Towards a Bacterial Biofertiliser for the Rehabilitation of Disturbed and Degraded Land

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Abstract

Agriculture, mining, industry, and human activity disturb, degrade, and pollute pristine environments and particularly the soil environment. Excessive land exploitation slows or disrupts the soil potential, rendering it incapable of playing its role leading to land degradation. In the mining sector, specifically coal mining, rehabilitation of disturbed and degraded land involves strategies that include importing topsoil and using fossil fuel-derived fertilisers. Both practices are unsustainable. To address the unsustainability, a myco-phytoremediation technology known as Fungcoal was developed to facilitate successful revegetation of mining-disturbed and degraded land following the bioconversion of waste coal into a soil-like humic-rich substrate. To offset the dependence on chemical-based fertilisers, efforts were/are focussed on finding mutualistic and cost-effective microbial resources with plant growth-promoting (PGP) activity as a bacterial biofertiliser. This study made use of 22 isolated bacteria and the three Fungcoal coal-degrading fungi viz., *Aspergillus* sp. ECCN 84, *Aspergillus* sp. ECCN 225 and *Penicillium* sp. ECCN 243 as the microbial resource. Initially, characterisation of the substrate waste coal and molecular identification of the selected bacterial isolates were carried out. Physicochemical analysis of the low-rank coal (LRC) substrate revealed a pH of 3.60 with background S content equivalent to 7.13 g L^{-1}, N at 20 mg L^{-1}, P at 7.8 mg L^{-1} and K at 3.3 mg L^{-1}. Energy-dispersive X-ray spectroscopy (EDX) analysis revealed a C and O content of 23.09 and 69.03 wt%, respectively. Metagenomic analysis of the microbial population associated with the LRC substrate showed that among the 96.32% of bacteria, 59.46 to 62.18% belonged to Bacillota (also called Firmicutes), a phylum of largely Gram-positive bacteria, and 33.01 to 35.74% to Pseudomonadota (synonymous with Proteobacteria), a phylum of mostly Gram-negative bacteria. Following purification of the selected bacterial isolates and molecular characterisation by PCR, phylogenetic relatedness to known plant growth-promoting bacteria (PGPB) contained in the GenBank database showed that these bacterial isolates clustered with high bootstrap values to the reference PGPB strains. Only *Pseudomonas* sp. ECCN 10b (MW672582) was outside of the tree and shared significant similarity (100%) with *Pseudomonas fluorescens* (CP015638). A biochemical study revealed that the two *Proteus* sp. strains, *Exiguobacterium* sp., *Enterobacter* sp., and *Ancylobacter*, tolerated high salt and a wide range of temperatures. Bacterial isolates showed a high pH tolerance between 3 and 11, with the best growth at pH around 7. Nine of the identified strains, four *Bacillus* sp., *Exiguobacterium* sp., *Enterobacter* sp., *Pseudomonas* sp., *Arthrobacter* sp., and *Aeromonas* sp., were able to grow and increase in a medium containing either glucose, mannitol, sodium

L-glutamate, sucrose, or fructose. Growth was highest in media containing either sodium L-glutamate, sucrose, or fructose. All the coal degrading strains and 83% of those isolated from municipal wastewater used more complex carbon sources such as high and LRC. The potential for PGP activity was quantified spectrophotometrically by measuring the production of auxins, as indole-3-acetic acid (IAA) equivalents; gibberellins, as gibberellic acid (GA_3) equivalents, along with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and siderophore activity. Additionally, nutrient mobilisation was evaluated by monitoring an ability to mineralise NH_4^+, PO_4^{3-}, and K^+. Competent PGP strains for the coal degrading isolates included *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b, and *Serratia* strain ECCN 24b. In response to L-trp supplementation, the concentration of indolic compounds (measured as indole-3-acetic acid) increased. Production of ammonium and solubilisation of insoluble P by these strains was also apparent. Only *Serratia* strain ECCN 24b could solubilise insoluble K. Production of indoles increased following exposure to increasing aliquots of LRC, suggesting no negative effect of this material on indole production and that these bacteria may possess PGP potential. Of the twelve bacterial strains isolated from wastewater MaB-flocs, three produced indoles, nine mineralised NH_4^+, seven solubilised P, and one K. Potential of isolated strains for PGP activity according to a one-way ANOVA on ranks was: ECCN 7b > ECCN 4b > ECCN 6b > ECCN 3b = ECCN 10b > ECCN 1b = ECCN 5b > ECCN 8b > ECCN 2b > ECCN 12b > ECCN 9b = ECCN 11b. Further study revealed that cell-free filtrate from indole-producing cultures of *Aeromonas* strain ECCN 4b, *Enterobacter* strain ECCN 7b, and *Arthrobacter* strain ECCN 6b promoted mung bean adventitious root formation. Based on a biochemical study and the outcome of the ranking of bacterial strains according to PGP-like activities, three bacteria, *Enterobacter* sp., strain ECCN 7b, *Proteus* sp., strain ECCN 20b and *Serratia* sp., strain ECCN 24b that showed great mutualistic relationship with the most effective Fungcoal biocatalyst, *A. fischeri* ECCN 84, were used to prepare a bacterial bio-fertiliser. This consortium grew well in NB supplemented with L-tryptophan and produced indole compounds that could activate the adventitious rooting of mung bean (*Vigna radiata* L.) hypocotyls. Finally, the consortium showed no antibiotic resistance activity; however, they produced better biofertiliser with good responses to root/plant biomass production of the same *Fabaceae*, mung bean (*Vigna radiata* L.). The further development of this consortium into a cost-effective, environmentally friendly biofertiliser may help reduce dependence on chemical-based fertilisers and improve the sustainability of Fungcoal and other land rehabilitation strategies. Further studies are therefore underway to investigate in greater detail the PGP activity of these isolates individually and in consortium under field conditions to support the Fungcoal myco-phytoremediation strategy.

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List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ALM	Aleksandrow liquid medium
AMF	Arbuscular mycorrhizal fungi
AR	Adventitious rooting
ANOVA	Analysis of variance
BC	Bituminous coal
BE	Backscattered electron
BLAST	Basic local alignment search tool
CAS	Chrome azurol sulfonate
CFU	Colony forming unit
CH	Casein hydrolysate
CK	Cytokinin
Cont-	Control negative
Cont+	Control positive
CO_2	Carbon dioxide
CS	Coal slurry
DCS	Diesel-contaminated soils
DM_{t10}	Final dry mass after 10 d
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EBRU	Environmental biotechnology, Rhodes University
EC	Electrical conductivity
ECCN b	EBRU culture collection number of bacteria
ECCN BL	EBRU culture collection number of low-ranking coal bacteria
EDS	Energy-dispersive X-Ray spectroscopy
EM	Excelsior mine
EMU	Electron microscopy unit
EPS	Extra polymeric substances
FAO	Food and Agriculture Organization of the United Nations
FM_{t0}	Initial fresh mass at 0 d
FM_{t10}	Final fresh mass after 10 d
GAs	Gibberellic acid

GHC	Good hope colliery
GR	Germination rate
H^+	Proton
HA	Humic acids
HDTMA	Hexadecyltrimethylammonium bromide
HFM_{t0}	Hypocotyl's initial fresh mass at 0 d
HFM_{t10}	Hypocotyl's final fresh mass after 10 d
HH_{t0}	Hypocotyl initial height at 0 d
HH_{t10}	Hypocotyl final height after 10 d
HRAOP	High-rate algal oxidation pond
HRC	High-rank coal
IAA	Indole-3-acetic acid
IAPS	Integrated algal pond system
IPD	In-pond digester
ITS	Internal transcribed spacer
LBB	Luria-Bertani broth
LRC	Low-rank coal
MaB-flocs	Microalgae-bacterial flocs
MCC	Microbial culture collections
MEGA	Molecular evolutionary genetics analysis
MM9	Minimal Medium 9
Mn(II)	Divalent manganese
NA	Nutrient agar
NB	Nutrient broth
NCBI	National centre for biotechnology information
N, P, K, S, Fe	Nitrogen, phosphorus, potassium, sulphur, and iron
NO_2^-	Nitrogen dioxide
NO_3^-/NH_4^+	Nitrate/ammonium
N_2O/NH_3	Nitrous oxide/ammonia
OD_{600}	Optical density measured at 600 nm
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Potatoes dextrose agar

PDB	Potato dextrose broth
pH	Potential of hydrogen
PGPB	Plant growth-promoting bacteria
PGPM	Plant growth-promoting microorganisms
PGP	Plant growth promotion
PGR	Plant growth regulators
PVK	Pikovskayas
RDP	Ribosomal database project
$\mathbf{RFM_{t0}}$	Root initial fresh mass at 0 d
$\mathbf{RFM_{t10}}$	Root final fresh mass after 10 d
$\mathbf{RH_{t0}}$	Root initial height at 0 d
$\mathbf{RH_{t10}}$	Root final height
rRNA	Ribosomal ribonucleic acid
$\mathbf{R\text{-}SO_3^-/R\text{-}SO_4^-}$	Sulfonates/Sulphate esters
SAM	Solid Aleksandrow medium
SE	Secondary electron
±SE	± standard error
SEM	Scanning electron microscope
UN	United Nations
USA/UK	United States of America/United Kingdom
OTU	Operational taxonomic units
WC	Waste coal
WWT	Wastewater treatment
YECA	Yeast coal agar
YEMA	Yeast mannitol

Chapter One: General Introduction

Globally, food supply has become a challenge due to limited arable land and the ever-increasing world population; estimated to exceed 9.7 billion by 2050 (Elferink and Schierhorn 2016; United Nations 2022). Alternatives to increasing crop yields lie in expanding or restoring agricultural land, improving the productivity of existing land through fertilisers and irrigation, or using other innovative farming methods, such as biofertilisation. Chemical fertilisers have partially offset this global scourge by improving crop yields (Reynolds et al. 2015; Yousaf et al. 2017). However, these fertilisers and harvested foods lead to significant environmental and health stresses (Reynolds et al. 2015; Yousaf et al. 2017) related to pollution and food toxicity. There is a need to explore more sustainable solutions to increase and improve the quantity and quality of food crops to meet the growing global food demand (Maheshwari 2011; Sala et al. 2017).

Studies have shown that the most dominant microbial communities in the biosphere are bacteria, fungi, algae, and protozoa. These organisms, when used as biofertilisers, can improve plant growth and development (Rashid et al. 2016; Jacoby et al. 2017; Mahanty et al. 2017; Przemieniecki et al. 2019). Biofertilisers are products composed of living microorganisms that promote plant growth through the production, solubilisation, mineralisation, and mobilisation of nutrients. When applied to soil, plant root systems or seeds, they colonise the rhizosphere/roots (endophytes) and promote plant growth through the production of plant growth regulators (PGR) and essential nutrients (Singh 2013; Souza et al. 2015). These cost-effective fertilisers ensure food security and agricultural sustainability, as recommended by the 2016 United Nations General Assembly (Singh 2013; Przemieniecki et al. 2019; A/RES/68/231). In addition to increasing crop yields and quality, biofertilisers have the potential to remediate degraded land caused by ecological imbalances such as mining, deforestation, and intensive agriculture (Stehman and Overton, 2014). They then play a vital role in the recycling of nutrients (N, P, K), a process necessary for life on this planet (Maheshwari 2011).

Industry needs to respond to the food shortage caused by a growing world population by applying ecological farming methods. Therefore, better exploitation of the potential of PGP organisms to increase crop yields and improve bioremediation and land restoration strategies is essential (Maheshwari 2011; Souza et al. 2015). Plant growth promoting bacteria suppress pathogens and have no adverse environmental effects (Przemieniecki et al. 2019). As a source of PGR including indoles and gibberellins, ACCs, strigolactones, along with siderophores and extra-polymeric substances (EPS) containing carbohydrates (Pandey 206; Vejan et al. 2016; Jimoh 2017; Backer et al. 2018), PGP organisms may provide a lasting solution to long-unresolved global food stress. Used as biofertilisers these organisms mineralise inorganic/organic P and K, oxidise Mn and S and mobilise the nutrients for plant uptake (Cerrato et al. 2010; Grayston and Germida 2011; Goldy 2013; Ghosh et al. 2014; Pandey 2015; Rashid et al. 2016; Johns 2017; Titilawo et al. 2020). *Pseudomonas* sp., *Enterobacter* sp., *Bacillus* sp. and *Rhizobium* sp. are among the strongest phosphate solubilisers. The primary solubilisation mechanism of mineral phosphate is the production of organic acids; however, acid phosphatases play a major role in organic phosphorus mineralisation (Rodríguez and Fraga 1999; Titilawo et al. 2020).

Some strains of bacteria can protect host plants from biotic and abiotic stresses through various mechanisms, including the production of antibiotics and antifungal compounds (Ahemad and Kibret 2014; Souza et al. 2015). To improve sustainable agriculture, these bacteria could then be used to develop sustainable biofertilisers (Mahanty et al. 2017; Souza et al. 2015; Reece et al. 2011; Przemieniecki et al. 2019).

Sustainable agriculture emphasises the long-term increase in agricultural yield by developing technologies or practices that conserve natural resources now and for future generations (Pretty 2008). Among the biological approaches necessary for sustainable agriculture, the use of environmental samples of bacteria and fungi seems to be gaining the upper hand. The selected samples involved in the present research include algal-based bacterial strains isolated from high-rate algal oxidation ponds (HRAOP) of the integrated algal pond system (IAPS) (Masudi 2020; Masudi et al. 2023) (Figure 1.1) as well as coal-degrading fungi and bacteria from coal slurry (CS) and diesel-contaminated soils (DCS) (Edeki 2015; Olawale et al. 2020).

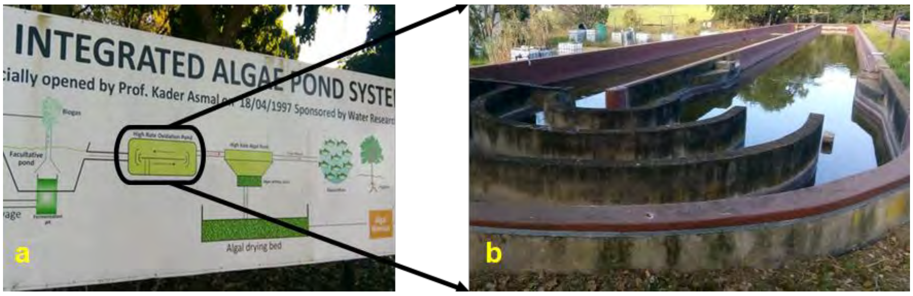


Figure 1. 1 Integrated algal pond system (IAPS) located at EBRU experimental station, Makhanda District, Eastern Cape Province, South Africa. Flow and outcome design (a), high-rate algal oxidation pond (HRAOP) containing microalgae-bacterial flocs (MaB-flocs) as sources of environmental samples (b).

The EBRU's IAPS consists of an in-pond digester (IPD) called the primary treatment unit, and two HRAOPs known as the secondary treatment unit. High-rate algal oxidation ponds exploit the mutualistic interactions between bacteria and photosynthetic microalgae to treat biological/domestic effluents to generate quality water suitable for reuse (Craggs et al. 2014; Mambo et al. 2014a; Jimoh et al. 2017). From the mutualistic interactions, algae-bacteria, a biomass containing microalgae and bacteria as flocs and called microalgae-bacterial flocs (MaB-flocs) is generated. The MaB-flocs have also been used to produce feed materials, nutrient fertilisers, soil improvers, amino acids, vitamins, polysaccharides, and EPS (Aaronson et al. 1980; Borowitzka 1988; Rose 2002; Craggs et al. 2014; Cowan et al. 2016; Butler et al. 2017; Jimoh et al. 2017). Previous studies conducted in the EBRU laboratories and elsewhere revealed that bacterial biomass generated from treated wastewaters was able to produce auxin, indole-3-acetic acid, and gibberellic acid as PGR, and to mineralise ammonium, phosphate, and potassium as plant essential nutrients (Masudi 2020; Titilawo et al. 2020).

Bacteria promoting plant growth are capable of antagonistic activity against pathogenic bacteria and fungi (*Fusarium culmorum* and *Fusarium graminearum* (Xu et al. 2014; Ramanan et al. 2016; Kadmiri et al.2018; Przemieniecki et al. 2019). Thus, the PGPB can be used to support novel rehabilitation strategies developed to offset the adverse impact of mining activities on local communities which has been working and materialised in the bioremediation, restoration, and rehabilitation of coal-mined lands since 2006 (Cowan et al. 2016; Sekhohola et al. 2022). In South Africa, the process of coal biodegradation has been intensively studied to develop practical methods that can be used for successful revegetation and rehabilitation, but many challenges remain (van Breugel et al. 2019; Olawale et al. 2020).

Studies have reported that certain strains of fungi and bacteria use the complex organic compounds of geological coal as the sole carbon source. Scientists could then consider exploring these potential organisms in terms of fertilisation and restoration strategy (Sekhohola 2014; Olawale et al. 2020) without using common and unsuccessful rehabilitation practices. Thus, coal-degrading bacteria can additionally produce ligninolytic enzymes (laccases) mediating the depolymerisation of lignin into humic substances (Nelson et al. 2014; Olawale et al. 2020; Sekhohola-Dlamini et al. 2022) known as major organic constituents of soil and often used as a soil amendment to fertiliser agriculture.

The present research has focused more on the development of a bacterial biofertiliser to support the phytoremediation strategy called Fungcoal. It aimed to use a collection of 22 microbial strains including ten bacterial isolates from diesel-contaminated sites (DCS) and coal slurry (CS) as well as twelve novel algae-based wastewater treatment bacteria promoting plant growth that have been gathered by EBRU. The PGP capability of these isolates was evaluated based on the production of plant essential nutrients (N, P, and K) PGR (IAA, GAs, ACC and Siderophores) and EPS.

1.1 Rehabilitation of Disturbed and Degraded Land

Rehabilitation of degraded land involves human intervention designed to provide geo-ecological improvement. It aims to mitigate or reverse land degradation caused by natural disasters or excessive land use practices that do not meet environmental protection standards, such as unsustainable agricultural and coal mining activities (Haigh 2000). The earth has always been victim of human activities that overexploit its natural resources causing harmful effects on ecological functions, services, and public health. Overexploitation includes unsafe agricultural practices, rapid industrialisation, mining operations, pastoralism, recreation, logging, and urbanisation (Azubuike et al. 2016; Mentis 2020). Soil remediation is necessary to restore its fertility and physicochemical and biochemical properties (Rashid et al. 2016; Sekhohola-Dlamini et al. 2020).

1.1.1 Causes of Land Degradations

The degradation or loss of the soil's productive capacity results from several parameters, such as natural disasters and overexploitation of energy reservoirs by men. This global challenge directly or indirectly affects current and future ecological products and services, causing many scourges, such as food insecurity, rising food prices, climate change, and biodiversity loss (Azubuike et al. 2016; Global Environment Facility 2022). According to the UN World Day held in New York in 2019, the world loses around 24 billion tons of fertile land annually. This degradation is the result of desertification, land degradation, and drought. Each year, land degradation affects negatively 3.2 billion people, especially women, and children whereas 10% of biodiversity and ecosystem services are lost (Scholes et al. 2018).

Land degradation releases enormous amounts of CO_2, NH_4, and N_2O into the atmosphere, increasing greenhouse gases that are the leading cause of global warming and climate change (Myhre et al. 2013; Global Environment Facility 2022). Climate change is exacerbating variations in crop yields and incomes, threatening the resilience of agroecosystems and the stability of food crop production (Global Environment Facility 2022). Land degradation can be a natural or human-induced process and has been described as unsustainable actions (Gabriels and Corneli 2023).

