. This approach brings with it specific problems that include acidification of the cover soil due to capillary rise of acid leachate formed gradually from the underlying discard. As a consequence, re-vegetation is often sporadic, substrate compaction ensues, and the cover vegetation eventually dies. This, coupled with a 'robbing Peter to pay Paul' approach to the sourcing and acquisition of topsoil, results in a rehabilitation strategy that is far from sustainable and outcomes that are less than desirable.

A major oversight in developing the above rehabilitation strategy is the apparent omission of some very important biotic factors that underpin successful and sustainable re-vegetation of land disturbed by mining. For example, coal mining not only visibly disrupts the aesthetics of a landscape; it disrupts all of the soil components including soil horizons and structure, soil microbe populations, and nutrient cycles that are crucial to sustaining a healthy ecosystem. All ecosystems function optimally as a process that is dependent on nutrient re-cycling, which is the balance between growth and decomposition of microbial, plant and animal matter. Soil provides the scaffold to sustain this process, so its structure, composition, and density directly affect the future stability of any plant community post rehabilitation. Recontouring the land once mining is complete returns the landscape to a natural looking form, but the replaced soils do not resemble a pattern akin to normal evolutionary development. Soil components may have been negatively affected by one or more events associated with coal mining: initial removal of soil from the site as mining activity started, storing or stockpiling soil, respreading soil upon completion of mining activities, and post-spreading circumstances including the application of remotely sourced topsoil. Reclamation strategies must therefore address soil structure, microbe populations, and nutrient cycling in order to transform the land from its disturbed condition to a self-sustaining ecosystem. Any rehabilitation protocol must therefore include strategies that rebuild soil structure, stimulate soil microbial populations and re-establish nutrient cycles if this system is to reassemble and become self-sustaining.

The present study, as far as I am aware, is unique in being the first to demonstrate bacterial utilisation of South African waste coal and, the use of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b results in the release of humic acid-like substances. *Citrobacter* sp, *Exiguobacterium* sp. and *Serratia* sp. are known to utilise and degrade hydrocarbons and promote plant growth. Thus, *in situ* treatment of waste coal dumps using these bacterial strains as a catalyst might be a possibility and lead to the release of products such as humic acid, capable of supporting re-vegetation of coal dumps. This will help to mitigate the deleterious effects of stockpiled waste coal and enhance waste coal dump rehabilitation.

Finally, although this study has demonstrated that the bacterial strains can biodegrade waste coal and release humic acid-like substances, an important area of research that is not explored in the present study is an investigation into whether the employed bacterial strains could produce methane gas and other industrially important chemical such as alcohol during the biodegradation process. It is therefore recommended that future study explore this area of research.

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APPENDICES

Appendix I. Gene sequences for the bacterial strains

ECCN 19b confirmed as *Citrobacter* strain

Accession number CP016952.1

CGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGG
CGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGCG
ATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAG
GTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGC
CCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTG
GCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGT
TGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCT
CASRGTTCCCGAAGGCACCAARSCATCTCTGSTAAGTTCYSTGGATGTCAAGAGTAGGTA
AGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTC
AATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAG
CTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTC
CAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACC
TGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGGATGCAGTTCCCAGGT
TGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTA
ATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGT
GCTTCTTCTGCGAGTAACGTCAATCGYTGCGGTTATTAACCACAACGCCTTCCTCCTCGC
TGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTT
GCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAG
TTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTT
ACCCCACCTACTAGCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAGGTCCC
CCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTATC

ECCN 21b confirmed as *Exiguobacterium* strain

Accession number KJ722475.1

CACTTTCGGCGGCTGGCTCCCTAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCG
TGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCAGTATGCTGACCTG
CGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAA
CGGCTTTCTGGGATTGGCTCCACCTCGCGGCTTCGCTGCCCTTTGTACCGTCCATTGTAG
CACGTGTGTAGCCCAACTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAACTGAATGGTGGCAACTAAGGACAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGC
ACCACCTGTCACCCCTGCCCCCGAAGGGGAAGGTACATCTCTGTACCGGTCAGGGGGATG
TCAAGAGTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGT
GCGGGTCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGACCGTACTCCCCAGGCGGAGTG
CTTAATGCGTTAGCTTCAGCACTGAAGGGCGGAAACCCTCCAACACCTAGCACTCATCGT
TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAG
CGTCAGTTATAGGCCAAAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCAT
TTCACCGCTACACGTGGAATTCCACTCTTCTCTCCTATACTCAAGCCTCCCAGTTTCCAA
TGGCCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAGGCCGCCTGCGCGCG
CTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCA
CGTAGTTAGCCGTGGCTTTCTCGCAAGGTACCGTCAAGGTGCCGCCATTGCCTGCGGCAC
TTGTTCTTCCCTTACAACAGAACTTTACGACCCGAAGGCCTTCATCGTTCACGCGGCGTT
GCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCT
GGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACC

ECCN 24b confirmed as *Serratia* strain

Accession number KC172028.1

GGTTAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGT
GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTA
CTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGTACTT
TATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATACGCCATTGTAGCACGTG
TGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTA
TCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCAC
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CCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACG
CGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAATCGACATCGTTTACAGCG
TGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTC
TTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGC
TACACCTGGAATTCTACCCCCCTCTACGAGACTCTAGCTTGCCAGTTTCAAATGCAGTTC
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CCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTA
GCCGGTGCTTCTTCTGCGAGTAACGTCAATTGATGAACGTATTAAGTTCACCACCTTCCT
CCTCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGCATC
AGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTG
TCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGA
GCCATTACCCCACCTACTAGCTAATCCCATCTGGGCACATCTGATGGCAAGAGGCCCGAA
GGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTATC
CCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCC

ECCN 23b confirmed as *Proteus* strain

Accession number KX058415.1

GCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGG
GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGC
GATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACAGACTTTATGA
GTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGCCATTGTAGCACGTGTGTAG
CCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACC
GGCAGTCTCCTTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGATAAGGGTTGCGCTC
GTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGT
CTCAGCGTTCCCGAAGGCACTCCTCTATCTCTAAAGGATTCGCTGGATGTCAAGAGTAGG
TAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCG
TCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACGCGTT
AGCTCCAGAAGCCACGGTTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGA
CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTG
TCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCACATCTCTACGCATTTCACCGCTACA
CATGGAATTCTACCCCCCTCTACAAGACTCTAGCCAACCAGTTTCAGATGCAATTCCCAA
GTTAAGCTCGGGGCTTTCACATCTGACTTAATTGACCGCCTGCGTGCGCTTTACGCCCAG
TAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCG
GTGCTTCTTCTGCGGGTAACGTCAATTGAYAAGGGTATTAACCTTATCACCTTCCTCCCC
GCTGAAAGTACTTTACAACCCTAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGC
TTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC
AGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTAGAGATCGTCGCCTAGGTGAGCCT
TTACCCCACCTACTAGCTAATCCCATATGGGTTCATCCGATAGTGCAAGGTCCGAAGAGC
CCCTGCTTTTGGTCCGTAGACATTATGCGGTATTAGCCACCGTTTCCAGTAGTTATCCCC
CTCTATCGGGCAGATCCCCATACATTACTCACCCCGTCCGCCGCTCGTCAGCAAGAAAG

ECCN 20b confirmed as *Proteus* strain

Accession number JF775423.1

CGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGG
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ATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACAGACTTTATGAG
TTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGCCATTGTAGCACGTGTGTAGC
CCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCG
GCAGTCTCCTTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGATAAGGGTTGCGCTCG
TTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTC
TCAGCGTTCCCGAAGGCACTCCTCTATCTCTAAAGGATTCGCTGGATGTCAAGAGTAGGT
AAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGT
CAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACGCGTTA
GCTCCAGAAGCCACGGTTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGAC
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CCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCACATCTCTACGCATTTCACCGCTACAC
ATGGAATTCTACCCCCCTCTACAAGACTCTAGCCAACCAGTTTCAGATGCAATTCCCAAG
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AATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGG
TGCTTCTTCTGCGGGTAACGTCAATTGAYAAGGGTATTAACCTTATCACCTTCCTCCCCG
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TGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCA
GTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTAGAGATCGTCGCCTAGGTGAGCCTT
TACCCCACCTACTAGCTAATCCCATATGGGTTCATCCGATAGTGCAAGGTCCGAAGAGCC
CCTGCTTTGGTCCGTAGACATTATGCGGTATTAGCCACCGTTTCCAGTAGTTATCCCCCT
CTATCGGGCAGATCCCCATACATTACTCACCCGTCCGCCGCTCGTCAGCAAGAAAGCAAG
CTTTCTCCTGTTACCG

ECCN 26b confirmed as *Bacillus* strain

Accession number KX242272.1

GGCTCCAAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGC
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CAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG
CAGTCACCTTAGAGTGCCCAACTTAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACT
CTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTTCAGAGGATGTCAAGACCTGGTAAG
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GAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAA
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TCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAG
TCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTT
ACCTCACCAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGCCGAAGCCGCCTT
TCAATTTCGAACCATGCAGTTCAAAATGTTATCCGGTATTAGCCCCGGTTTCCCGGAGTT
ATCCCAGTCTTATGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTA

ECCN 18b confirmed as *Bacillus* strain

Accession number KM114617.1

AAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA
CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCT
TCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGATTAGCTCC
ACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCAT
AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACC
TTAGAGTGCCCAACTTAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTT
AACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCTCCC
GAAGGAGAAGCCCTATCTCTAGGGTTTTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCG
CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTG
AGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACT
AAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTA
TCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTC
GCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTCC
ACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTG
GGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGAT
AACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGG
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TGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACC
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CTTATGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACTTCTTGAGAGCAAG
CTCTCAATCCATTCGCTCGACTTGCAT

ECCN 41b confirmed as *Bacillus* strain

Accession number KJ127191.1

GGTAAGCGCCCTCCCGAARGGTWACCCCCCCACTTYTKTTGGA-CCCACTTCTATGGGGT
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GSTTTAAGAAGACCASTTGCTCCTCSSGGGYTTG-TTSTTTTTGGATCCGYCCTTGGAAS
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GKTTAGYMCCGGSAAGYTCCCTTAAAGTGCCCAACTTACCGATGG-AACTAARATCAAGG
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TMAGYTACRGACCAGARAGYSGCCTTCGCCACTGGWGTTCCTCCAWATCTCTACGMATTT
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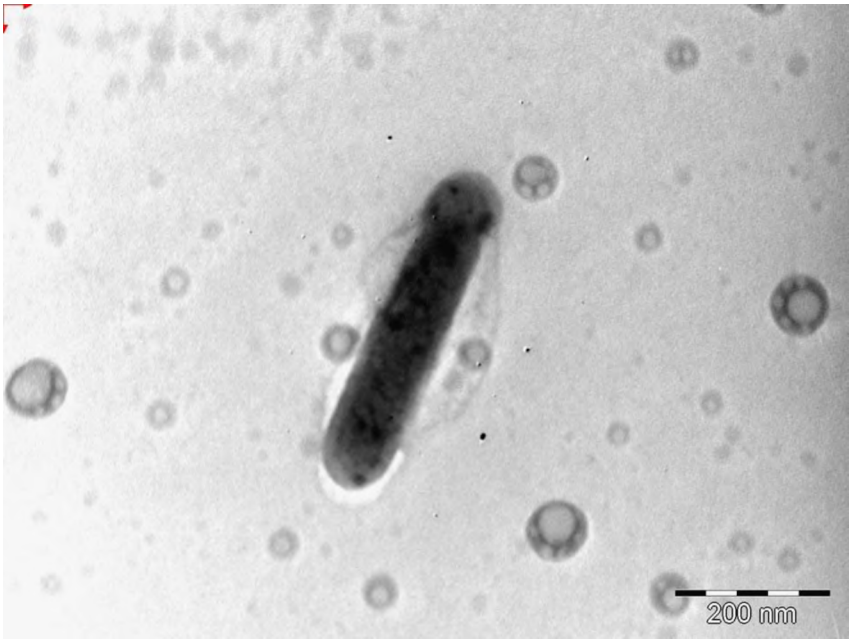
GTAG-TAGCCGTGCTTYTCYTGTYAGGTA-CGTCAATCGTG-CAGCTTATTAMMMCTAGC
RCTTGTCCTTCCGTAGCAACRGAGTTTTACGACCKRARAGCCTTTCATCACTCACGCGAC
TGTKCTMCGTACGACTGTCGTCAATTGCAGA

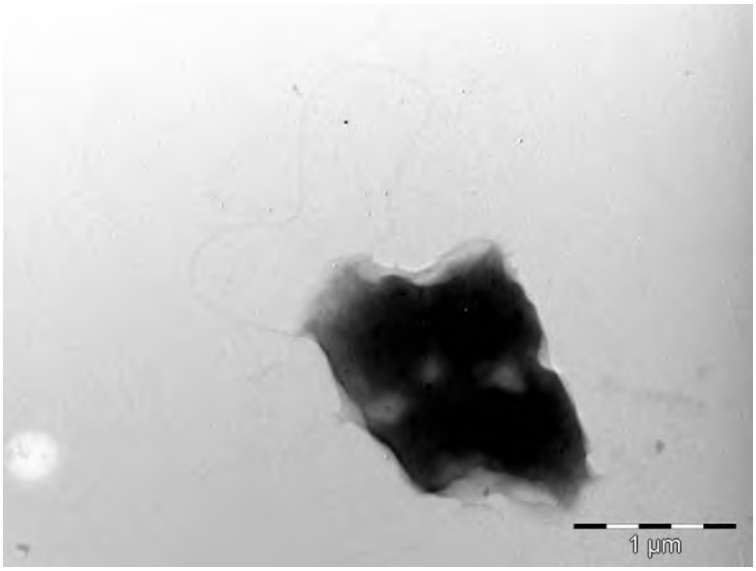
Appendix II. Growth of bacterial strains on nutrient agar plate