1.1.1.1 Effects of Natural Disasters

No place on Earth is immune to natural disasters; however, some disasters strike specific geographic regions more frequently than others (Metych 2023). In South Africa, the province of Kwazulu-Natal has repeatedly suffered from floods and landslides. Depending on their occurrence natural disasters can be caused by effects familiarly linked to soil, air, and water disturbances (Bisht et al. 2020) which can lead to significant loss of biotopes or biodiversity, even human lives. Thus, the destruction of land and the natural environment, private property, and public infrastructure (Srivastava 2010; Metych 2023) are among the consequences linked to natural disasters.

Due to the unsustainable use of natural resources, the intensity of disasters continues to increase. Disaster mitigation plans are followed to protect the environment and reduce the adverse effects of natural disasters (Bisht et al. 2020), although with meagre results.

1.1.1.2 Effects of Human-Induced Activities

Overuse of natural resources harms soil biological processes; therefore, various types of land degradation arise (Emadodin and Bork 2012). Human-induced land degradation activities include unsafe agricultural practices, deforestation, pastoralism, mining, overgrazing, poor land management, overexploitation of vegetation cover, rapid urbanisation, and activities fast industrial processes (Azubuike et al. 2016; Mentis 2020; Gabriels and Corneli 2023). Water erosion occurring mainly in Asia, ranks first in land degradation, with 56% of the global land area affected, followed by wind erosion at 28% (Asia and Africa) (Abdel-Rahman 2023; Gabriels and Corneli 2023). Salinisation and loss of plant nutrients (mainly in Asia) are the consequences of soil degradation by chemical fertilizers. At the same time, compaction is the main consequence of physical soil degradation (Gabriels and Corneli 2023).

- **Unsafe Agricultural Practices**

If no solution is found, the global cost of land degradation from unsafe agricultural practices will reach $23 trillion by 2050 (FAO 2022). However, excessive use of chemical-based fertilisers, pesticides and insecticides is among the unsafe agricultural practices known to threaten soil biology related to biogeochemical nutrient cycling (Patidar and Patidar 2015; Jallow et al. 2017; Bisht et al. Chauhan 2021; Naylor et al. 2022) as well as restoration processes. Once degraded, the land becomes deficient in nutrients and beneficial microorganisms, causing soil salinisation and saturation (Suresh and Himanshu 2015). Additionally, chemical fertilisers and pesticides may contain heavy metals comprising Cr, Mn, Co, Ni, Cu, Zn, Cd, etc. which can contaminate soil and eventually end up in fruits and vegetables, causing additional risks to human and animal health (Srivastava et al. 2017; Rahman and Zhang 2018).

The economic fortunes of Southern African countries depend on the state of their natural resources including soil, water, and biomass. Overexploitation of these resources without considering their interdependence has depleted the land, especially the fertile topsoil (Pelser and Kherehloa 2000). However, farmer knowledge is another issue that needs to be addressed. In Madagascar, Kuwait region, the indiscriminate use of pesticides by unskilled agricultural workers has posed significant risks to the environment and human health, including the destruction of non-target species, soil contamination, as well as waterways and the air (Andréa et al. 2000; Bedos et al. 2002; Arias-Estévez et al. 2008). About 42% of farmers were unaware of the prohibited or restricted use of pesticides or insecticides such as dichloro-diphenyl-trichloroethane (DDT), permethrin, methiocarb and methamidophos (Jallow et al. 2017).

- **Unsafe Coal-Mining Operations**

Coal mining activities are applied in many countries, including Australia, the United States, the United Kingdom, Mongolia, India, China, and South Africa (Measham et al. 2013). These mining operations are essential to global economic growth and job creation. However, coal mining operations are responsible for adverse ecological effects that deteriorate soil structures and their biochemical properties (Laisani and Jegede 2019). In South Africa, the gold and diamond mines are in the first rank; however, the country has iron ore, platinum, manganese, chromium, copper, uranium, silver, beryllium, titanium, and platinum as reserve mines (Mokrani 2022; Qin and 2023).

South African coalfields exist in many provinces, especially in Mpumalanga (Olufemi et al. 2018; Olawale et al. 2020). About 51% of South African coal mines are underground, and 49% are surface (Munnik 2010). Of the country's coal mines, 64 are active, while 400 are abandoned. The five dominant and producing up to 80% of the country's coal include Anglo American Thermal Coal, BHP Billiton's Energy Coal, Exxaro Resources, Xstrata Coal, and Sasol Mining. However, Eskom power plants are significant local users of coal (Mathu and Chinomona 2013; Shiao and Maddocks 2014; Wang 2023).

Significant open pit deposits are found in Mpumalanga and the Free State North. Of the total production of South African coal, 25% is exported to East Asia and Europe, and 75% is used for local consumption to generate electricity and supply the chemical and steel industries, to process liquid fuels and petrochemicals (Olufemi et al. 2018; Shongwe 2018; Qin et al. 2023; Mokrani 2022; Wang 2023). Therefore, coal production is beneficial and developmentally relevant in creating employment and improving economic growth as expressive effects of exports, domestic consumption, and gross domestic product. (Mathu and Chinomona 2013; Shongwe 2018; Wang 2023).

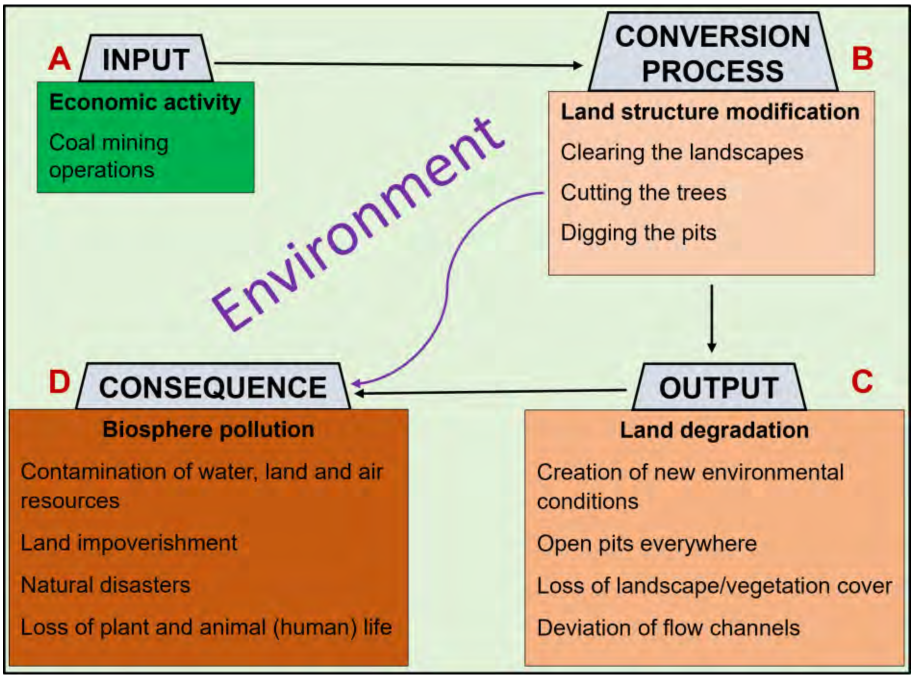


Figure 1. 2 Adverse environmental effects of unsafe coal-mining operations (A). It starts with modifying soil and landscape structure (B), followed by the gradual disruption of ecosystems that causes land degradation (C). The maximum stage of degradation disrupts biogeochemical cycles causing land impoverishment, natural disasters, and the disappearance of vulnerable species (D) (Adapted from Mbaya et al. 2013).

Studies have shown that coal mining operations destroy ecological ecosystems, including animal and plant health, agriculture, landscapes, and forests (Mbaya 2013; Olufemi et al. 2018; Wang 2023). They are the primary cause of the removal of topsoil, trees, and vegetation, processes that deplete the land of its nutrients and lead to the loss of its fertility (Laisani and Jegede 2019). Also, coal mining has exposed the land to wind and water erosion, water pollution, and other environmental hazards that threaten biotic and abiotic resources in several ways (Mbaya 2013). In addition, coal mining pollutes the air with emissions of dust and toxic gases, such as SO_2, CH_4, NO_X, and H_2S, generated during blasting, wind erosion, delivery, or transport and machining (Shongwe 2018; Xia et al. 2022; Wang 2023).

Some coal miners develop cardiovascular and pulmonary diseases such as dust inhalation pneumoconiosis (Perret et al. 2017; Laisani and Jegede 2019). To avoid stomach ulcers, Kendal and Ogies, Mpumalanga residents no longer eat citrus fruits (oranges) from orange trees planted close to coal mines because of possible contamination with toxic chemicals (Laisani and Jegede 2019). Acid mine drainage (AMD) affects surface, groundwater, and rivers (Olufemi et al. 2018). As highly acidic water from coal mines, AMD can seep into the ground or run off into waterways. Some coals contain sulphide minerals such as pyrites. When exposed to water and air, oxidation takes place and produces large amounts of H_2SO_4 (equation 1).

$$4\,FeS_2 + 15\,O_2 + 14\,H_2O \longrightarrow 4\,Fe(OH)_3 + 8\,H_2SO_4 \qquad (1)$$

Due to runoff and rainwater infiltration, sulfuric acid and toxic heavy metals will contaminate groundwater and surface waterways (Gaikwad and Gupta 2007; Hamidovic et al. 2016; Olufemi et al. 2018; www.sahrc.org.za).

1.1.2 Rehabilitation Strategies

The mining sector is a source of minerals needed for industrialisation, economic expansion, and urban sprawl. It also presents a socio-environmental and public health concern due to the increase in environmental pollutants (Azubuike et al. 2016; Bandyopadhyay and Maiti 2022). Some of the pollutants include nuclear and coal waste, pesticides, greenhouse gases and hydrocarbons (Azubuike et al. 2016; Munawer 2018; Sekhohola-Dlamini et al. 2022). There is little knowledge that can lead researchers to find a better remediation strategy coupled with potential carbon sequestration capacity in degraded mining areas (Bandyopadhyay and Maiti 2022). Since the emergence of polluted land remediation techniques, none have been entirely practical due to the varying nature and type of pollutants.

However, some indigenous organisms from degraded environments (coal basins) have been able to degrade the coal. This potential is needed to solve most land degradation challenges (Verma and Jaiswal 2016; Olawale et al. 2020). These native organisms have played an essential metabolic role in coal's biogeochemical dynamics and ecological ecosystems' functioning (Olawale et al. 2020; Akimbekov et al. 2022). Microbial bioremediation should be prominent in rehabilitating degraded sites (Gerhardt et al. 2009; Sekhohola et al. 2013; Karaca et al. 2018; Karn et al. 2022).

1.1.2.1 Microbial Bioremediation Processes

Microorganisms are essential for natural sanitation, nutrient cycling, and stabilisation of aggregates. They impact the physicochemical properties, chemistry, and structure of the soil.

This aspect has always been neglected in rehabilitating polluted sites that can improve mining spoils (da Silva et al. 2023). The microbial biocatalysts primarily involved in coalfield biological remediation include bacteria and fungi (Sekhohola and Cowan 2017; Olawale et al. 2020). Most of these organisms have the potential to solubilise, convert and degrade low-rank coal, coal waste, coal fines, and other coal residues into value-added products with new potential for reuse (Yang et al. 2023). These potentials are due to the ability of bacteria and fungi to produce ligninolytic enzymes, which are biological mediators that degrade lignin polymers known as the main component of low-ranking coal (Mönkemann et al. 1997; Pawlik et al. 2016). Additionally, ligninolytic enzymes, namely laccase, lignin peroxidase, manganese peroxidase, and polyvalent peroxidase, play crucial roles in the removal and detoxification of environmental lignocellulosic wastes (Kumar and Chandra 2020).

As low-rank coal (LRC) has high lignin content, its biological degradation produces an important number of humic acids (HA) making this coal type valuable for soil carbon amendment and organic fertiliser production (Giannouli et al. 2009; Titilawo et al. 2020). Microbial strains including *Achromobacter* sp., *Arthrobacter* sp., *Azotobacter* sp., *Azospirillum* sp., *Bacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Aspergillus* sp. (*Neosartorya fischeri*) and *Penicillium* sp., have been shown to degrade carbonaceous pollutants including low-rank and bituminous coals. They have also demonstrated the ability to solubilise or mineralise insoluble inorganic potassium and phosphate rocks. Biological processes release essential nutrients for plant growth and development, also becoming practically useful for the bioremediation strategy of specific polluted environments including coalfields (Gray and Smith 2005; Igbinigie et al. 2008; Olawale and al. 2020; Akimbekov et al. 2022).

1.1.3 Fungcoal Rehabilitation Process Strategy

Before the enactment of the Minerals Act 1991, mining companies operating in South Africa shirked their responsibility to rehabilitate mined land, leaving it unrehabilitated. This negative legacy concerned the long-term residual effects on the social, health, and environmental well-being of the communities located near these unrehabilitated and abandoned mining areas (Swart 2003; Šourková et al. 2005). Additionally, factors limiting soil fertility, as well as physicochemical properties, microbial composition, and interactions, need to be explored to properly select appropriate plant species capable of maintaining successful soil cover over time (Katzur and Haubold-Rosar 1996; Kumar and Gopal 2015; Lima et al. 2016; van Breugel et al. 2019). Consequently, South African mine closure legislation has recommended that mining company operators restore and rehabilitate mined lands to mitigate the negative environmental impacts and facilitate the transition to reuse (Limpitlaw and Briel 2014; Land Rehabilitation Society of Southern Africa, Coaltech, Minerals Council of South Africa 2018).

1.1.3.1 Ex Planta Phytoremediation

In South Africa, the "ex planta phytoremediation" process remains the most widely used (Salt et al. 1998; Wang et al. 2004). It consists of covering the coal dumps with a 30 to 100 cm layer of soil (topsoil), followed by an application of fertilizer before sowing annual and perennial grasses (Salt et al. 1998; Wang et al. 2004; Cowan et al. 2016). This process has many limitations, including the quality and quantity of topsoil (da Silva et al. 2023), and insufficient macronutrient (N, P, K) production and mobilization processes. The acidity, high temperatures and concentrations of toxic metals that characterize coal dumps do not allow the degradation of the underlying carbonaceous layer (Maiti 2007; Cowan et al. 2016). Additionally, accessing, excavating, and transporting many tons of topsoil is expensive and can lead to other negative environmental impacts (Cowan et al. 2016).

1.1.3.2 Fungcoal Process

Using microbial inoculants as feedstock is more beneficial to reactivate and reorganize soil biochemistry and structure. This innovative approach has the potential to positively alter the chemical and physical limitations caused by the degradation of mined lands and can improve the rehabilitation of polluted mine sites (da Silva et al. 2023). Industrial coal mining leaves behind tons of LRC coal waste with little value. Their handling and accumulation may present adverse environmental impacts (Sekhohola 2015) such as soil, air, and waterways pollution. The Fungcoal process was innovated in South Africa for sustainable rehabilitation goals (Cowan et al. 2016; Mentis 2020; Wang et al. 2022).

Sustainable rehabilitation consists of meeting the present needs of local, regional, or even international communities, without compromising the capacity of future generations (World Commission on Environment and Development 1987; Pavloudakis et al. 2020). Sustainable rehabilitation should be able to balance economic growth, environmental protection, and community well-being (www.becas-santander.com). The Fungcoal process involves exploring coal-fungi degradation capabilities to produce soil amendment compounds in the form of organic fertilisers, the cornerstone of soil fertility and plant life (Cowan et al. 2016; Sekhohola-Dlamini et al. 2022).

This new patented bioprocess is used in the *in-situ* treatment of coal waste. It is based on a mutualistic interaction between the fungus *Aspergillus* sp. or *Neosartorya fischeri* (ECCN 84) and the grass species *Cynodon dactylon*, commonly known as Bermuda grass; *Eragrostis tef*, and *Pennisetum clandestinum*. Thus, a suite of root exudates, AMF including *Glomus clarum*, *Paraglomus occultum*, *Gigaspora gigantea*, and *Glomus mossea* (Sekhohola 2015; Sekhohola-Dlamini et al. 2022). This mutualistic process facilitates the rapid conversion of coal waste into a soil-like material to stimulate vegetation establishment for eventual coal dump rehabilitation. Furthermore, the Fungcoal process improves the water-holding capacity of the substrate, reduces its ash content, and mitigates its acidification and salinity (Kundu and Ghose 1997; Cowan et al. 2016).

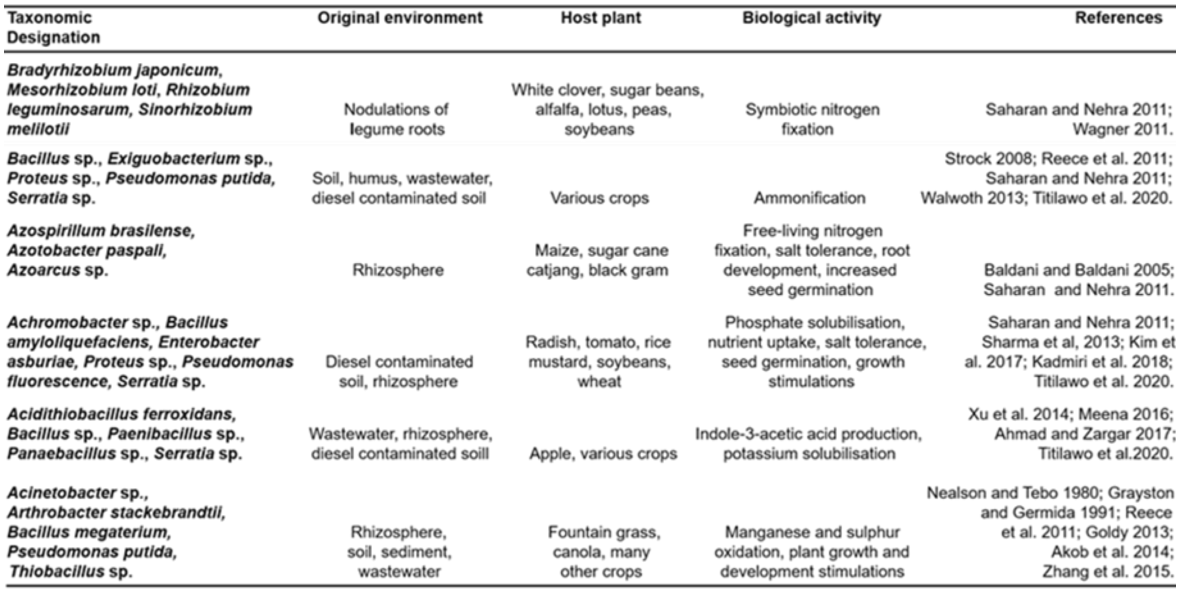
Starting in 2006, small-scale trials were set up on a Roofcoal waste landfill at the Kleinkopje mine, Landau Colliery, to assess the effectiveness of the Fungcoal bioprocess in the field. Once materialised, this sustainable waste and coal spoil rehabilitation would facilitate revegetation without needing topsoil. The protocol was established to ensure the biological degradation of the carbonaceous substrate and produce a humus-enriched soil-like material capable of supporting vegetation. However, from 2012 to 2014, commercial-scale one-hectare trials were initiated at Kromdraai Mine, Landau Colliery, to corroborate the small-scale results (Cowan et al. 2016; Sekhohola and Cowan 2017). The results showed that an increase in humic acid-like substances was observed in the substrate. For commercial scale trials, it was observed that all cultivated grass species were present, and diversity was maintained with approximately 100% canopy cover and over 40% basal cover, indicating that degradation has resulted in successful rehabilitation (Sekhohola 2015; Cowan et al. 2016).