Growth of *Citrobacter* strain ECCN 19b, *Exiguobaterium* strain ECCN 21b and *Serratia* strain ECCN 24b on nutrient agar plate

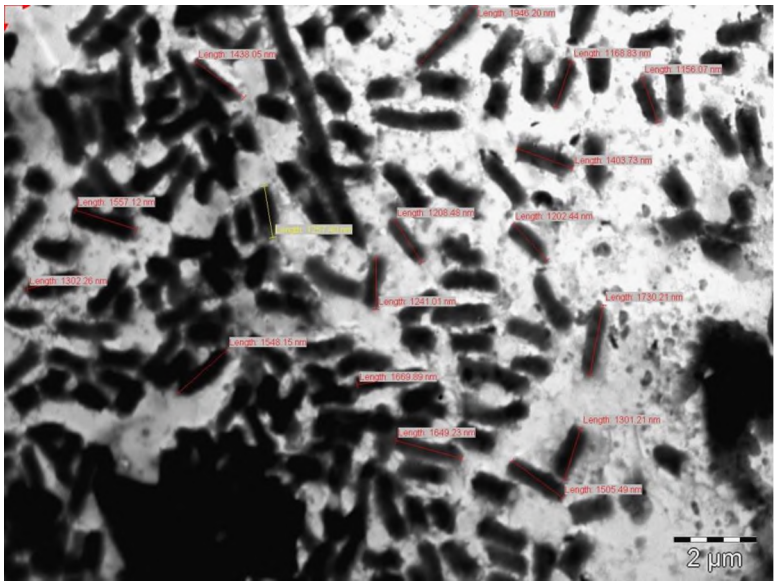
Appendix III. TEM micrographs of bacterial strains on coal



a. TEM image showing *Citrobacter* strain ECCN 19b flagella

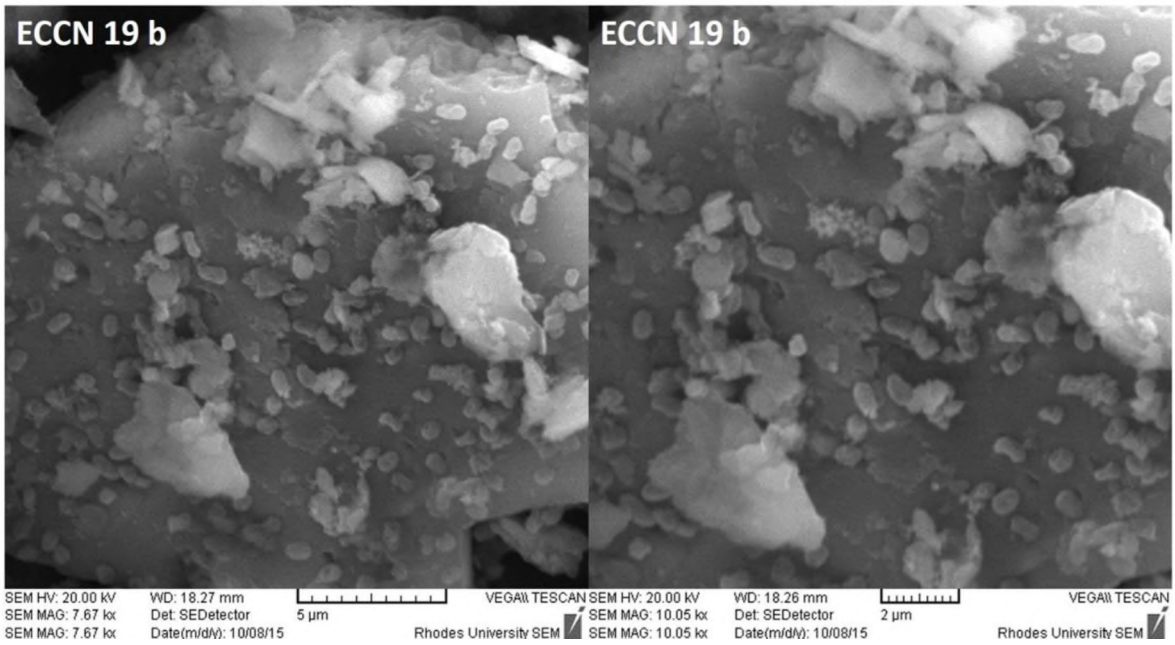


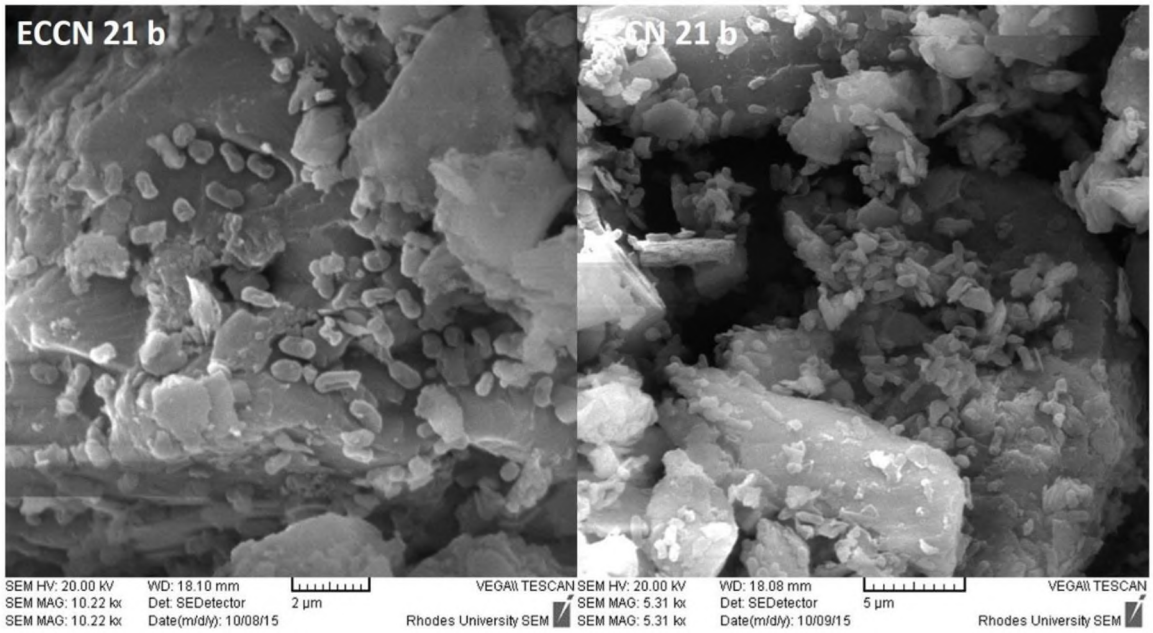
b. TEM image showing *Serratia* strain ECCN 24b flagella