While biodegradation of carbonaceous pollutants by bacterial/fungal strains is demonstrated, it is then possible to state that PGP rhizobacteria, microalgae (Lukešová 2001; Titilawo et al. 2020; Feng et al. 2021), and plants also contribute to the desired biotransformation processes, revegetation, and land restoration. The topsoil-based phytoremediation process, which is unlikely to be sustainable and cost-effective in the long term (Cowan et al. 2016), faces many limitations, as mentioned previously, and prevents it from being successful.

1.2. Plant Growth Promoting Microorganisms as Fertiliser in Rehabilitation Bioprocess

As chemical-based fertilisers negatively affect most ecosystems, researchers should develop sustainable and environmentally friendly fertilisation technologies to produce healthy and clean food globally (Majeed et al. 2018; Ji et al. 2019). Beneficial soil organisms promoting plant growth offer excellent opportunities due to their broad potential in soil bioremediation by mineralising and mobilising plant nutrients (Majeed et al. 2018). These organisms include endophytes, free-living bacteria, and fungi as tools for sustainable food production that do not compromise ecological ecosystems (Abhilash et al. 2016; Elferink and Schierhorn 2016).

Table 1. 1 List some plant growth-promoting microorganisms used to develop bacterial biofertilisers (modified from Vessey 2003; Vejan et al. 2016; Majeed et al. 2018).



Taxonomic Designation	**Original environment**	**Host plant**	**Biological activity**	**References**
Bradyrhizobium japonicum, Mesorhizobium loti, Rhizobium leguminosarum, Sinorhizobium meliloti	Nodulations of legume roots	White clover, sugar beans, alfalfa, lotus, peas, soybeans	Symbiotic nitrogen fixation	Saharan and Nehra 2011; Wagner 2011.
Bacillus **sp.,** ***Exiguobacterium*** **sp.,** ***Proteus*** **sp.,** ***Pseudomonas putida, Serratia*** **sp.**	Soil, humus, wastewater, diesel contaminated soil	Various crops	Ammonification	Strock 2008; Reece et al. 2011; Saharan and Nehra 2011; Walwoth 2013; Titilawo et al. 2020.
Azospirillum brasilense, Azotobacter paspali, Azoarcus **sp.**	Rhizosphere	Maize, sugar cane catjang, black gram	Free-living nitrogen fixation, salt tolerance, root development, increased seed germination	Baldani and Baldani 2005; Saharan and Nehra 2011.
Achromobacter **sp.,** ***Bacillus amyloliquefaciens, Enterobacter asburiae, Proteus*** **sp.,** ***Pseudomonas fluorescence, Serratia*** **sp.**	Diesel contaminated soil, rhizosphere	Radish, tomato, rice mustard, soybeans, wheat	Phosphate solubilisation, nutrient uptake, salt tolerance, seed germination, growth stimulations	Saharan and Nehra 2011; Sharma et al, 2013; Kim et al. 2017; Kadmiri et al. 2018; Titilawo et al. 2020.
Acidithiobacillus ferroxidans, Bacillus **sp.,** ***Paenibacillus*** **sp.,** ***Panaebacillus*** **sp.,** ***Serratia*** **sp.**	Wastewater, rhizosphere, diesel contaminated soill	Apple, various crops	Indole-3-acetic acid production, potassium solubilisation	Xu et al. 2014; Meena 2016; Ahmad and Zargar 2017; Titilawo et al.2020.
Acinetobacter **sp.,** ***Arthrobacter stackebrandtii, Bacillus megaterium, Pseudomonas putida, Thiobacillus*** **sp.**	Rhizosphere, soil, sediment, wastewater	Fountain grass, canola, many other crops	Manganese and sulphur oxidation, plant growth and development stimulations	Nealson and Tebo 1980; Grayston and Germida 1991; Reece et al. 2011; Goldy 2013; Akob et al. 2014; Zhang et al. 2015.

The best-known endophytes are *Allorhizobium* sp., *Azorhizobium* sp., *Bradyrhizobium* sp., *Mesorhizobium* sp., *Rhizobium* sp., *Paraconiothyrium* sp. and *Neotyphodium* sp. (Afzal et al. 2019; Ju et al. 1998; Vejan et al. 2016; Bärenstrauch et al. 2020). However, the free-living organisms are known as, *Agrobacterium* sp., *Arthrobacter* sp., *Azotobacter* sp., *Azospirillum* sp., *Bacillus* sp., *Burkholderia* sp., *Caulobacter* sp., *Chromobacterium* sp., *Erwiniasp*., *Flavobacterium* sp., *Micrococcus* sp, *Pseudomonas* sp., *Serratia* sp, *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. (Goswami et al. 2016; Vejan et al. 2016; Titilawo et al. 2020; Imran et al. 2021; Sekhohola et al. 2022). By colonising the rhizosphere and root system, these organisms can enhance plant growth and development while protecting host plants and native microbiota against pathogens and abiotic stresses (Beneduzi et al. 2012; Vejan et al. 2016; Kumar et al. 2022).

1.2.1 Mechanisms Involved in Plant Growth Promotion

Organisms exert beneficial effects on plants through direct and indirect mechanisms; however, their modes of action are often not yet fully understood. The molecular basis of plant-microbiota interaction is beginning to shed light through emerging “omics” approaches (Ahmad et al. 2014; Backer et al. 2018).

1.2.1.1 Direct Mechanisms

The direct PGP activities include producing and mineralising nutrients like N, P, K, S, Fe, and Mn from organic decaying matter or mineral rocks. These nutrients are necessary for plant growth and development without any chemical input to the soil (Cerrato et al. 2010; Etesami et al. 2017; Titilawo et al. 2020; Basu et al. 2021). The PGPMs such as *Bacillus* sp., *Serratia* sp., *Pseudomonas* sp., *Arthrobacter* sp. and *Enterobacter* sp., provide host plants with PGR such as indole-3-acetic acid (IAA), gibberellic acids (GA), ethylene, and cytokinin. These regulators are known to increase plant root area (Jangu and Sindhu 2011; Kapoor et al. 2016; Khan et al. 2020; Titilawo et al. 2020).

Beneficial organisms can supply carbohydrates in the form of organic polymers, the EPS (Vejan et al. 2016; Jimoh 2017; Backer et al. 2018). EPS are beneficial for the metabolism of both organisms. They provide a suitable soil environment by maintaining its moisture for microbial bioactivity and facilitating particle aggregation, allowing nutrient trapping by microbiota and host plant root systems (Costa et al. 2018).

1.2.1.2 Indirect Mechanisms

Each year, 20-40% of the overall productivity of cereals and legumes is reduced due to plant diseases (Oerke 2006; Sehrawat and Sindhu 2019; Nazarov et al. 2020; Pathak et al. al. 2022). Chemical pesticides have been deployed to contain this scourge but have caused adverse environmental consequences, such as soil and waterway pollution (Chandler et al. 2011). In the rhizosphere, by protecting the host plant against pathogenic bacteria, fungi, and viruses, biopesticides promote plant health and indirectly plant growth and development, increased yield, and overall sustainable agriculture (Chandler et al. 2011; Pathak et al. 2022). The most effective biological pesticides involved in plant growth promotion/stimulation include antagonistic compounds (siderophores, antibiotics, volatile organic compounds, hydrocyanic acid), hydrolytic enzymes (chitinases, glucanases and proteases) and induction of systemic resistance in plants against a broad spectrum. root and foliar pathogens (Narasimhan et al. 2003; Santoyo et al. 2012; Sharma et al. 2019; Kumar et al. 2022).

Studies have shown that certain bacterial strains from the rhizosphere of common bean (*Phaseolus vulgaris*), *Bacillus* sp., *Sinorhizobium* sp., and *Pseudomonas* sp., inhibited the growth of pathogenic fungi, *Sclerotinia* sp., *Fusarium* sp., and *Rhizoctonia* sp., due to the production of hydrolytic enzymes, siderophores, and antifungal lipopeptides (Kumar et al. 2010; Vejan et al. 2016; Memenza-Zegarra and Zúniga-Davila 2021; Kumar et al. 2022).

1.3 Biofertiliser Formulations

Biofertilisers are products containing PGP organisms with the potential to mineralise and mobilise nutrients from insoluble sources for plant uptake and assimilation (Vessey 2003; Selvakumar et al. 2009). Biofertilisers can be formulated as a gel or an emulsion (Malusá and Vassilev 2014). Like the mixture of living and latent cells ensuring the fixation of N_2, the solubilisation of P and K rocks, oxidation of S and Mn, and production of PGR (IAA and GA), bio-fertilising organisms are also part of the microbial component of compost/manure that can accelerate vital soil processes to mobilise and increase plant nutrient availability; while protecting them from pathogens (Grayston and Germida 1991; Vessey 2003; Mishra et al. 2013; Zhang et al. 2015).

1.3.1 Soil Microbial Interactions

Interactions between arbuscular mycorrhizal fungi (AMF) and PGPB may be required for phytoremediation and rehabilitation of contaminated/degraded land for reuse (Santoyo et al. 2021; Sekhohola-Dlamini et al. 2022). These direct or indirect activities beneficial to host plants can occur synergistically and without abiotic and biotic stress (Gamalero et al. 2009; Santoyo et al. 2021).

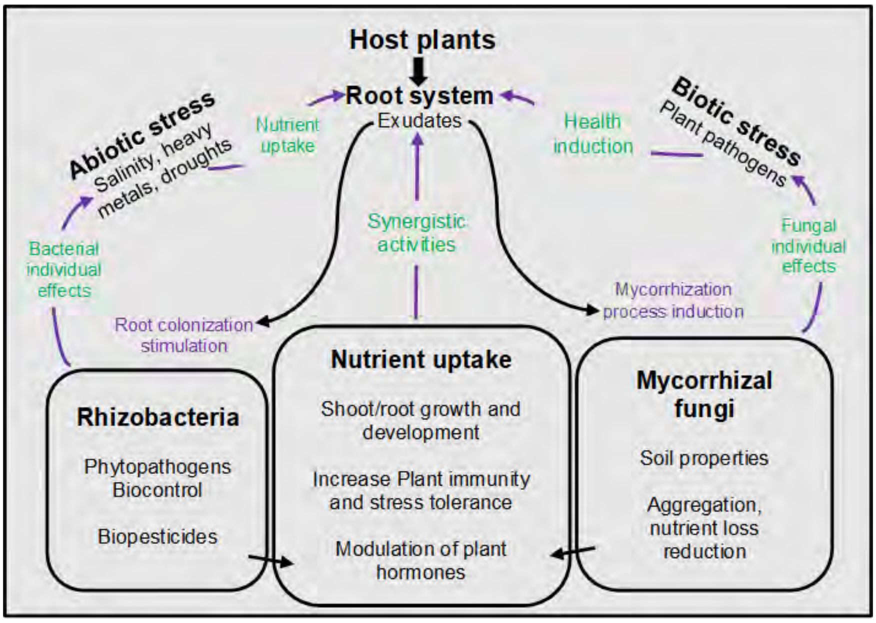


Figure 1. 3 Individual and synergistic mechanisms exerted by mycorrhizal fungi and PGPB to facilitate the phytoremediation process and promote plant growth and development in metal-contaminated soils (adapted from Malero et al. 2009; Santoyo et al. 2021).

Plant growth-promoting bacteria are mainly known as biological control agents against plant pathogens, while AMFs the excellent soil property improvers. Host plant root exudates also stimulate PGPB colonisation and mycorrhization, which improve plant growth and development and increase food crop yields (Santoyo et al. 2021).

1.3.2 Plant Microbial Interactions

Microbiome exists in terrestrial and aquatic ecosystems; however, due to the rapid development of molecular biology, sequencing technology, and omics analysis, the critical role of the rhizosphere microbiota is being revealed (Mendes et al. 2013; Li et al. 2021). Known as the plant root-soil zone, the rhizosphere is influenced by the release of rhizodeposits (exudates) from the plant root systems (Lynch 1990; Hinsinger et al. 2005).

In the rhizosphere, mutualistic nutrient exchanges occur between host plants and organisms (Burdman et al. 2000; Barea et al. 2005). Plants invest 20 to 40% of photosynthetic carbon in root exudates as rhizodeposits (Hinsinger et al. 2005; Badri and Vivanco 2009). The invested rhizodeposits are mainly used as a source of carbon by soil microbial communities, which, in turn, promote plant growth and development by producing, mineralizing, and mobilizing essential nutrients (Kloepper et al. 1989; Burdman et al. 2000). The rhizodeposits consist of organic compounds including sugars and polysaccharides, amino acids and peptides, proteins, and extra-polymeric substances (EPS) (Bowen and Rovira 1999; Nguyen 2003; Jones et al. 2004; Jimoh and Cowan 2017).

Interacting microorganisms in the rhizosphere include bacteria, fungi, nematodes, protozoa, algae, viruses, and arthropods. However, the number of bacteria appears to be higher than other soil organisms. A single gram of soil contains up to 10^{10} bacteria, 10^{9} viruses, 10^{8} fungi, 10^{5} protozoa, and 10^{5} arthropods (Torsvik et al. 1990; Glick 2012; Mendes et al. 2013; Raynaud and Nunan 2014). A peculiarity is observed, especially with the rhizosphere microorganisms; in addition to the promoted ecological interactions, they also participate in the global transformation and bioremediation of aquatic and terrestrial ecosystems (Raynaud and Nunan 2014). This potential should be explored and explained to increase plant growth and productivity and facilitate the recycling and restoration of polluted environments for reuse (Raynaud and Nunan 2014; Li et al.2021; Deng et al. 2022).

1.3.3 Types of Biofertilisers

Biofertilisers are classified into microbial functional groups as they promote plant growth and development and boost agricultural yield. Bacteria, fungi, and algae are the most known biofertilisers; however, their impacts in promoting plant growth are highlighted in this study.

Some endophytes and free-living bacterial biofertilisers include *Azotobacter* sp., *Azospirillum* sp., *Azoarcus* sp., *Klebsiella* sp., *Bacillus* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Enterobacter* sp., *Burkholderia* sp., *Serratia* sp., *Proteus* sp. and *Rhizobium* sp. (Lata and Prasad 2011; Elavarasi et al. 2020; Titilawo et al. 2020). Among fungal biofertilisers, AMFs facilitate mutualistic exchanges of about 80% with plants. They provide water and nutrients to host plants and suppress abiotic stress while protecting them against pathogens (Berruti et al. 2016; Odoh et al. 2020). Some fungal strains used in biofertiliser formulation include *Mycorrhiza* sp., Trichoderma sp., *Chaetomium* sp., *Gigaspora* sp., *Acaulospora* sp., *Scutellospora* sp., *Gliocladium* sp. (Berruti et al. 2016; Odoh et al. 2020). These strains, individually or in consortium interact with plants to enhance the uptake of P, N, Zn, Cu, Fe, S, and B as plant growth-promoting compounds to increase plant physiological activity that improves growth and development (Rai et al. 2013; Odoh et al. 2020; Lestari et al. 2021).

Algae-based biofertilisers such as *Gloeothece* sp., *Cyanothece* sp., *Gloeocapsa* sp., Synechocystis sp., Chamaesiphon sp., Cyanobacteria sp., Oscillatoria sp., Spirulina sp., *Arthrospira* sp., *Lyngbya* sp., *Microcoleus* sp., *Pseudanabaena* sp., *Anabaena* sp., *Nostoc* sp. and *Nodularia* sp., can provide NH_3 to soil and plants as they perform atmospheric N_2 fixation (Marschner et al. 2011; Elavarasi et al. 2020). Since the 1939s, algal biofertilisers have been used to boost rice production in tropical regions (Elavarasi et al. 2020). Therefore, research has demonstrated the plausibility of using *Spirulina platensis* in domestic wastewater treatment and reusing treated water in rice cultivation to stimulate increased grain germination rate, plant development (Nguyen et al. 2022) and crop yields.

1.3.3.1 Indole Compounds Production Biofertilisation Process

Indole-3-acetic acid (IAA) affects and induces primary and adventitious rooting as well as root hair production; the structures facilitating the root system to access nutrients (Spaepen et al. 2007; Overvoorde et al. 2010; Vejan et al. 2016; Suliasih and Widawati 2020). Plants and some microorganisms synthesize IAA; however, the production of IAA by microorganisms in media with/without L-tryptophan is essential in determining the plant growth-promoting activity (Suliasih and Widawati 2020; Lestari et al. 2021). Some IAA-producing organisms include *Azospirillum* sp., *Agrobacterium* sp., *Bacillus* sp., *Enterobacter* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Serratia* sp., *Proteus* sp., *Rhizobium* sp., *Aspergillus* sp. and *Penicillium* sp. (Ahmed and Hasnain 2010; Vejan et al. 2016; Lestari et al. 2021). Research has, however, revealed how *Bacillus* sp. promoted the growth and development of potato plants, *Solanum tuberosum* (Ahmed and Hasnain 2010; Vejan et al. 2016).

1.3.3.2 Gibberellic Compounds Production Biofertilisation Process

Gibberellins (GAs) are products of some strains of fungi and bacteria. This phytohormone was first discovered in 1935 from a fungal plant pathogen, *Gibberella Fujikuroi* (Hedden et al. 2001; Gupta et al. 2015). This fungus still plays a critical biological role as most of the marketed gibberellic compounds (GAs) remain the products of *Gibberella Fujikuroi* (Hedden et al. 2001). GAs compounds stimulate flower and fruit induction, seed germination processes, and stem and leaf growth (Bottini et al. 2004). However, the most dominant physiological effect of GA is root elongation (Tanimoto 1991). Many species of the *Acetobacter*, *Bacillus* and *Promicromonospora* genera have demonstrated the potential to produce GAs (Bastian et al. 1998; Joo et al. 2004). Tomato (*Solanum lycopersicum*) plants previously inoculated with GA-producing *Sphingomonas* sp. showed significantly increased growth and development (Khan et al. 2014) rate.

1.3.3.3 Cytokinin and Ethylene Production Biofertilisation Process

Cytokinin (CK) is known to regulate root gravitropism and sensitivity, cambial vascular differentiation, and root hair proliferation while inhibiting lateral root formation and primary root elongation (Aloni et al. 2006; Riefler et al. 2006; Hussain and Hasnain 2009). Chinese white cedar (*Platycladus orientalis*) seedlings inoculated with CK-producing *Bacillus subtilis* were more drought-resistant (Liu et al. 2013). Moreover, CK products of *Bacillus licheniformis*, *Bacillus subtilis* and *Pseudomonas aeruginosa* improved cell division, fresh weight, and cotyledon size of cucumber plants (Hussain and Hasnain 2009).

Ethylene regulates their growth development and stress tolerance processes (Chang 2016). However, the 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an ethylene precursor, is sometimes synthesised by plants and some PGP organisms in response to tolerance to environmental stresses comprising cold, drought, flood, infections, salinity, and heavy metals (Glick 2012; Vejan et al. 2016).

Bacterial isolates (*Pseudomonas* sp. and *Rhizobium* sp.) with ACC-deaminase activities acted as PGPMs when they improved the growth, physiology, and quality of mung bean (*Vigna radiata*) grown in saline soil (Ahmad et al. 2013; Glick 2014). *Oryza sativa* exhibited some plant growth-promoting attributes in response to *Methylobacterium oryzae* inoculants (Yim et al. 2010).