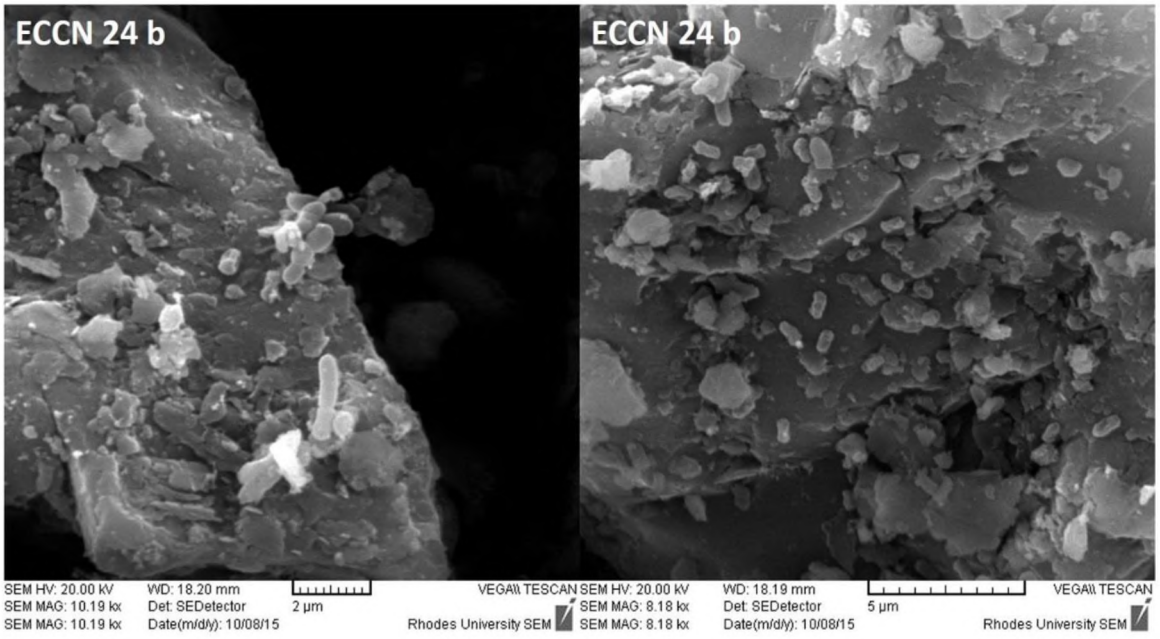


c. TEM of *Citrobacter* strain ECCN 19b cells

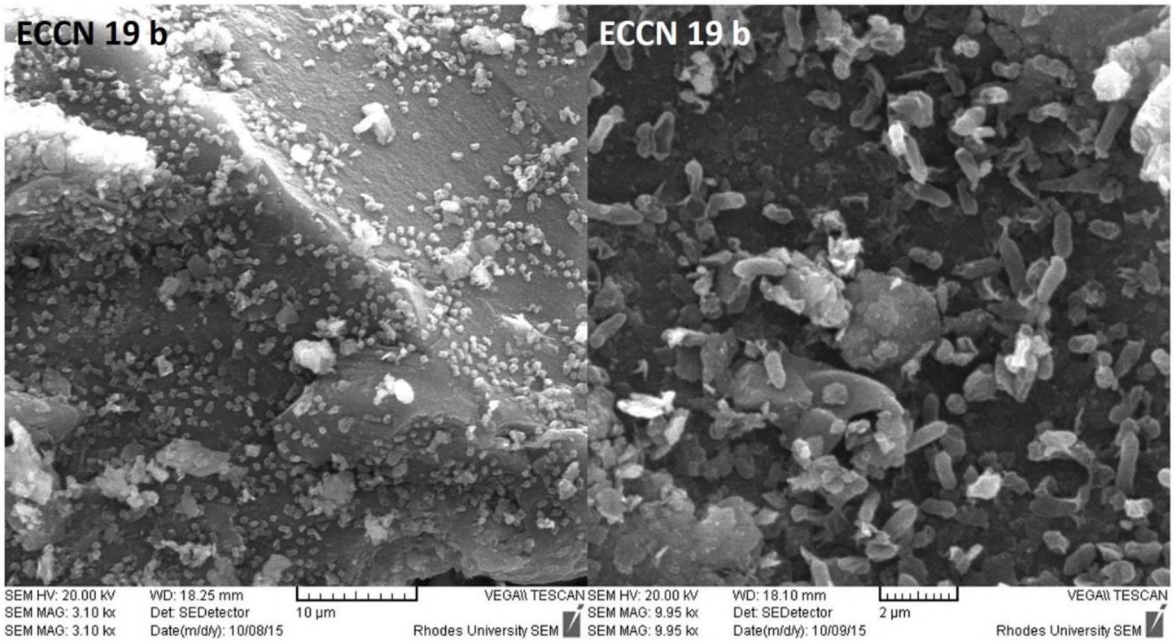
Appendix IV. SEM micrographs of bacterial strains on coal