1.3.3.4 Siderophore Production Biofertilisation Process

Plants and microorganisms absorb iron in the form of Fe(II). However, it is present in various insoluble ferric mineral rocks as Fe(III) hydroxide and oxyhydroxide compounds (Wang et al. 2014; Pahari and Mishra 2017; Ghazy and El -Nahrawy 2021; Kumar et al. 2022). Iron is involved in chlorophyll synthesis, and maintenance of chloroplast structure and function (Zuo and Zhang 2011; Gyana and Sunita 2015). Plants and microorganisms can produce siderophores in soil with low iron resources. These small organic molecules with 200-2000 Da, have iron-chelating activities (Ahmed and Holmström 2014). Siderophores solubilise ferric rocks by complexing with Fe(III) and reducing it to Fe(II) needed by plants and other microorganisms as nutrients (Wang et al. 2014; Ahmed and Holmstrom 2014).

The contribution of siderophore-producing organisms in PGP has manifested itself in several ways; in particular, *Pseudomonas fluorescens* improved iron absorption in certain grasses and broadleaf weeds. *Bacillus subtilis* in pepper, showed significant suppression of *Fusarium* wilt caused by *Fusarium oxysporum* (Shirley et al. 2011; Yu et al. 2011; Santoyo et al. 2012). Inoculants of *Penicillium citrinum*, *Aspergillus niger*, and *Trichoderma harzianum* increased the growth parameter of the annual legume chickpea, *Cicer arietinum* (Yadav et al. 2011).

1.3.3.5 Nitrogen Cycle Biofertilisation Process

Nitrogen is vital for the plant as a major component of chlorophyll, the pigment most necessary for photosynthesis. A major component of amino acids, the fundamental building blocks of proteins, vitamins, and nucleic acids (Wagner 2011).

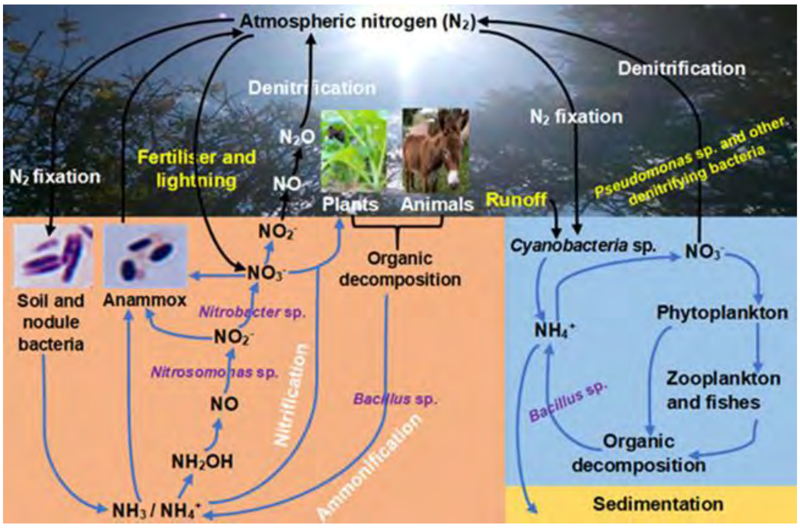


Figure 1. 4 The biogeochemical cycle of nitrogen. The process includes fixation of atmospheric nitrogen, mineralisation of organic nitrogen (ammonification), nitrification and uptake of NO_3^-/NH_4^+ by plants, denitrification (comprising anammox) and some cases of ammonium immobilisation (sedimentation) in hydrosphere environments (adapted from Walworth 2013; Lehnert et al. 2018; Norton and Ouyang 2019).

The biogeochemical process known as the nitrogen cycle (Figure 1.5) that results in soil nitro-fertilisation has four stages comprising N_2 fixation, ammonification, nitrification, and denitrification (Walworth 2013; Lehnert et al. 2018; Norton et al. Ouyang 2019).

- **Nitrogen fixation**

Chemical fertilisers damage soil health by releasing significant amounts of nitrous oxide (N_2O) and ammonia (NH_3) capable of disrupting biological cycles causing climate change and pollution (Girkin and Cooper 2022; Kumar et al. 2022; Pan et al. 2022). The Earth's atmosphere accounts for about 79% (V: V) of N_2 (Bennett 1999; Reece et al. 2011; Wagner 2011), which can only be used by plants in reduced forms of NH_3 or NO_3^- (Wagner 2011). The process of N_2 fixation by diazotrophic cells (bacteria and archaea) and cyanobacteria is necessary (Girkin and Cooper 2022; Kumar et al. 2022) because it provides the soil with nitrogen compounds.

Lightning allows sudden dissociation and oxidation of N_2 to form nitrogen oxides (Bennett 1999; Wagner 2011; Vance 2001; Cloete and Atlas 2006; Reece et al. 2011), which are not sufficient to meet the nitrogen requirements of soil/plant. The process of atmospheric N_2 fixation occurs via the molybdenum cofactor requiring nitrogenase synthesised in all diazotrophic organisms (Bishop and Jorerger 1990; Ahemad and Kibret 2014). These organisms live in intimate association with leguminous plants or freely in soil and aquatic environments (Wainwright 1999). The conversion of N_2 to NH_3 (Equation 2), requires the chemical energy of ATP molecules (Reece et al. 2011). N_2 fixation utilises carbon resources provided by the high carbohydrate intake from decaying material and root exudates or vascular tissues of the plant root system (Reece et al. 2011; Berg et al. 2012).

$$N_2 + 8\,e^- + 8\,H^+ \xrightarrow[16\,ATP \;\curvearrowright\; 16\,ADP]{\text{Nitrogenase}} 2\,NH_3 + H_2 + 16\,Pi \tag{2}$$

- **Ammonification**

Ammonifying organisms are mainly bacteria capable of degrading proteins and other organic nitrogen compounds, such as urea (Strock 2008; Reece et al. 2011), to release NH_3. At the same time, nitrogen-fixing organisms produce NH_3.

$$R{-}CH{-}NH_2{-}COOH + H_2O \xrightarrow{\textit{Bacillus}\ \text{sp.}} R{-}CHOH{-}COO^- + NH_4^+ \tag{3}$$

$$NH_2 - CO - NH_2 + 2\,H_2O \xrightarrow{\text{Urease}} (NH_4)_2CO_3 + H_2O \longrightarrow 2\,NH_4^+ + 2\,OH^- + CO_2 \tag{4}$$

Therefore, NH_3 from either decaying material or atmospheric fixation captures H^+ and makes up NH_4^+ (equations 3 and 4) needed by plants and other living organisms as nitrogen sources (Lim 1989; Reece et al. 2011).

- **Nitrification**

Nitrification is an aerobic process with two main steps, including the oxidation of NH_4^+/NH_3 to NO_2^- (equation 5) and NO_2^- to NO_3^- (equation 6). It is a biologically energy-producing process carried out by *Nitrosomonas* sp., *Nitrosococcus* sp., and *Nitrobacter* sp. (Cloete and Atlas 2006; Norton and Ouyang 2019). Plants take up the most nitrogen in the form of NO_3^- and then reduce it to NH_4^+ before being assimilated into amino acids.

$$2\ NH_4^+ + 2\ NH_3 + 3\ O_3 \xrightarrow{\textit{Nitrosomonas}\ \text{sp.}} 2\ NO_2^- + 2\ H_2O + 4\ H^+ \longleftrightarrow 2\ H^+ \qquad (5)$$

$$2\ NO_2^- + O_2 \xrightarrow{\textit{Nitrobacter}\ \text{sp.}} 2\ NO_3^- \qquad (6)$$

To reduce the toxicity caused by ammonia/nitrates accumulated in the soil and water sludges, other microbial strains carry out anaerobic ammonium oxidation (anammox). In this anammox process, bacteria transform NH_4^+ and NH_3 or NO_3^- (Figure 1.4) into N_2 (Kuenen 2008; Reece et al. 2011; Norton and Ouyang 2019) which is lost to the atmosphere. The bacterial anammox process can reduce N_2 by up to 50% (Kuenen 2008).

- **Denitrification**

The process protects ecological environments from harmful nitrogen compounds (Cloete and Atlas 2006; Reece et al. 2011). Microorganisms performing denitrification include *Paracoccus* sp., *Pseudomonas* sp., *Moraxella* sp., *Spirillum* sp., *Thiobacillus* sp., *Achromobacter* sp. and *Bacillus* sp. They release nitrogenous intermediate products such as NO_2^-, and N_2O to form N_2. About 9.3 10^7-19 10^7 Kg N_2/year diffuses into the atmosphere (Hopkins 1999; Cloete and Atlas 2006).

1.3.3.6 Symbiotic Nitrogen-Fixation Biofertilisation Process

The N_2-fixing bacteria in the root nodule of legumes include *Allorhizobium* sp., *Azorhizobium* sp., M*esorhizobium* sp., *Rhizobium* sp., and *Sinorhizobium* sp. (Sindhu and Dadarwal 1997; Mus et al. 2016; Kumar et al. 2022; Kawaka 2022). However, approximately 80% of N_2 is fixed in symbiotic form, which makes leguminous plants less dependent on nitrogen fertilisers (Goyal et al. 2021). Furthermore, 30-80 Kg N/ha/year is left each year in the soil after harvest (Sindhu et al. 1992) as organic nitrogen fertiliser. *Acetobacter diazotrophicus* is known as an N_2-fixing organism present in symbiotic relationships with sugarcane, *Saccharum officinarum*

(Kannaiyan 1993; Kumar et al. 2022). Cyanobacteria, including *Anabaena azollae* fix atmospheric N_2 and are effective bio-fertilising strains. *A. azollae* frequently found in rice fields, fixes 40-60 Kg N/ha/year of rice crops (Kannaiyan 1993).

1.3.3.7 Associative Nitrogen-Fixation Biofertilisation Process

This mutualistic interaction typically involves non-leguminous plants such as cereals, rice, wheat, maize, barley, and *Poaceae,* switchgrass (*Panicum virgatum*) (Roley et al. 2018; Haskett et al. 2022). Atmospheric N_2 is biologically converted to NH_3 by bacterial species mostly *Azospirillum*, *Glucenobacter*, *Acetobacter*, *Herbaspirillum*, and *Azoarcus* in occasional rhizosphere association with non-leguminous plants (Roley et al. 2018; Chaudhary et al. 2022). It is estimated that *Azospirillum* alone fixes up to 20-40 Kg N/ha/year (Fibach-Paldi et al. 2011).

1.3.3.8 Free-Living Nitrogen-Fixation Biofertilisation Process

Rhizobacteria in symbiotic forms achieve about 80% of the biological N_2 fixation (Peoples et al. 1995; Orr et al. 2011). *Azospirillum* sp., *Azotobacter* sp., *Clostridia* sp., and *Pseudomonas* sp.) can fix significant amounts of N_2 estimated at 60 Kg/ha/year (Kahindi et al. 1997; Bürgmann et al. 2004; Orr et al. 2011; Walworth 2013; Ramanan et al. 2016). Blue-green algae and fungal strains, namely *Nostoc* sp., *Oscillatoria* sp., *Plectoneme* sp., *Rhodospirillum* sp., *Saccharomyces* sp. and *Rhodotorula* sp. can also fix atmospheric N_2 in the non-symbiotic form (Berman et al. 1985; Knoth et al. 2013). These organisms draw energy from the degradation of organic matter and root secretions, and, in return, fix the nitrogen that the plant needs. The fixed nitrogen is then taken up by the root system and, after assimilation, forms new tissues, the process leading to plant growth and development (Reece et al. 2011; Berg et al. 2012; Aczel 2019).

1.3.3.9 Phosphates Solubilisation Biofertilisation Process

Phosphorus (P) accounts for approximately 0.2-0.8% of plant dry weight and plays a vital role in energy production and cell structure (Sharma et al. 2013; Bechtaoui et al. 2019). It regulates protein synthesis, growth, and development of new tissues, cell division, seed formation, and branching of plant roots. These processes stimulate plant vitality, disease resistance, and stress tolerance (Widawati and Suliasih 2006; Sharma et al. 2013; Gizaw et al. 2017). However, P deficiency reduces plant height (Sharma et al. 2013).

To increase agricultural production, adding phosphate to the soil in the form of chemical fertiliser has proven to be the best way. However, due to the processes of adsorption, immobilisation (Figure 1.5), and precipitation by metal-cation complexes, only 10-15% of the P fertiliser is available for plant uptake (Sharma et al 2013; Lehnert et al. 2018).

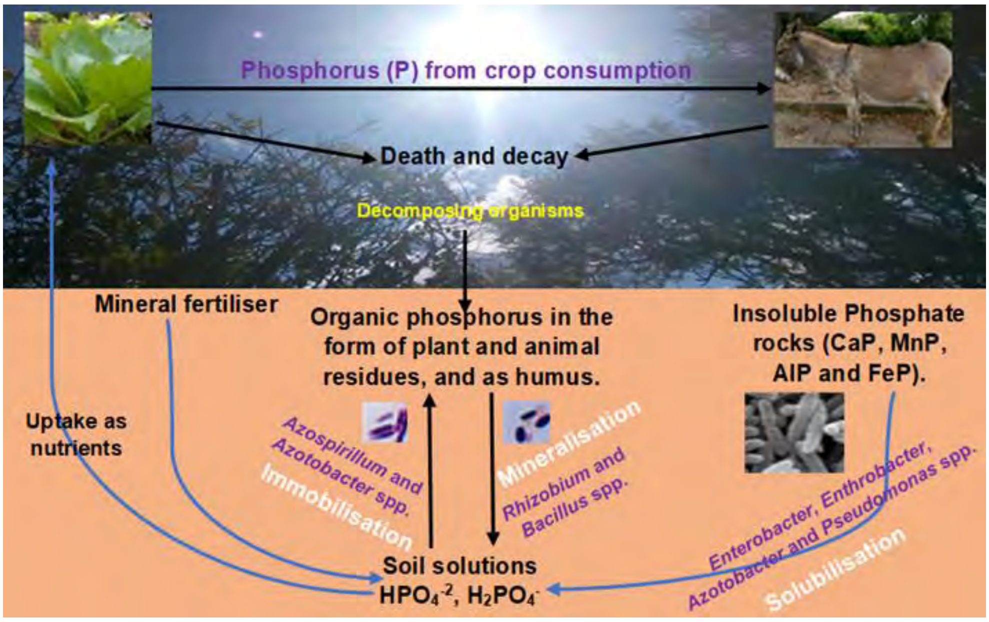


Figure 1. 5 Biological solubilisation of phosphate. The process includes the solubilisation of phosphate rocks, mineralisation of organic phosphorus and mobilisation of nutrients for plant uptake (adapted from Khan et al. 2009; Ahemad and Kibret 2014; Sharma et al. 2013; Lehnert et al. 2018).

Bacterial/fungal solubilising phosphates include Penicillium sp., Pseudomonas sp., *Azospirillum* sp., *Azotobacter* sp., *Bacillus* sp., *Enterobacter* sp., *Exiguobacterium* sp., *Klebsiella* sp., *Alcaligenes* sp., *Arthrobacter, Burkholderia* sp., *Rhizobium* sp., *Serratia* sp., *Aspergillus* sp., *Pichia* sp., *Cryptococcus* sp., *Rhodotorula* sp. and *Penicillium* sp. (Kloepper et al. 1989; Sundara et al. 2002; Saharan and Nehra 2011; Walpola and Yoon 2012; Gizaw et al. 2017; Titilawo et al. 2020). Research has shown that some of these effective PGPR are isolates of polluted environments such as diesel-contaminated soil and coal sludge (Titilawo et al. 2020).

1.3.3.10 Potassium Solubilisation Biofertilisation Process

More than 90% of this soil component exists in the fixed form of rocks and silicates (Parmar and Sindhu 2013). K-minerals composed of mica, feldspar, potassium aluminium silicate, phosphorite, K-rich shale, microcline, orthoclase and muscovite are 98% insoluble (Parmar and Sindhu 2013; Rajawat and 2014; Bahadur et al. 2019). The use of K rocks could be more beneficial to the environment than K as a chemical fertiliser; however, the release of nutrients from rocks is slow, making this practice insignificant in agriculture (Rajan et al. 1996; Sindhu et al. 2014). Research then revealed that combining K rocks and microbial inoculants would increase the release and mobilise this macronutrient for plant uptake (Parmar and Sindhu 2013; Etesami et al. 2017).

Potassium participates in many cellular metabolic activities, namely selective cell permeability, enzyme activation, photosynthesis, sugar and water transport, stomatal activity, crop yields as well and protein synthesis. and starch (Van Brunt and Sultenfuss 1998; Thomas and Thomas 2009; Patil et al. 2011; Reece et al. 2011; Pajapati and Modi 2012; Rawat et al. 2016; Etesami et al. 2017).

K-solubilising bacteria and fungi include *Acidithiobacillus* sp., *Bacillus* spp., *Paenibacillus* spp., *Proteus* sp., *Serratia* sp., as well as *Aspergillus* spp. (Prajapati et al. 2012; Meena et al. 2016; Etesami et al. 2017; Dhiman et al. 2019; Titilawo et al. 2020). These organisms solubilise inorganic K to mobilise mineralised K^+ in plants through biological processes called acidolysis, chelation, exchange reactions, complex lysis, and organic acid production (Calvaruso et al. 2006; Meena et al. 2016).

1.3.3.11 Sulphur Oxidisation Biofertilisation Process

Sulphur (S) accounts for about 0.1% of the plant's dry mass and is taken up by the roots as SO_4^{-2} (Howarth et al. 1992; Zhao et al. 2008; Reece et al. 2011). More than 95% of the S is found in organic forms as sulphate esters, R-SO_4^- and sulfonates, R-SO_3^- resulting from the subsequent humification of biological matter deposition (Gahan and Schmalenberger 2014). This macronutrient is of paramount importance in the formation of protein structure and enzyme/coenzyme activities while boosting plant disease resistance and stress tolerances (Grayston and Germida 1991; Zhao et al. 2008; Reece et al. 2011). The biological assimilation of S is preceded by its reduction to cysteine, known as the main precursor of plant S metabolites (Zhao et al. 2008).

Soil S availability depends on the physicochemical processes and microbial activities (Yesmin and Banerjee 2000; Awad et al. 2011). The oxidation of S to SO_4^{-2} and mobilisation of sulphated esters are carried out by bacterial and fungal strains known as *Pseudomonas* sp., *Klebsiella* sp., *Salmonella* sp., *Enterobacter* sp., *Serratia* sp., *Comamonas* sp., *Thiobacillus* sp., *Aspergillus* sp., *Scolecobasidium* sp., *Myrothecium* sp. and *Trichoderma* sp. (Hummerjohann et al. 2000; Awad et al. 2011; Chaudhary and Goyal 2019). Certain plants such as canola (*Brassica napus*) have high S requirements during their vegetative growth and generative development periods. But, during this period, S may be deficient in the rhizosphere. Seed inoculation with certain sulphur-oxidising bacteria or fungi may be of utmost importance.

1.3.3.12 Manganese Oxidation Biofertilisation Process

Manganese (Mn) participates more in lipid biosynthesis, photosynthesis, phosphorylation, decarboxylation, hydrolysis, and redox reactions (Iranzo 2001; Socha and Guerinot 2014; Schmidt and Husted 2019). This micronutrient promotes plant growth and development as well as agricultural productivity. Mn deficiencies reduce plant growth and development and increase susceptibility to disease and freezing temperatures. As Mn(II) is more soluble and in large quantities in the soil, it becomes a more preferred form by plants (Ocha and Guerinot 2014; Keiluweit et al. 2015). Mn-oxidizing organisms are mostly ubiquitous bacteria such as *Bacillus* and *Pseudomonas* spp. (Burger et al. 2008; Tebo et al. 2005; Cerrato et al. 2010).

- **Cross Inoculation**

Cross-inoculation is based on the ability of bacteria to selectively form nodules with their host plant following specific recognition of signal molecules produced by both partners (Datta et al. 2015; Mendoza-Suárez et al. 2020). However, this statement remains relative as some legumes have been nodulated with rhizobia which were not part of their groups (Pankievicz et al. 2019).

- **Consortium Inoculation**

Plant-microbial mutualistic interactions use variable biological processes to colonize root systems and enhance growth (Vílchez et al. 2015). Some can be effective as individuals or as consortia. The success of beneficial microbes in improving plant growth and crop-up food quantity and quality depends on adequate plant-microbial interactions that enhance nutrient uptake and stress tolerances (Du Jardin 2015; Vílchez et al. 2015; Molina-Romero et al. 2021). However, when used as individuals or consortia, they must be harmless and safe for soil microbiota, host plants and animals (Vílchez et al. 2015; Molina-Romero et al. 2021). Research has indicated that multiple inoculations stimulated more plant growth and increased crop yield compared to single inoculation (Sivaramaiah et al. 2007; Souza et al. 2015; Chaudhary and Sindhu 2016). Sugarcane (*Saccharum officinarum*) cultivated in 75% P-depleted soil, showed a 38% increased dry biomass, total P, and stem production after double inoculation *Azospirillum brasilense* and *Bacillus subtilis* (Rosa et al. 2020). However, about a 20% increase in wood yield was observed when the *Fabacaea*, *Schizolobium parahyba* (*amazonicum*) was inoculated with the rhizobacterial-mycorrhizal fungi (Cely et al. 2016).

1.3.4 Commercial Biofertilisers

In developing countries, the biofertiliser industry is still underdeveloped due to several challenges, including weak support for research or innovation. The adoption of biofertilisation technology has yet materialised compared to developed countries (Alori and Babalola 2018; Raimi et al. 2021). Biofertilisers can be made in solid or liquid form with single or mixed microbial inoculants (Reddy and Saravanan 2013). Thus, to preserve microbial viability during storage, inoculants must be kept in carriers, structures designed to provide a favourable microenvironment for microbial inoculants (Malusá et al. 2012). Carriers are made up of natural or synthetic polymers (gum Arabic and xanthan, gelatin, polyvinyl pyrrolidone), glycerol, horticultural oil, glucose, and lactose (Lee et al. 2016; Valetti et al. 2016; Bernabeu et al. 2018; Dey 2021).

1.3.4.1 Liquid Biofertiliser

As liquid inoculants are easy to prepare and cost-effective, they dominate the commercial market (Lee et al. 2016; Dey 2021). Liquid biofertilisers are ready as broths, suspensions of humic acids, or mineral/organic oils (Reddy and Saravanan 2013; Lee et al. 2016; Dey 2021). Liquid inoculants can be useful for seed coating, root dipping when transplanting, and foliar spraying (Reddy and Saravanan 2013).

1.3.4.2 Solid Biofertiliser

Solid biofertilisers contain microbial inoculants in organic/inorganic powder/granular carrier molecules (Malusá et al. 2012; Lee et al. 2016) as wet and dry formulations. For the damp formulation, the drying process does not occur, and thus, the water content remains high even during storage and application periods (Joe et al. 2014; Liffourrena and Lucchesi 2018). However, different drying processes, including freeze-drying, air-drying, desiccation, and spray-drying, are used (Ruíz-Valdiviezo et al. 2015; Berninger et al. 2017; Basheer et al. 2018). Previous studies have found that applying biofertilisers containing complex microbial inoculants can establish more balanced and beneficial plant-microbe interactions (Zhang et al. 2022). Microbial consortia containing multiple biological control agents overcome some plant disease problems caused by mixed active pathogens (Guetsky et al. 2001).

1.3.5 Challenges Limiting Biofertiliser Production

Biofertilisers have several advantages over agricultural chemicals. They are environmentally friendly renewable sources of nutrients, soil biology enhancers and fertility restorers (Bharti et al. 2016; Sharma et al. 2016; Timmusk et al. 2017). Despite these advantages, biofertilisation technologies are still limited by challenges. These agricultural techniques are not feasible on a large scale (Callaghan 2016; Timmusk et al. 2017) because laboratory results are still difficult to verify in the field.

Constraints also include differences in the physicochemical properties of soils, possible competition between soil organisms and the inoculated strain, abiotic stress, and likely fertiliser contamination during periods of seed preparation, inoculation, and sowing. Additionally, lack of equipment for microbial storage, a lack of awareness, and weak regulatory standards for microbial safety (Cuddeford and Kabaluk 2010; Bharti and Suryavanshi 2021).

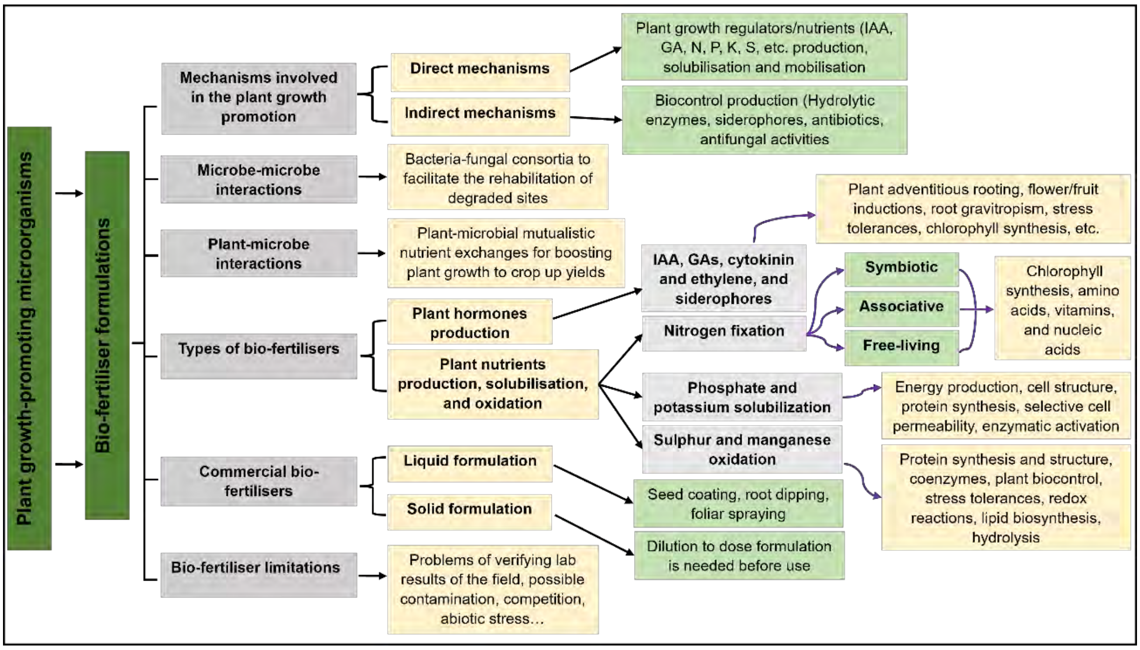


Figure 1. 6 Biofertiliser formulation classification based on the activity promoting plant growth, advantages, and some limitations (adapted from Azubuike et al. 2016; Kumar et al. 2022).

To develop and produce effective biofertilisers, manufacturers should select resistant species from microbial strains that have been tested under different soil environmental conditions, using many crop varieties. However, the claim that mixed microbial inoculants could provide the best result in terms of plant nutrient uptake, plant growth development, and yield increase (Mamnabi et al. 2020; Zeffa et al. 2020; Santoyo et al. 2021) should also be explored.

1.4 Aim and Objectives

This study aimed to develop a bespoke biofertiliser for use in the land rehabilitation bioprocess known in South Africa as Fungcoal. To achieve this, several sources of bacteria were used to find candidate strains to incorporate into the biofertiliser. One source was a suite of previously isolated bacteria known to degrade waste and weathered coal, which possess plant growth-promoting (PGP) activity. The second was a group of novel aerobic bacteria isolated from microalgal-bacterial flocs (MaB-flocs) formed in high-rate oxidation ponds (HRAOP) of an integrated algae-based wastewater treatment process (IAPS). A third source was a group of newly isolated bacterial strains from coal obtained from the Mpumalanga Coal Fields. To date, and as far as can be determined, no known PGP bacteria have been isolated either from MaB-flocs or from mined coal. Here, the study reports on the biochemical and molecular characterisation of PGP bacterial isolates from MaB-flocs formed in HRAOP of an IAPS treating municipal wastewater and mined coal and efforts to establish a consortium capable of replacing fossil fuel-derived and chemically synthesised fertilisers and plant growth regulators.

The specific objectives, of the research described in this thesis, are as follows.:

1. Secure the viability and bioactivity of all Fungcoal biocatalysts, coal degrading and plant growth promoting bacteria and background characterisation of microbial flora in a typical South African coal discard waste dump in the eMalahleni Coalfields.
2. Screening all organisms for abiotic/environmental stress tolerance (pH, salinity, and temperature) and carbon substrate utilisation
3. Setting up bespoke bioassays for rapid nutrient bio-mineralisation (N, P, K, and coal) potential, PGR (IAA, GAs, and ACC) production and siderophores activity.
4. Formulate the biocatalytic component of a biofertiliser containing PGPB to support the Fungcoal myco-phytoremediation strategy for revitalising and restoring soils affected by coal mining to the low-cost sustainable cover of waste landfills.

Chapter Two: Coal Substrate, Physicochemical Properties and Biology

2.1 Introduction

Coal is essentially a sedimentary organoclastic rock generated by the coalification of woody or halophilic plant debris. Coalification involves decomposing, compressing, and consolidating plant debris between rock strata, followed by microbial transformation and thermal interactions over geological times (Schweinfurth 2009; O'Keefe et al. 2013; Keshinro 2021; Australian Government 2023). Geologically, coal is composed of inorganic and organic substances. Its inorganic part includes ash, volatile mineral compounds, carbon (C), nitrogen (N), sulphur (S), and trace elements. However, its main inorganic S is pyrite. These volatile compounds are often responsible for environmental pollution linked to greenhouse gases, acid rain, and global climate change (Finkelman and Brown 1991; Finkelman and Gross 1999; Zhang et al. 2015; Australian Government 2023). The coal organic fractions consist of a complex mixture of aromatic and aliphatic hydrocarbons as well as heterocyclic compounds containing N, S, and O_2, such as lignin. The major organic S components of coal include thiol, sulphide, disulphide, and thiophene rings (Kabe et al. 2004; Zhang et al. 2015; Olawale et al. 2020).

Coal is broadly divided into two groups comprising low-rank coal (LRC) or waste coal (WC) and high-rank coal (HRC). The LRC known also as brown coal includes lignite and sub-bituminous whereas the HRC, black coal includes bituminous and anthracite (Sekhohola et al. 2013). As better fuels, only bituminous and anthracite are exploited for energy production because of their higher carbon contents. The rest of the coal known as WC is then accumulated thus becoming a permanent danger to the environment. Having a low calorific value, LRC has a high lignin content, which microbial decomposition may release humic and fluvic acids, essential components in soil carbon amendments and the production of organic fertilisers (Olawale et al. 2020). Previous research has shown how certain strains of bacteria and fungi isolated from coal environments such as coal slurry (CS) could biologically solubilise/degrade LRC, coal fines, and other coal residues into value-added products (humic acid), providing a new reuse potential (Yang et al. 2023). These potentials were due to the ability of bacteria and fungi to produce ligninolytic enzymes known as biological mediators that break down lignin polymers (Mönkemann et al 1997; Giannouli et al 2009; Pawlik et al. 2016; Olawale et al. 2020). Ligninolytic enzymes (laccase, lignin peroxidase, manganese peroxidase, and multivalent peroxidase), are utilised in the removal and detoxification of environmental lignocellulosic wastes (Kumar and Chandra 2020). The WC, known as LRC used in this study was provided by Thungela Coal Company, a South African thermal coal exporter with is located at Rosebank, Johannesburg, in the Gauteng Province.

This chapter aimed to determine the physicochemical properties, including pH value, electrical conductivity, dissolved oxygen, and essential plant nutrients (N, P, K, S) of LRC samples. Energy-dispersive X-ray spectroscopy analysis assessed the chemical concentration and composition in a spectrum. The biological properties of coal consisted of conducting metagenomic analysis to examine the structure and microbial diversity interacting in this environment with bacterial and fungal strains as targets. Finally, the molecular identity of some bacterial strains isolated from LRC samples was determined.

2.2 Materials and Methods

2.2.1 Coal Substrate

Waste coal (WC), colloquially known as low-rank coal (LRC), was provided by Thungela Coal. The company is a South African thermal coal exporter with its head office located at Rosebank (26° 08' 25.80" South, 28° 02' 15.60" East), Johannesburg in the Gauteng Province. The supplied coal was partially crushed and sourced from the waste generated by the Navigation Processing Plant, Khwezela mine, Emalahleni, Mpumalanga Province (Figure 2.1A). Subsamples of coal were sieved using an Endecott test sieve (BS 410-1, England) to yield the finest particles of 250 microns (Figure 2.1B), which were added (1 g L^{-1}) to the culture medium prepared for microbial biodegradation assays.

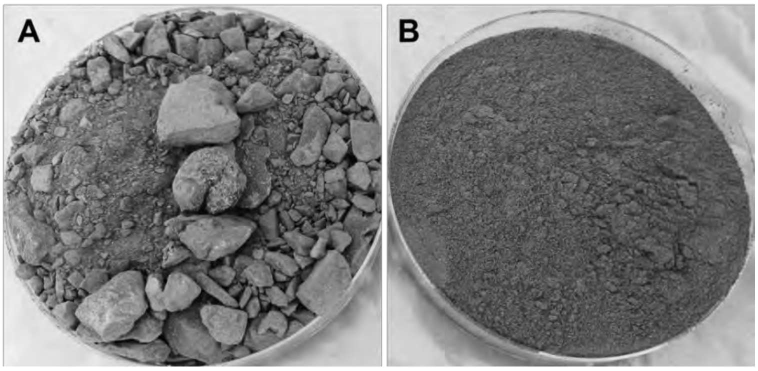


Figure 2. 1 Waste coal as supplied by Thungela (a) and the finest particles after being crushed and sieved (b).

2.2.2 Physicochemical Properties of Coal Substrate

To carry out the physicochemical properties of the coal, the suspensions were made accordingly using Mill-Q water to estimate the pH, electrical conductivity (EC) and dissolved oxygen (DO). The availability of essential plant nutrients (EPN), including N, P, K, and S, has also been studied (Nair et al. 2020; Pang et al. 2021).

2.2.2.1 pH, Electrical Conductivity and Dissolved oxygen

A coal slurry (1:10 WV^{-1}) was prepared beforehand, and pH estimation was monitored using a measuring device (EUTECH Instruments, 2136506, Singapore). The portable meters (Flexi HQ30d and Multimeter HQ40d, Malaysia) were also used for EC and DO measurements.

2.2.2.2 Nutrient Content Quantification

The nutrient content of WC was measured as NH_3, phosphorus as PO_4^{-3}, potassium as K^+ and sulphur as SO_4^{-2} equivalents (Nair et al. 2020). To proceed with quantification assays, the coal suspension was filtered through Munktell Filtrak^{TM}, filter papers (Grade 3, Disc h, 150 mm, Germany) from which 2 mL was used for quantification of NH_3-N using a purchased test kit (HACH 2604545, Set LR, USA). A five mL filtrate was used for the analysis of available phosphates using the PO_4^{-3}-P test kit protocol (HACH 2604545, Set LR, USA); however, potassium was determined using the test kit method (Merck KGaA, 1.14562.0001, Germany) followed by interpolation from a standard curve prepared with KCl. The availability of SO_4^{-2} was determined by transferring 50 mL filtrate into a 100 mL Erlenmeyer flask. 10 mL solution 1 (24 g NaCl and 2 mL HCl to make up 100 mL with Milli-Q water), 10 mL solution 2 (25 mL glycerol in 50 mL absolute ethanol), and 0.15 g BaCl2 were added. The sample was stirred for an hour, and then absorbance (A_{420}) was measured (Nair et al. 2020), followed by interpolation from a standard curve prepared from Na_2SO_4 (Univar, Merck).

2.2.3 Energy-Dispersive X-Ray Spectroscopy of Coal Substrates

Energy-dispersive X-ray spectroscopy (EDS) was carried out at the Electron Microscopy Unit (EMU), at Rhodes University. Coal samples of about 1.3 cm^2 and 2 mm thick were prepared. Then, secondary electron (SE) images, backscattered electron (BE), and EDS images were collected under a high vacuum on a scanning electron microscope (Tescan Vega 2) with a W filament. The EDS data of uncoated coal samples were collected on an Oxford INCA (Penta-FET-X3) and Si as a detector at 20 kV.

2.2.4 Biology of Coal Substrates

This section consisted of performing the metagenomic analysis and investigating the microbial content (bacteria and fungi) of coal substrates.

2.2.4.1 Metagenomic Analysis and Sanger Sequencing

Low-rank coal sample was submitted to Inqaba Biotechnical Industries (Pty) Ltd P.O. Box 14356, Hatfield 0028, South Africa, for metagenomic sequencing analysis of the coal microbial community. Genomic DNA was directly extracted from the filters (Zhou et al. 1996), and the extraction steps were carried out with 50 mL proteinase K (10 mg mL^{-1}) after precipitation with isopropanol. The nucleic acid pellets were obtained by centrifugation at 50000 xg for 30 min at ambient temperature. Extracted genomic DNA was stored at - 20°C until PCR amplification, and then metagenomic sequencing was performed (Klindworth et al. 2013).

Polymerase chain reaction (PCR) amplifications were performed to test primer pairs for rDNA. The primer pairs included: (a) S-D-S-17, 5'-CCTACGGGNGGCWGCAG-3'and (b) S-D-A-21, 5'-GACTACHVGGGTATCTA ATCC-3' (Herlemann et al. 2011). The reaction was carried out in 50 µL volumes containing 0.3 mg mL-1 Bovine Serum Albumin (BSA), 250 µM dNTPs, 0.5 µM of each primer, 0.02 U Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland) and 5x Phusion HF Buffer containing 1.5 mM MgCl2. Initial denaturation was performed at 95 °C for 5 min, followed by 25 cycles consisting of denaturation (95 °C for 40 s), annealing (2 min), and extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The annealing temperature for primers was set at 55°C and 44°C. PCR products were purified with a Qiaquick kit (Qiagen, Hilden, Germany). Two full PTPs per sample were sequenced and published by the MIMAS project for metagenomics and can be obtained from INSDC with an accession number (Teeling et al. 2012).

Samples were sequenced on an Illumina system (www.illumina.com). Reads were processed via search (https://drive5.com/usearch), and taxonomic information was determined based on the Ribosomal Database (http://rdp.cme.msu.edu/index.jsp) 16s database v16 reference or, in the case of the Internal Transcribed Spacer (ITS)1F, the Ribosomal Database Project (RDP), ITS V2 database.

2.2.4.2 Microbial Content of Coal Samples

Efforts were made to analyse the microbial content of the low-rank coal used in this study; 50 g of coal powder was suspended in 100 mL of phosphate buffered saline (PBS) followed by incubation overnight at 30°C. Before microbial isolation, serial dilutions were prepared by measuring 1 mL of the sample in a 10 mL test tube and topping up to 10 mL of labelled volume with Milli-Q water to form a solution dilution stock 10^{-1}. Gradient concentrations 10^{-2} to 10^{-4} were prepared from the stock solution and the 10^{-3} dilution was chosen as best for streak-plate techniques due to the expected number of colonies obtained.