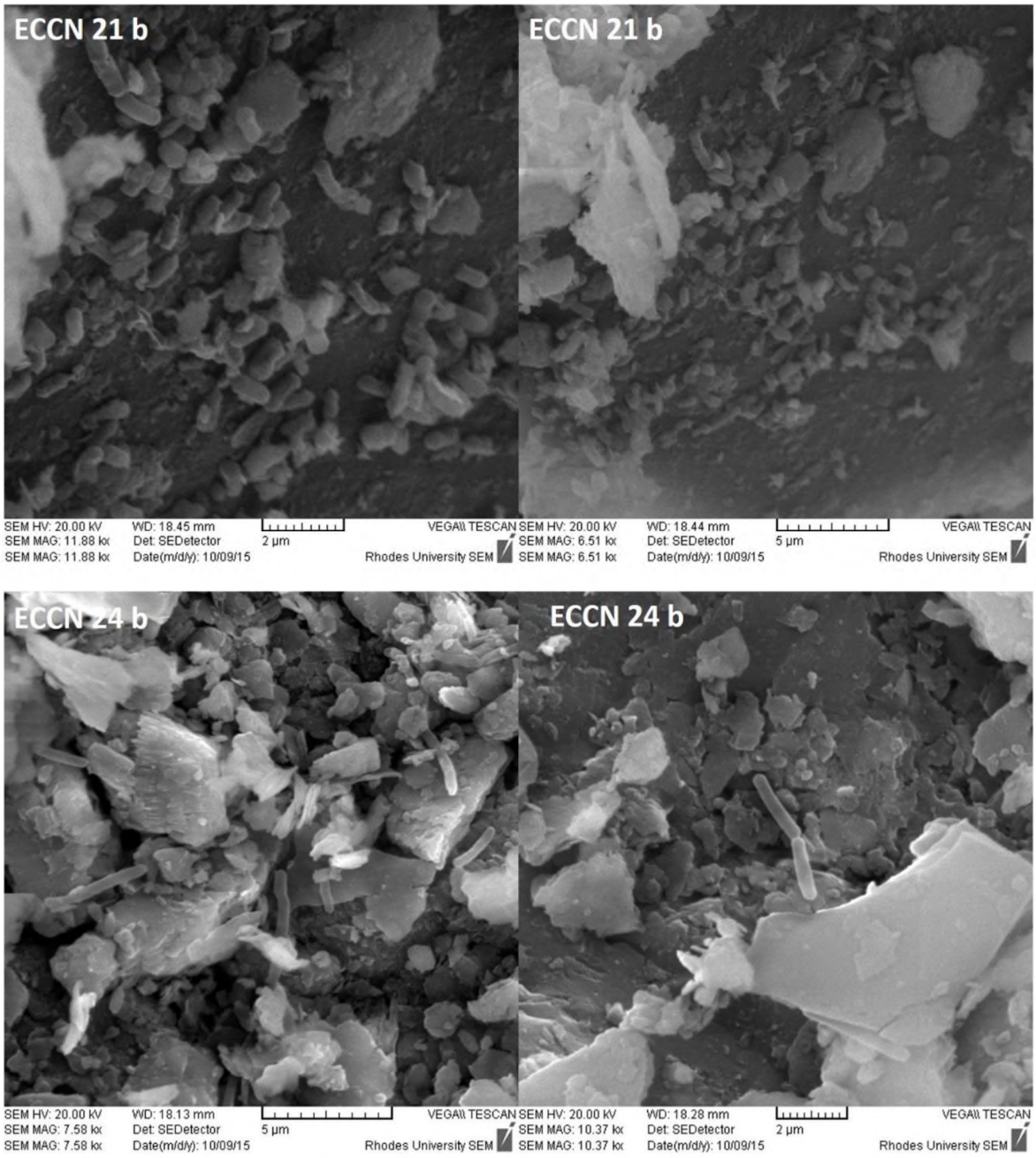




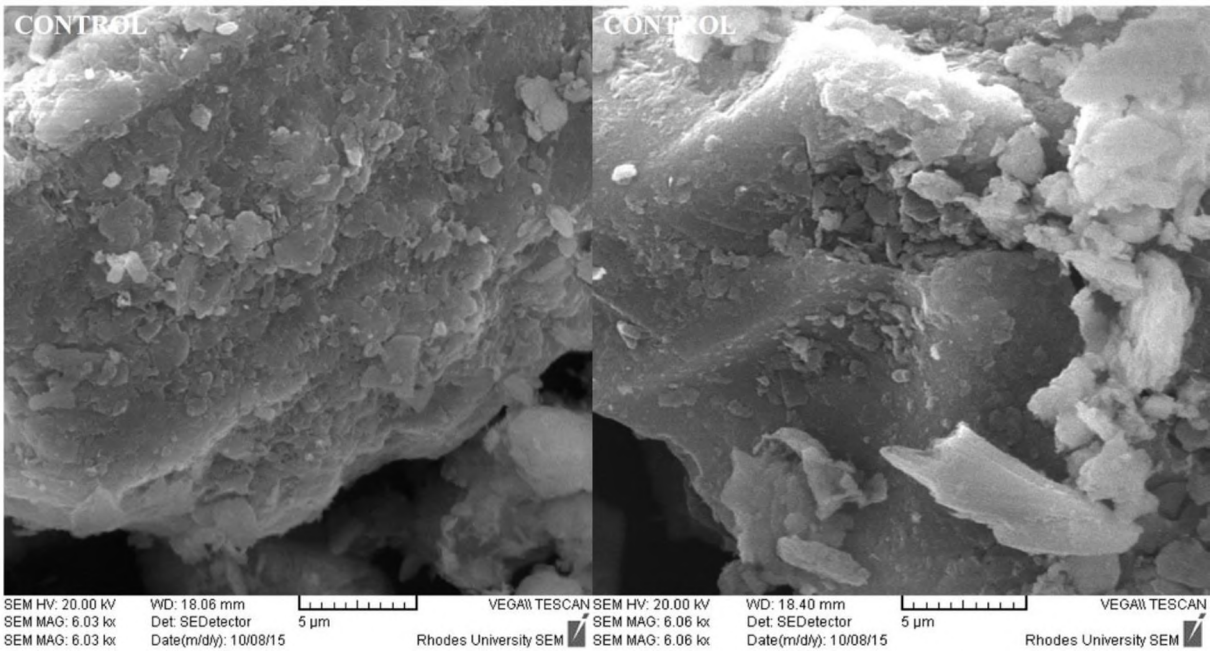


a. Representative images of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b cells attaching on discard coal