The spread plate method involved pipetting 0.1mL of the 10^{-3} dilution into the centre of the agar plate and using a glass L-rod, the sample was spread over the agar and incubated at 30 °C for 3 d (NA) and 5 d (PDA). After incubation, the purification process was carried out and once the colonies were confirmed pure, morphological characterisation was assessed (Ruangpan and Tendencia 2004) before submitting all isolated microbials as plate samples to Inqaba Biotechnical Industries (Pty) Ltd P.O. Box 14356, Hatfield 0028, South Africa for Sanger sequencing and molecular identification.

2.3 Results

2.3.1 Physicochemical Properties

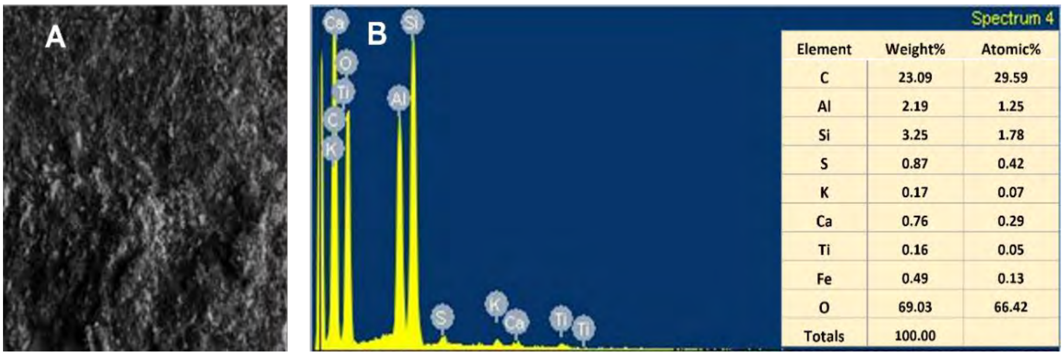
The results in Table 2.1 showed an acidic pH of 3.60 with an important dissolved S background of 7130.43 mg L^{-1}, and some amounts of N (20 mg L^{-1}), P (7.8 mg L^{-1}) and K (3.3 mg L^{-1}).

Table 2. 1 Analyses of the geological WC substrates

Physicochemical Properties of Coal Substrates							
Parameter Analysed	**pH**	**EC (mS Cm^{-1})**	**DO (mg L^{-1})**	**Essential Plant Nutrients (mg L^{-1})**			
				N	**P**	**K**	**S**
Mean Values (MV)	3.46	2.92	6.77	20.00	7.8	3.3	7130.43
Standard Error (± SE)	0.10	0.32	0.44	0.30	0.50	0.05	1.5.44

2.3.2 Energy-Dispersive X-Ray Spectroscopy

The EDX results of the solid coal samples (Figure 2.2) revealed a high concentration of carbon (C) 23.09% and oxygen (O) 69.03% taking up about 92.12% of the coal surface mineral composition.



Element	Weight%	Atomic%
C	23.09	29.59
Al	2.19	1.25
Si	3.25	1.78
S	0.87	0.42
K	0.17	0.07
Ca	0.76	0.29
Ti	0.16	0.05
Fe	0.49	0.13
O	69.03	66.42
Totals	100.00	

Figure 2. 2 Energy-disperse X-ray spectroscopy analysis showing the electron image of the coal samples (A) and the surface chemical composition in a spectrum (B) containing a table of weight and atomic percentages.

Other components like aluminium (Al) 2.19%, silicon (Si) 3.25%, sulphur (S) 0.87%, potassium (K) 0.17 %, calcium (Ca) 0.76%, titanium (Ti) 0.16% and iron (Fe) 0.49% are all present but in low proportions about 7.88%. However, the solid coal samples found no nitrogen (N) and phosphorus (P).

2.3.3 Metagenomic Analysis

According to the Inqaba Biotech Metagenomic Report, the 16s metagenomic analysis was sequenced on an Illumina system (www.illumina.com) and reads were processed via usearch (https://drive5.com/usearch). Taxonomic information was determined based on the Ribosomal Database Project 16s v16 database (http://rdp.cme.msu.edu/index.jsp) or, in the case of ITS1F, the RDP database ITS V2 (table 2.2).

Table 2. 2 Summary of metagenomic analysis of the microbial community of low-rank coal samples. 16s/ITS1F gene sequences of organisms listing essential taxonomic information at the phylum (A) and species (B) level.

Phylum Classification of the LRC Microbial Community Analysis					
Inqaba Report (A)			**Mothur Generated Report (B)**		
Nomenclature	Reads	%	Nomenclature	Reads	%
Firmicutes	17451.0	59.46	*Firmicutes*	26492.0	62.18
Proteobacteria	9687.0	33.01	*Proteobacteria*	15229.0	35.74
Unknown	1807.0	6.16	*Bacteroidota*	401.0	0.94
Bacteroidota	337.0	1.15	Unclassified	373.0	0.88
Actinobacteriota	50.0	0.17	*Actinobacteriota*	67.0	0.16
Planctomycetota	9.0	0.03	*Planctomycetota*	16.0	0.04
Gemmatimonadota	6.0	0.02	*Gemmatimonadota*	16.0	0.04
Verrucomicrobiota	3.0	0.01	*Verrucomicrobiota*	5.0	0.01
			Other Phyla	9.0	0.02

The operational taxonomic units (OTU) were excluded as they contributed less than 1% of the total data. The report generation command used $create_usearch_single_sample EM-5_S23 showed that the microbial community was dominated by the Kingdom classification of Bacteria (96.32 %). The Kingdom classification of Unknown was found in enormously minor amounts (3.68%). Eight Phylum classifications dominated by *Firmicutes* (59.46%), *Proteobacteria* (33.01%), and unknown (6.16%), as shown in Table 2.2A and Figure 2.3. However, 29 Order and 38 Family classifications were dominated by *Bacillales* (57.29%), *Acetobacterales* (19.84%), Unknown (6.96%) and *Pseudomonadales* (4.98%); as well as *Sporolactobacillaceae* (57.06%), *Acetobacteraceae* (19.85%), Unknown (7.29%), and *Pseudomonadaceae* (4.95%).

Finally, 43 genera classifications and 18 species. The top 4 ranked are *Sporolactobacillus* (57.07%), *Acetobacter* (16.30%), Unknown (14.36%) and *Pseudomonas* (4.95%) as well as

Unknown (50.97%), *Sporolactobacillus putidus* (47.972%), *Niveibacterium beta proteobacterium* (0.74%), and *Pseudomonas spumella* (0.04%).

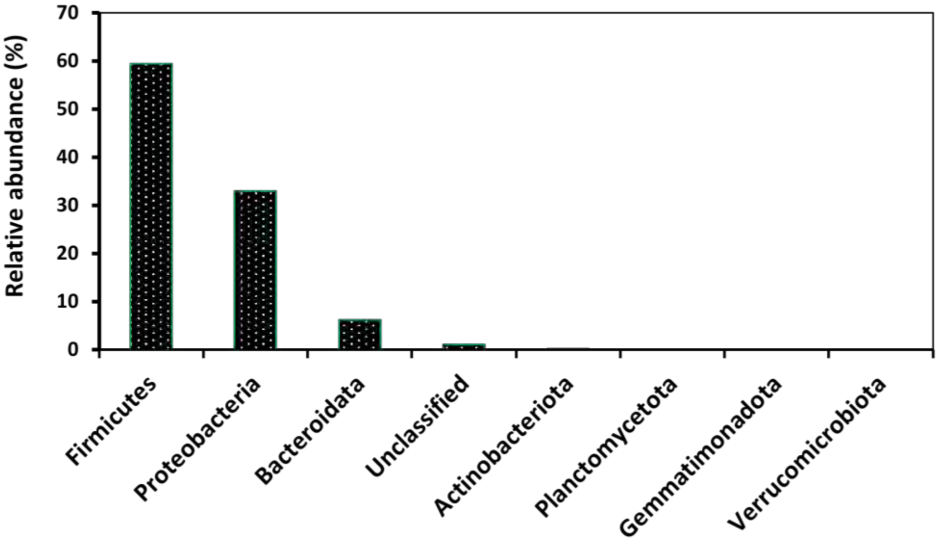


Figure 2. 3 Relative abundance of microbial biodiversity and community structure associated with LRC sample from a coal waste dump from the eMalahleni Coalfields. Phylum classification reports from Inqaba were compared using the Mothur database report. Results are expressed as a relative % of the total sequence of the coal microbial component.

After analysing the raw data from Inqaba Biotech using mothur database software and summarised in Table 2.2B and Figure 2.3, the sequencing result gives similar classifications. Considering microbial Phylum, 7 taxa, including *Firmicutes*, *Proteobacteria*, *Bacteriodata*, *Actinobacteriota*, *Planctomycetota*, *Gemmatimonadota* and *Verrucomicrobiota*, top the rankings in both cases, accounting for 93.85 to 99.50% of the sequenced microbial genomic component of the LRC sample. However, *Firmicutes* and *Proteobacteria* were the most representative organisms with a relative abundance of 92.47% (Inqaba report results) and 97.92% (mother-generated results).

2.3.4. Microbial Content

After performing a plate streaking procedure, five bacterial colonies were isolated based on their morphology. These colonies were assigned EBRU culture collection low-ranking coal bacteria (ECCN LB). They were labelled accordingly as ECCN LB-1, ECCN LB-2, ECCN LB-3, ECCN LB-4, and ECCN LB-5 (Table 2.3). However, all organisms had circular, smooth, opaque, and homogeneous appearances. Some of these gram-positive and catalase-positive bacterial isolates were observed as white colonies (ECCN LB-1, ECCN LB-3, and ECCN LB-4), and beige (ECCN LB-5), with colony diameters between 1 and 4 mm and raised elevations.

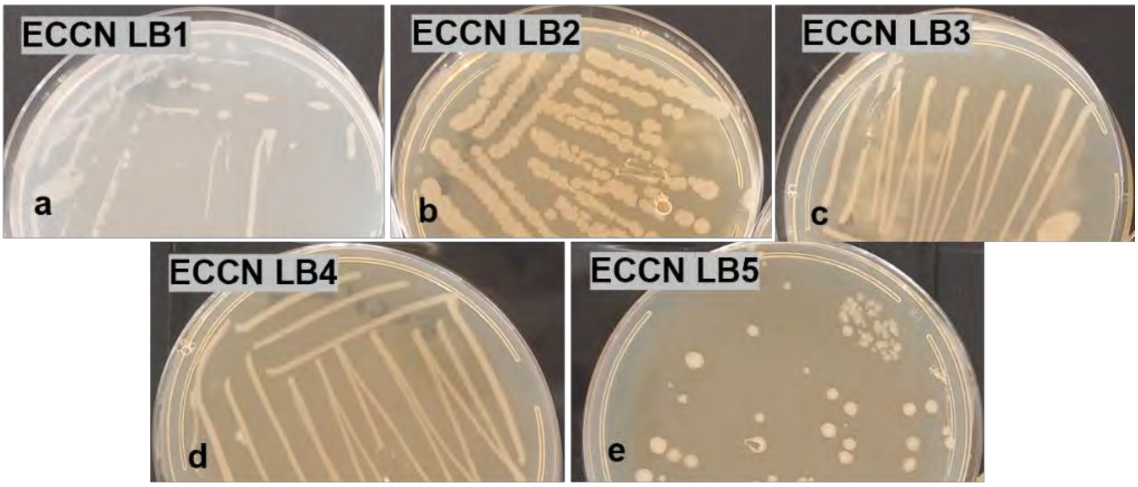


Figure 2. 4 Morphological characteristics of bacterial strains isolated from LRC samples. The organisms were cultured in NA and incubated for 3 d and at 30° C.

Isolates were also identified using molecular characterization with 16S rRNA and recorded from the genetic analyser. The BLAST analysis confirmed the taxonomic identity of each strain at the species level using the online database, *Margalitia camellia* being the taxonomic identity for ECCN LB-1, ECCN LB-3, and ECCN LB-4. However, ECCN LB-2 and ECCN LB-5 have been identified as *Priestia megaterium* and *Neobacillus cucumis*.

Table 2. 3 The molecular identity of bacterial strains isolated from WC samples sequenced by Inqaba Biotechnical Industries (Pty) Ltd, P.O. Box 14356, Hatfield 0028, Pretoria, South Africa. *Margalitia* sp*., strains* ECCN LB-1, ECCN LB-3, and ECCN LB-4, *Priestia* sp., strain ECCN LB-2 and *Neobacillus* sp., strain ECCN LB-5.

Strains Number	Source	Microbial Blast Prediction	Identity (%)	GenBank Accession Number	Length (bp)
ECCN LB-1	Thungela	*Margalitia camelliae*	96.36	NR_159341.1	1099
ECCN LB-2	Thungela	*Priestia megaterium*	99.17	NR_116873.1	961
ECCN LB-3	Thungela	*Margalitia camelliae*	98.01	NR_159341.1	1003
ECCN LB-4	Thungela	*Margalitia camelliae*	98.65	NR_159341.1	960
ECCN LB-5	Thungela	*Neobacillus cucumis*	99.53	NR_148626.1	850

Based on the information recorded in Table 2.3, the % identity of each microbial strain was 96.36% matched with *Margalitia camelliae* ECCN LB-1, 99.17% corresponded with *Priestia megaterium* ECCN LB-2, 98.01% matched with *Margalitia camelliae* ECCN LB-3, 98.65% corresponded with *Margalitia camelliae* ECCN LB-4 and 99.53% matched with *Neobacillus cucumis* ECCN LB-5. All these organisms belonging to the Phylum classification *Firmicutes* or *Bacillota* provided insight into the active microbial population interacting in the geologically WC ecological niches.

2.4 Summary

This chapter set up the physicochemical properties and biology of coal substrate. The results revealed how the LRC substrates showed an acidic pH of 3.60 with an important dissolved S background of 7130.43 mg L^{-1}, and some amounts of N (20 mg L^{-1}), P (7.8 mg L^{-1}) and K (3.3 mg L^{-1}). The EDX analysis revealed the concentration of carbon (C) at up to 23.09% and oxygen (O) at 69.03% taking up about 92.12% of the coal surface mineral composition.

The metagenomic analysis report of the coal samples showed the predominance of bacteria at 96.32%. However, eight phyla were at the top of the ranking but dominated by *Firmicutes* (59.46 to 62.18%) and *Proteobacteria* (33.01 to 35.74%). The microbial contents of the WC after performing a plate streak assay showed five bacterial isolates which were assigned EBRU culture collection low-ranking coal bacteria (ECCN LB). These organisms were molecularly identified as *Margalitia camellia* being the taxonomic identity of ECCN LB-1, ECCN LB-3 and ECCN LB-4. However, ECCN LB-2 and ECCN LB-5 were identified as *Priestia megaterium* and *Neobacillus cucumis*. This work revealed how geological WCs remain a valuable microbial/ecological niche, as they provided insight into active microbial interaction that can be explored biologically as a source of soil compound amendments and plant nutrients for sustainable agricultural practices.

Chapter Three: Biological Properties of Candidate Bacterial Isolates for Biofertiliser Preparations

3.1 Introduction

Microorganisms have many advantages, including degradation of xenobiotic compounds, bioremediation, soil restoration, and biofertilisation that promotes plant growth and clean crop production (Wang et al. 2022; Sekhohola et al. 2022). Bacterial isolates that possess plant growth-promoting (PGP) activity may provide an additional agroecological advantage that might be expected to impact food security and sustainable agriculture (Cowan et al. 2016; Sekhohola and Cowan 2017; Sekhohola et al. 2014; 2022; Van Breugel et al. 2019; Titilawo et al. 2020). These soil-associated organisms play a major role in the recycling of nutrients (N, P, K), organic matter decomposition, processes related to biofertilisation (Naranjo-Ortiz and Gabald T 2019; Odoh et al. 2020), and restoration of degraded environmental sites (Igbinigie et al. 2008; Brodt et al. 2011; Jacoby et al. 2017; Olawale et al. 2020). As microbial isolates have significant biodiversity activities among living organisms (Luciana et al. 2019), there is a global need to find methods and techniques to harness these potentials for the multiple benefits of communities namely, land restoration, and increased crop production (Sekhohola-Dlamini et al. 2022).

Previous studies have discovered and continue to isolate new taxa of microbial strains with biological potentials that improve soil fertility, plant health and development, induce plant tolerance to biotic and abiotic stresses and restore land degraded by natural disasters or human impacts related to mining activities and other direct and indirect mechanisms (Sharma and Shouche 2014; Gull et al. 2019; Olawale et al. 2020; Díaz-Rodríguez et al. 2021). Considerable potential has been identified by academic and commercial players in the bioconversion of coal as the sole source of carbon. This microbial conversion of low-rank coal (LRC) and other coal residues like waste bituminous coal (BC) into value-added products with new potential for use in promoting plant growth and development (Titilawo et al. 2020; Olawale et al. 2020; Keshinro 2021; Sekhohola-Dlamini et al. 2022) is critically necessary and need to be explored.

Therefore, characterisation and exploration of the potential of these beneficial organisms and their storage as microbial culture collections (MCC), for future use, are expected to become crucial to maintaining their bioactivity or future biotechnological research related to land rehabilitation/restoration and sustainable agriculture (Sharma and Shouche 2014). These organisms can be isolated from any ecological sample, region, or country; however, most of the world's microbial genetic resources come from developed/developing countries, such as the United States, Europe, Japan, and China. Minimal capacity exists in low and middle-income countries, which are often rich in biodiversity but lack resources for conservation and exploitation (Whitman et al. 1998; Ryan et al. 2019).

This study aimed to ensure the viability and biological properties of these 22 bacteria as microbial culture collections (MCC) comprising ten isolated strains from diesel-contaminated soil (DCS) and coal sludge (CS) and possessing coal degradation activity. The twelve new PGP bacteria from the high-rate algal oxidation pond (HRAOP) of the integrated algal pond system (IAPS) treating Makhanda municipal wastewater. Together, these MCC represent the microbial biocatalysts that underpin Fungcoal as a land reclamation bioprocess technology. Morphology, molecular properties, and phylogenetic relationship of all PGP bacteria were studied to achieve the objectives of this study. However, tolerance to abiotic stress, pH, salt, and temperature, has been examined. Finally, four carbohydrates (fructose, glucose, sucrose, and mannitol), one monosodium salt (sodium L-glutamate), and two geological coal substrates (LRC and BC) were selected and used to assess the preferred carbon source for each isolate.

3.2 Material and Methods

3.2.1 Bacterial Isolates

The bacterial isolates used in this study consisted of 22 strains referred to as EBRU Culture Collection Numbers (ECCN b). The ECCN strains included ten isolates and partially classified as coal-degrading (CD) bacteria and 12 isolates and partially characterised as plant growth-promoting bacteria (PGPB). Some of the CD bacteria were isolated from coal mines (CM)/coal slurry (CS) (*Citrobacter* sp., ECCN 19b, *Escherichia* sp., ECCN 25b and *Bacillus* sp., ECCN 26b) obtained from coal discard dumps (Greenside Colliery, Klippan Dump, and Kromdraai Opencast), Mpumalanga, others from slurries material of the diesel-contaminated sites (DCS) (*Bacillus* spp., ECCN 18b and ECCN 41b, *Proteus* spp., ECCN 20b and ECCN 23b, *Exiguobacterium* sp., ECCN 21b, *Microbacterium* sp., ECCN 22b, and *Serratia* sp., ECCN 24b) collected from the soil at mechanical workshops and old tractor engines on a farm in the Makana Local Municipality, Eastern Cape (Olawale et al. 2020).

Putative PGPB (ECCN 1b to ECCN 12b) identified as *Bacillus* sp. (ECCN 1b and ECCN 3b), *Fictibacillus* sp. (ECCN 2b), *Aeromonas* sp. (ECCN 4b), *Exiguobacterium* spp. (ECCN 5b and ECCN 8b), *Arthrobacter* sp. (ECCN 6b), *Enterobacter* sp. (ECCN 7b), *Microbacterium* spp. (ECCN 9b and ECCN 12b), *Pseudomonas* sp. (ECCN 10b), and *Ancylobacter* sp. (ECCN 11b) were isolated from the high-rate algal oxidation pond (HRAOP) of the integrated algal pond system for Municipal Wastewater Treatment (WWT) located in Makhanda, Eastern Cape. The details of the molecular characterisation of these isolated bacteria are provided in Table 3.1.

Table 3. 1 Details of ECCN bacteria sourced either from CS, DCS or from HRAOP of the algae-based WWT process. Where indicated, strains are on deposit at the Microbial Culture Collection (MCC), Maharashtra, India (Modified from Masudi 2019; Olawale et al.2017; Titilawo et al. 2020; Keshinro 2021).

Strains Number	Source	Microbial Blast Prediction	Identity (%)	GenBank Accession Number	Length (bp)	Microbial Culture Collection
CS/DCS bacteria						
ECCN 19b	CS	*Citrobacter sp.*	99	KC700328	372	MCC0033
ECCN 25b	CS	*Escherichia sp.*	99	KC700329	382	MCC0041
ECCN 26b	CS	*Bacillus sp.*	99	KC700330	450	MCC0062
ECCN 18b	DCS	*Bacillus sp.*	98	KC620473	560	MCC0034
ECCN 20b	DCS	*Proteus sp.*	94	KC620475	553	MCC0027
ECCN 21b	DCS	*Exiguobacterium sp.*	99	KC620476	551	MCC0016
ECCN 22b	DCS	*Microbacterium sp.*	99	KC620477	543	MCC0042
ECCN 23b	DCS	*Proteus sp.*	91	KC620478	439	MCC0022
ECCN 24b	DCS	*Serratia sp.*	99	KC620474	525	MCC0021
ECCN 41b	DCS	*Bacillus sp.*	99	KC758162	392	MCC0039
HRAOP bacteria						
ECCN 1b	WWT	*Bacillus sp.*	99	MW672568	1420	No deposit
ECCN 2b	WWT	*Fictibacillus sp.*	100	MW672569	1471	No deposit
ECCN 3b	WWT	*Bacillus sp.*	100	MW672570	1510	No deposit
ECCN 4b	WWT	*Aeromonas sp.*	99	MW672571	1533	No deposit
ECCN 5b	WWT	*Exiguobacterium sp.*	99	MW672573	1529	No deposit
ECCN 6b	WWT	*Arthrobacter sp.*	100	MW672575	1522	No deposit
ECCN 7b	WWT	*Enterobacter sp.*	99	MW672577	1389	No deposit
ECCN 8b	WWT	*Exiguobacterium sp.*	99	MW672579	1504	No deposit
ECCN 9b	WWT	*Microbacterium sp.*	98	MW672580	1488	No deposit
ECCN 10b	WWT	*Pseudomonas sp.*	100	MW672582	1547	No deposit
ECCN 11b	WWT	*Ancylobacter sp.*	100	MW672584	1428	No deposit
ECCN 12b	WWT	*Microbacterium sp.*	91	MW672585	1486	No deposit

3.2.2 Cultivation and Morphological Properties of Bacterial Isolates

3.2.2.1 Cultivation, Morphology, and Inoculum Preparation

The microbial isolates were previously stored in a glycerol medium at -80 °C. From the glycerol stock. isolates were seeded on a modified nutrient broth (NB) containing per litre of distilled water, 5 g of peptone (uniLAB, Merck, RSA), 3 g of yeast extract (Biolab, Merck, RSA), and 8 of NaCl (uniVAR, Merck, RSA) and incubated at 30 °C, 120 rpm for 3 d. To check bacterial purity, isolates were subcultured on nutrient agar (NA) for 3 d. Then, after confirming microbial purity, some plates were stored at 4 °C and served as seed cultures while others have been used for the study of morphological properties.

The morphological properties of colonies consisted of macroscopic (pigmentation, form, margin, etc.) (Sousa et al. 2013) and microscopic observations. Therefore, photos were taken directly from the freshly cultured agar plates. For the preparation of liquid inoculant, a portion of pure bacterial colony was transferred into a 40 mL NB. Cultures were allowed to grow at 30 °C, 120 rpm for 3 d. The bacterial inoculum concentration of each seed culture in the form of colony-forming units (CFU) was maintained at approximately 10^9 mL^{-1} (Sieuwerts et al. 2008 and Sanders 2012). Then, 10 mL aliquots were kept in a 10 mL test tube, labelled, and stored at 4 °C.

3.2.2.2 Scanning Electron Microscopy

Twenty-two bacterial isolates were cultured in NB for scanning electron microscope (SEM) analysis. From each freshly grown culture, 1 mL was transferred to 2 mL microtubes (SSIBio, 1310-00, 23 USA) and centrifuged at 3920xg for 5 min using a Costar mini centrifuge (10 MVSS-07343, USA) in 316 EBRU laboratory. The resulting pellets were washed three times in phosphate-buffered saline (PBS) and then transported to the Electron Microscopy Unit (EMU), at Rhodes University for SEM analysis. At the EMU laboratory, samples were placed overnight in 2.5% glutaraldehyde fixative solution at 4°C. The samples were immersed twice in 0.1 M sodium phosphate buffer for 10 min; then, the supernatant was discarded. Dehydration was followed by washing the pallets with 30, 50, 70, 80, 90%, and 100% ethanol. The samples were suspended in absolute ethanol and coated with evaporated carbon (CO_2) using the critical point dryer for 2 h. With the dry point complete, the samples were mounted on stubs with double-sided adhesive tapes and gold-coated (Quorum Q15ORS, England). The SEM views were carried out on an analytical Tescan Vega 2 (Figure 2.3).

3.2.3 Molecular Characterisation

Since the molecular identification of CS and DCS bacterial strains had previously been carried out (Olawale et al. 2020; Titilawo et al. 2020), the present study focused only on the twelve new bacterial isolates from HRAOP. However, Inqaba Biotechnical Industries (Pty) Ltd P.O. Box 14356, Hatfield 0028, South Africa, was selected to do this molecular analysis. Freshly grown NA plates containing pure colonies were subjected to 16S rDNA sequencing analysis. Genomic DNA extraction was performed using the Quick-DNATM Fungal/Bacterial Miniprep kit (Zymo Research, Catalogue No. D6005). Amplification of the 16S target region (Lane et al.

1991; Turner et al. 1999) was performed using the OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the universal primers:

(a) 16S-27F 5’-AGAGTTTGATCMTGGCTCAG-3’and,

(b) 16S-1492R 5’-CGGTTACCTTGTTACGACTT-3’. The PCR products were run on a gel and a gel extracted with the Zymoclean™ DNA Gel Recovery Kit (Zimo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse directions (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRB3- 100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500XL Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

A CLC Bio Main Workbench v7.6 was used to analyse the ab1 files generated by ABI 3500XL Genetic Analyser and results as the accession numbers and the closest taxonomic designation assigned were obtained by a BLAST search (http://blast.ncbi.nlm.nih.gov/).

3.2.4 Phylogenetic Analysis

Previous studies already carried out phylogenetic analyses of coal-degrading bacterial strains (Titilawo et al. 2020); however, it seemed meaningful to carry out the phylogenetic analysis of the novel potential PGPB. The phylogenetic relatedness of WWT bacteria to known PGP rhizobacteria was determined using MEGA version 6. The 34 reference nucleotide sequences were sourced from the NCBI GenBank database, and multiple alignments were deduced using the Neighbour-Joining method. The trees in which associated taxa clustered were bootstraps of 1000 replications, and the distances were calculated using the Jukes-Cantor method (Tamura et al. 2013).

3.2.5 Abiotic Stress Tolerance Potential

Extreme environmental conditions limit the metabolism of soil microbiota controlling symbiotic interactions involved in plant growth and development (Shrivastava and Kumar 2015; Lata et al. 2018). However, knowing whether the microorganisms can cope with environmental stresses and develop tolerances to extreme conditions is imperative before being used as biofertilisers (Shukla et al. 2012; Lata and Gond 2019). Therefore, the abiotic stress tolerances assessed in this work included pH, salinity, and temperature.

3.2.5.1 pH Tolerances

A modified NB containing per litre of distilled water, 5 g of peptone (uniLAB, Merck), 3 g of yeast extract (Biolab, Merck, RSA) and 8 g of NaCl (uniVAR, Merck) was prepared. Incubation was fixed for 3 d, at 30 °C and 120 rpm. The pH range between 3 and 11 was adjusted using NaOH, 5 M and HCl, 35% and a portable pH meter, HANNA Instruments (HI 8314). Bacterial inoculant (40 µL) was added to each 40 mL of autoclaved NB. Twelve Erlenmeyer flasks were prepared for the novel algae-based WWT organisms while ten were for CS/DCS bacteria, both in triplicate. Uninoculated flasks were used as controls. After incubation, 1 mL of freshly grown culture was transferred to a cuvette, and optical density was measured at 600 nm (Shimadzu UV-1280, Japan).

3.2.5.2 Salt and Temperature Tolerances

Bacterial inoculants were added to 40 mL NB medium with or without NaCl (0 to 1.2 M). Incubation was performed on a rotary shaker for 3 d at 30 °C and 120 rpm. After incubation, 1 mL of freshly grown culture was transferred to a spectrophotometer cuvette (Shimadzu UV-1280, Japan), and the OD_{600} was measured. The same protocol was used for temperature stress tolerances. However, the temperature was set between 4 and 45 °C.

3.2.6 Carbon Substrate Utilisation

3.2.6.1 Carbohydrate Substrate

Exploring information on microbial metabolisms regarding the carbonaceous substrate in an ecosystem is essential for understanding microbial environmental impacts (Preston-Mafham et al. 2002; Lundgren et al. 2021; Suzuki et al. 2022). In this study, five available carbohydrates, including fructose (Sigma-Aldrich, USA), glucose (Sigma-Aldrich, Macedonia), sucrose (Sigma-Aldrich, Germany), mannitol (Saarchem, Merck, RSA), and sodium L-glutamate (Sigma-Aldrich, Switzerland) (Preston-Mafham et al. 2002; Lundgren et al. 2021), were selected to study how these compounds could affect the growth or metabolic activity of bacterial isolates.

The NB was used as a bacterial culture medium, and pH was set at 7.35 before autoclaving. Aseptically, 25 g L^{-1} of each carbon substrate was added to the prepared NB and stirred until completely dissolved. About 6 mL broth was aseptically transferred into a 20 mL bottle, inoculated, and incubated at 30 °C, 120 rpm for 3 d. The carbon substrates' utilisation capacities were assessed by monitoring growth as optical density (OD_{600}).

3.2.6.2 Coal Substrate

The solid screening assay was selected as the best way to study the potential of bacterial isolates to utilise geological coal substrates as carbon sources. Two types of coal, including low-rank coal (LRC) (Thungela Ltd, RSA) and HRC or bituminous coal (BC) (Landau Colliery, RSA) were selected in terms of assessing how these carbonaceous compounds could affect bacterial growth using yeast coal agar (YECA) protocol. Yeast coal agar (YECA) was preferred and prepared following the purchased yeast mannitol (YEMA) agar protocol (*Himedia* M715) with the substitution of mannitol by the same amount (10 g L^{-1}) of LRC or BC. The YECA ingredient composition per litre of Milli-Q water was 1 g yeast extract (Biolab, Merck, RSA), 10 g coal, 0.5 g K_2HPO_4 (Sigma-Aldrich, USA), 0 .2 g of $MgSO_4$ (Unilab, Saarchem, RSA), 0.1 g NaCl (Sigma-Aldrich, RSA), 1 g $CaCO_3$ (Pal Chemicals) and 15 g agar (Biolab, Merck, RSA) then autoclaved.

Some bacterial isolates such as *Bacillus indicus* strain ECCN 1b, *Fictibacillus* sp., strain ECCN 2b, *Exiguobacterium* spp., strains ECCN 5b and 8b, and *Microbacterium* spp., strains ECCN 9b and 12b appeared sensitive to long storage conditions. Plates were then inoculated using old pure seed culture to determine whether coal substrates could affect the rapid dormancy breaking (Aanderud et al. 2016). Then, a series of non-overlapping parallel streaks were made on the agar and incubated at 30°C for 5 d. Growth proliferation was considered as the ability of bacterial isolates to utilise LRC and BC as carbon sources. Yeast mannitol agar suitable for the cultivation of soil microorganisms and PGPB such as Rhizobium species (Purwaningsih et al. 2021) was preferred and prepared to serve as a positive control.

3.3 Data and Statistical Analysis

All quantitative data were analysed in Excel (Version 16, Microsoft Corporation, Redmond, WA). However, the results were analysed using a one-way analysis of variance (ANOVA). Significant differences between measures for treatments were determined at $p < 0.05$. Data are presented as the mean of at least 3 determinations ± standard error (SE).

3.3 Results

3.3.1 Morphological Properties

3.3.1.1 Colony Morphology

As illustrated in Figure 3.1, the viability of all organisms comprising CS (a-c) and DCS (d-j) as well as the novel HRAOP (k-v) strains, has been secured. Morphological characteristics (pigmentation, shape, margin, etc.) were apparently with differences.

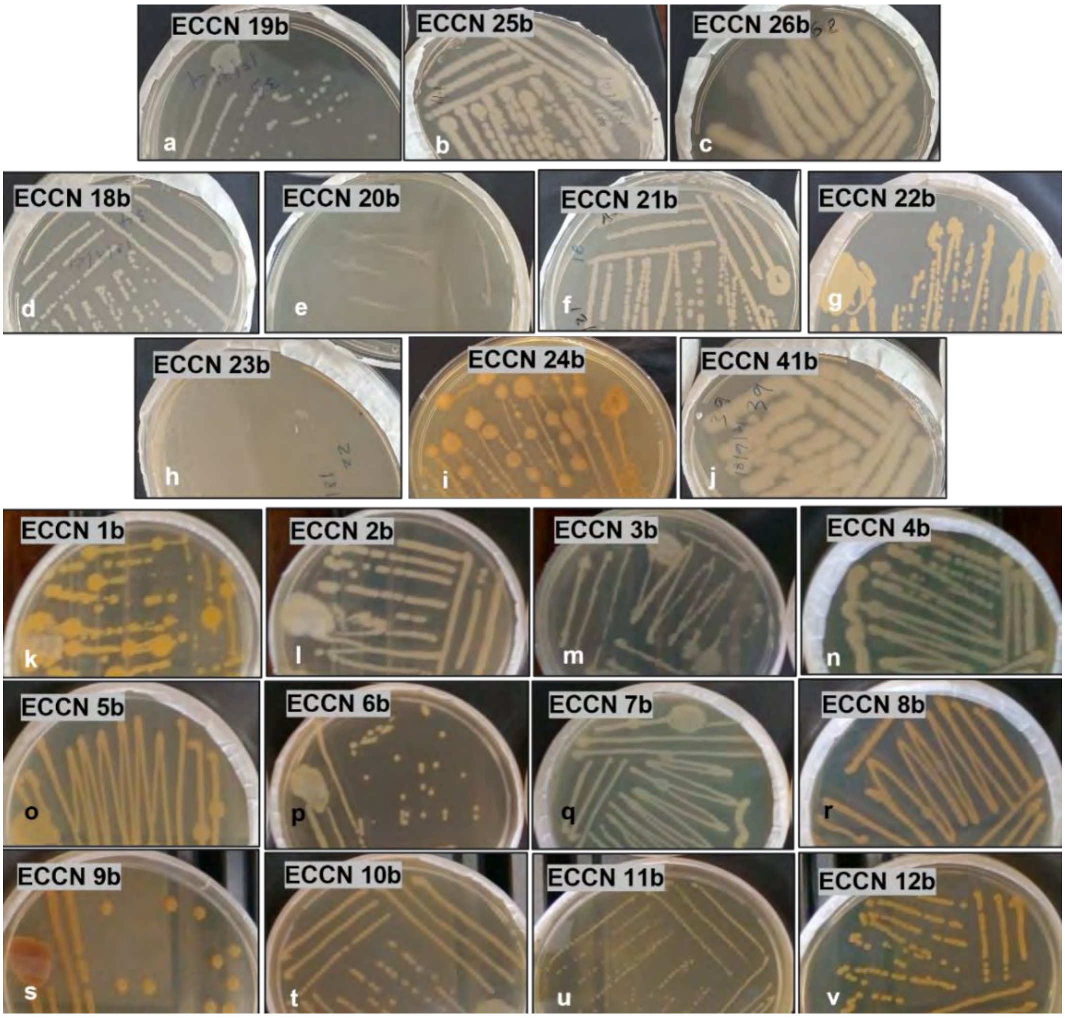


Figure 3. 1 Cultivation of 22 bacterial biocatalysts demonstrating strains purity and morphology. Some organisms were isolated from CS (ECCN 19b, 25b and 26b) and DCS (ECCN 18b, 20b,21b, 22b, 23b, 24b and 41b). Others (ECCN 1b to 12b) were generated from the HRAOP of an algal-based WWT process. They were previously identified as *Exiguobacterium* spp. (ECCN 21b, 5b and 8b), *Serratia* sp. (24b), *Proteus* spp. (ECCN 23b and 20b), *Citrobacter* sp. (ECCN 19b), *Bacillus* spp. (ECCN 18b, 41b, 26b, 1b, 2b and 3b), *Escherichia* sp. (ECCN 25b), *Microbacterium* spp. (ECCN 22b, 9b and 12b), *Aeromonas* sp. (4b), *Arthrobacter* sp. (ECCN 6b), *Enterobacter* sp. (ECCN 7b), *Pseudomonas* sp. (ECCN 10b) *Ancylobacter* sp. (ECCN 11b).

The colony diameter varied from 1 mm (*Ancylobacter* sp., strain ECCN 11b) (Figure 3.1u) to 3.5 mm (*Bacillus* spp. strains ECCN 1b and ECCN 41b) (Figure 3.1k, j). Bacterial colonies were circular, smooth, raised, and opaque. The orange, yellow or brown pigments were observed in *Bacillus indicus* (ECCN 1b), *Exiguobacterium* strains (ECCN 5b and ECCN 8b) and *Microbacterium* strains (ECCN 9 b, ECCN 12b and ECCN 22b). Undulating margins around the colonies were found in some *Bacillus* strains (ECCN 3b, ECCN 18b, ECCN 26b and ECCN 41b).

3.3.1.2 Scanning Electron Microscopy

The SEM micrographs revealed how the organisms were not the same size even though they could all be rod-shaped. *Bacillus* sp., ECCN 1b as well as *Fictibacillus* sp., ECCN 2b, appeared as long and multi-chain rod-shaped cells, , *Aeromonas* sp., ECCN 4b, and *Proteus* sp., ECCN 20b were small rod-shaped, while *Ancylobacter* sp., ECCN 11b small rod-shaped curved cells (Figure 3.2u).