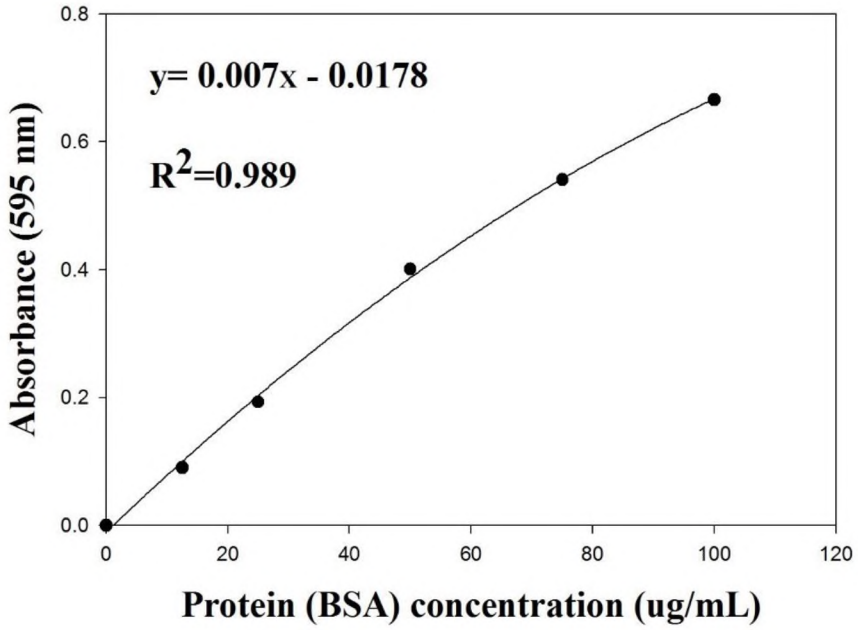




b. Representative images of *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 21b and *Serratia* strain ECCN 24b cells attaching on geologically weathered coal

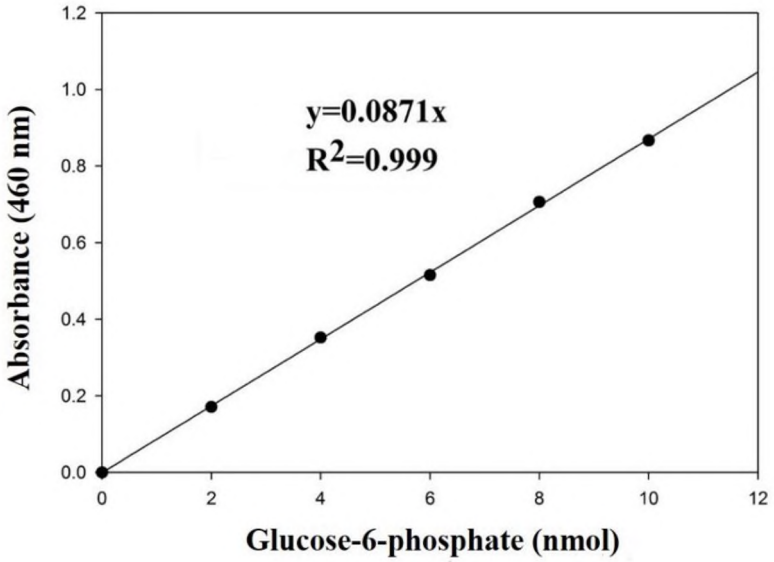


Appendix V: Protein standard curve using Bovin Serum Albumin (BSA)



Bovine serum albumin protein assay standard curve produced at the points of 20, 40, 60, 80 and 100 µg/mL. The data are fit with a linear regression by the line y= 0.007 x- 0.0178 with an R^2 value of 0.989.

Appendix VI: Glucose-6-phosphate standard calibration curve.



Glucose-6-phosphate standard calibration curve. One mole of G6P corresponds to one mole of β-$NADP^+$ reduced to NADPH, which subsequently generates one mole of reduced substrate. The data are fit with a linear regression by the line y=0.0871x with an R^2 value of 0.999