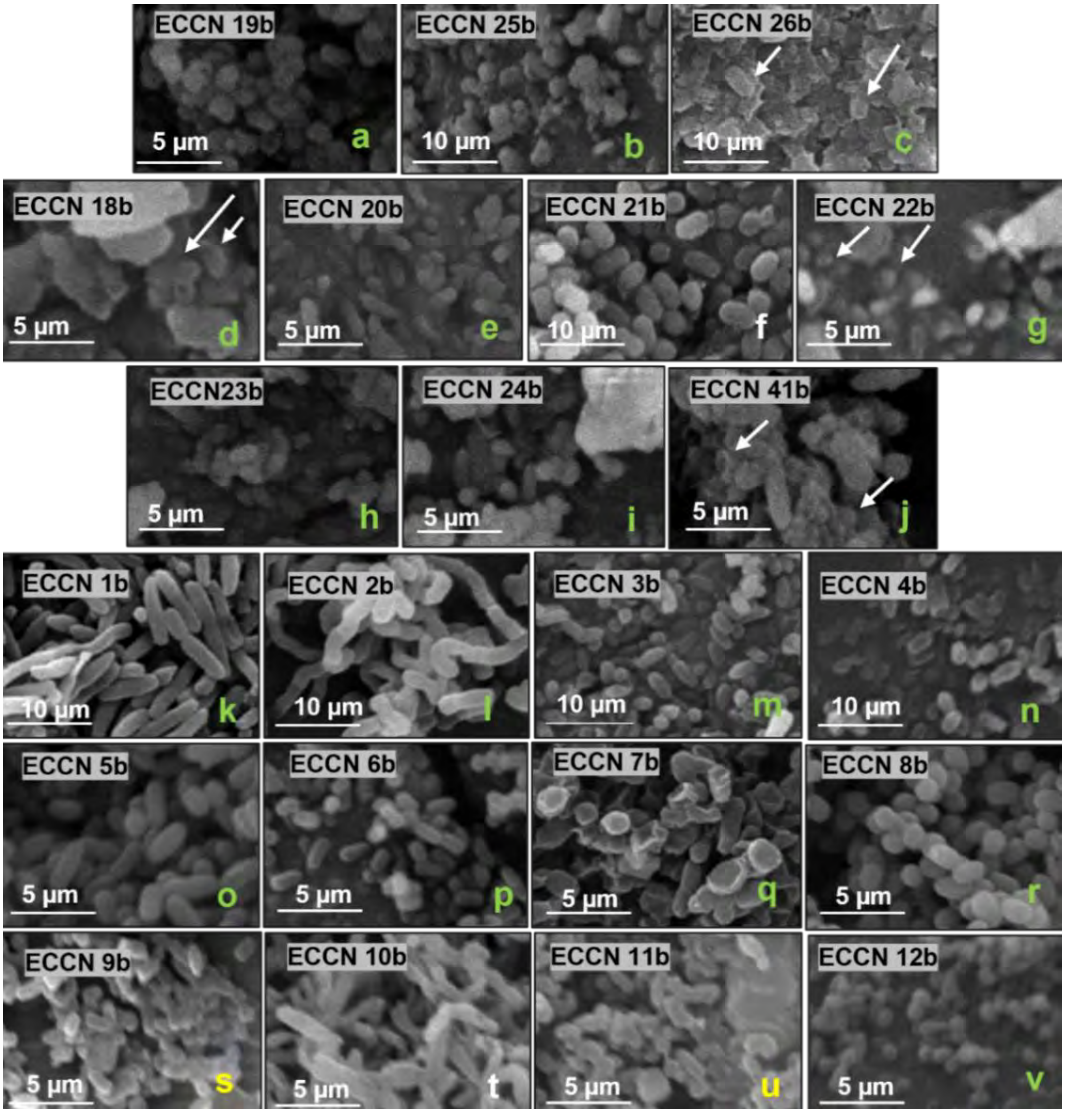


Figure 3. 2 Scanning electron micrographs showing cell morphology of 22 bacterial isolates. Organisms were viewed from an analytical Tescan Vega 2 (LMU -TPD 011, Germany).

3.3.2 Confirmation of Molecular Identity

The molecular identification of the 12 bacterial isolates promoting plant growth and isolates of HRAOP of the IAPS treating Makhanda-Municipal wastewater was carried out. Partial 16S rRNA gene sequences from each isolate, ranging from 1389 to 1547 bp, were amplified and taxonomic identity was determined following the BLAST analysis, as seen in Table 3.1. From the twelve bacterial strain samples, nine taxonomic genera comprising seven families were formed; however, *Bacilliaceae* (41.67%) and *Microbacteriaceae* (16.67%) dominated.

Results of sequence similarities showed that five isolates referred to as *Ancylobacter* sp., strain ECCN 11b (MW672584), *Arthrobacter* sp., strain ECCN 6b (MW672569), *Bacillus* sp., strain ECCN 3b (MW672570), *Fictibacillus* sp., strain ECCN 2b (MW672569) and *Pseudomonas* sp., strain ECCN 10b (MW672582) had 100% sequence homology to reference strains.

Five other isolates, comprising *Aeromonas* sp., strain ECCN 4b (MW672571), *Bacillus* sp., strain ECCN 279 1b (MW672568), *Enterobacter* sp., strain ECCN 7b (MW672577), and two *Exiguobacterium* spp., strains ECCN 5b (MW672573) and ECCN 8b (MW672579) showed 99% sequence homology to reference strains. The remained *Microbacterium* spp., strains ECCN 9b (MW672580) and ECCN 12b (MW672585) showed 98% and 91% homology respectively. However, *Bacillus* was the most represented genus accounting for 41.67%, followed by *Microbacterium,* 16.67%. *Aeromonas* sp., *Ancylobacter* sp., *Arthrobacter* sp., *Enterobacter* sp., and *Pseudomonas* sp. accounted for 8.33%.

3.3.3 Phylogenetic Relatedness to PGPB

Twelve novel bacterial biocatalysts from HRAOP were identified by PCR amplification and 16S rRNA gene sequence analysis. They were characterised after phylogenetic analysis of the sequencing relationship with the known PGP bacteria reference strains deposited in the NCBI GenBank database (Figure 3.3). Using the 34 NCBI GenBank-generated reference nucleotide sequences for PGPB, strains were grouped into eight distinct, but related clusters assigned as; *Bacillus* (A); *Exiguobacterium* (B); *Arthrobacter* (C), *Microbacterium* (D); *Ancylobacter* (E); *Aeromonas* (F); *Enterobacter* (G) and *Pseudomonas* (H). However, 15 sequences were grouped individually with respective PGPB relatives and showed 100% bootstrap support at the corresponding master nodes.

The clusters showed all high bootstrap values and many PGPB references from the GenBank database. Only one cluster, containing *Pseudomonas* sp., strain ECCN 10b (MW672582), was outside of the tree and shared significant similarity (100%) with *Pseudomonas fluorescens* (CP015638) as a reference GenBank PGPB sequenced strain.

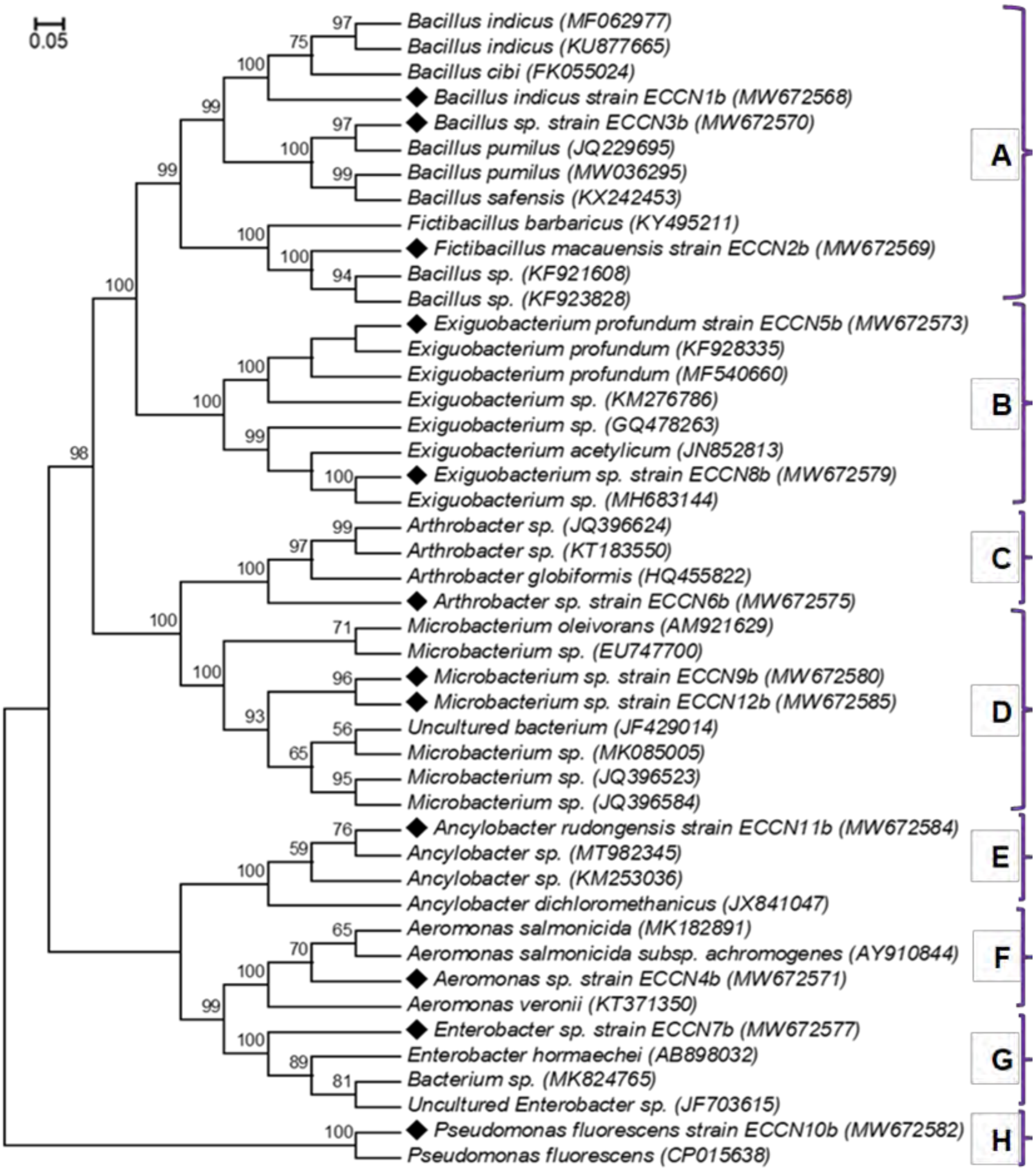


Figure 3. 3 Dendrogram showing the genetic relationship between the twelve potential PGPR strains (♦) isolated from MaB-flocs generated in HRAOP and reference strains contained in the GenBank database. Assigned strains and GenBank accession numbers are located to the right of each strain. Bootstrap test (1000 replicates) above branches, bootstrap values ≥56% at nodes and bar, substituting 5 nucleotides per 100.

3.3.4 Abiotic Stress Tolerances

3.3.4.1 pH Tolerances

The pH stress tolerance of all 22 bacterial strains was assessed by monitoring growth as a change in optical density at 600nm using NB with a fixed pH range from 3 to 11. Then the results showed that out of the 22 bacterial strains screened, 7 (31.82%) CS/DCS and 3 (13.64%) HRAOP organisms were found to be excellent pH-tolerant bacteria, showing great growth in both acidic and alkaline environments (Figure 3.4a-d, f).

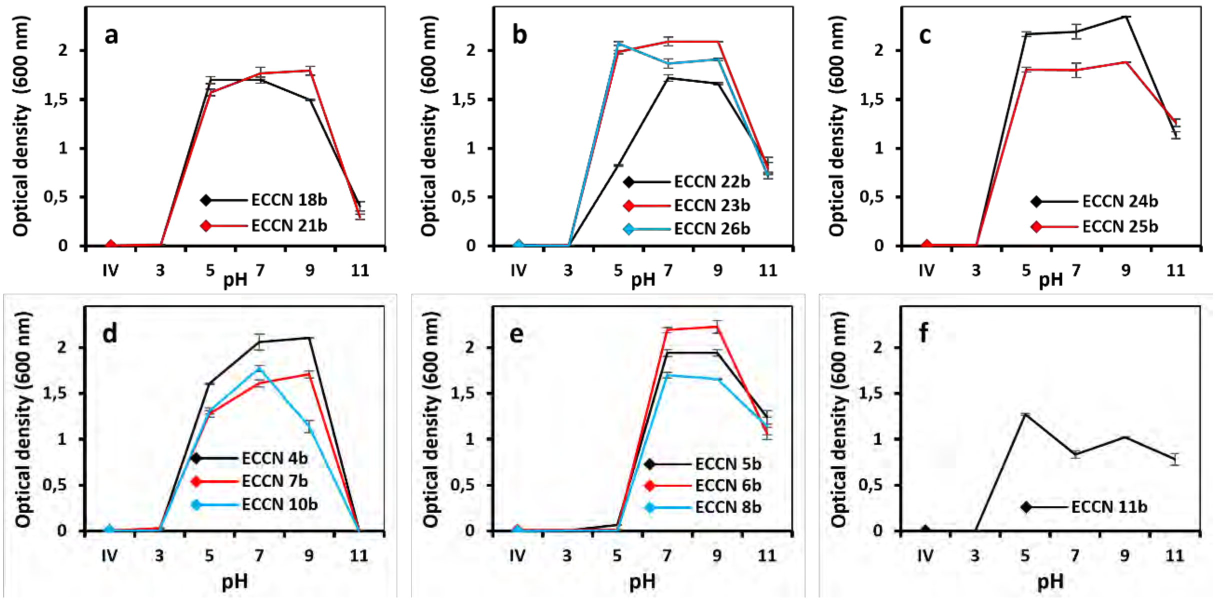


Figure 3. 4 pH stress tolerances of CS/DCS (a-c) and HRAOP (d-f) bacteria. Organisms were cultured in NB with pH values ranging from 3 to 11 at 30 °C and 120 rpm for 3 d. Culture density was determined at OD_{600} and results are expressed as the mean and standard error (SE) of at least 3 replicates.

The growth curves were optimal between pH 5 and 10 and much better at a pH around 7 (OD_{600} 1.854 for CS/DCS and 1.729 HRAOP bacteria). Of the 10 high-performing isolates related to pH stress tolerances, 7 (31.82%), namely *Serratia* strain ECCN 24b*, Proteus* strain ECCN 23b, *Escherichia* strain ECCN 25b, *Bacillus* strain ECCN 26b, *Exiguobacterium* strain ECCN 21b, *Aeromonas* strains ECCN 4b and *Enterobacter* strain ECCN 7b was ranked as the most effective with OD_{600} range between 1.311 and 0.908. These results showed the effectiveness of organisms from waste or polluted environments in tolerating extreme pH. As a result, they can be used in practices related to recycling and bioremediation of highly polluted acidic or alkaline environments.

3.3.4.2 Salt and Temperature Tolerances

Twenty-two bacteria biocatalysts were screened for abiotic stress tolerance using NB supplemented with or without NaCl in range of 0.1 to 1.2 M and at temperatures in the range 4 to 45 °C. For temperature (T) tolerance determination, cultures in NB were exposed to increasing temperatures from 4 to 45 °C (Figures 3.5 and 3.6). Salt tolerance curves showed that 17 bacterial biocatalysts, 7 (31.82%) from CS/DCS and 10 (45.46%) from HRAOP could tolerate low salinity estimated at 0.1 M. However, 12 organisms including 7 CS/DCS and 5 WWT could tolerate high salinity up to 0.7 M. Therefore, 10 (45.46%) bacteria showed salt tolerance up to 1M. Finally, 5 (23.81%) strains comprising of *Proteus* strains ECCN 20b and ECCN 23b (OD_{600} 1.368 and 1.273), *Enterobacter* strain ECCN 7b, *Ancylobacter* strain ECCN

11b and *Exiguobacterium* strain ECCN 8b (OD_{600} 0.631, 0.617 and 0.542), tolerated the maximum salinity stress of 1.2 M (70.13-72.00 g L^{-1}) (Figure 3.5a, d). NaCl concentration around 0.1 M (6-8 g L^{-1}) showed the best growth rate with a maximum OD_{600} of 1.748 for CS/DCSM and 1.744 for WWTM.

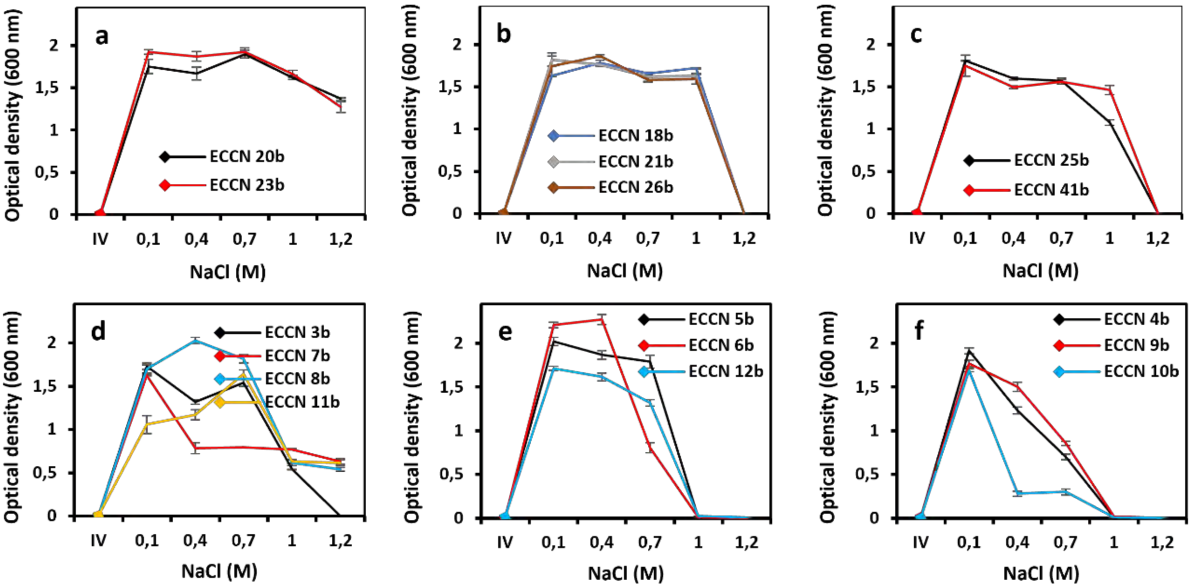


Figure 3. 5 Saline stress tolerances of CS/DCS (a-c) and HRAOP (d-f) bacteria. Organisms were grown in NB with NaCl concentrations ranging from 0.1 to 1.2 M at 30 °C, 120 rpm for 3 d. Culture density was measured at OD_{600} and results are expressed as the mean and standard error (SE) of at least 3 replicates.

Regarding the temperature stress tolerance, 5 bacterial isolates comprising 2 from DCS (*Exiguobacterium* strain ECCN 21b and *Proteus* strain ECCN 23b) and 3 from HRAOP (*Bacillus* strain ECCN 3b, *Exiguobacterium* strain ECCN 8b and *Ancylobacter* strain ECCN 11b) bacteria showed high-temperature tolerance potential up to 45 °C (Figure 3.6 a, d).

However, the growth parameter monitored as the change in optical density at 600 nm (OD_{600}) for each of the five salt-tolerant organisms was spectrophotometrically measured as 0.749 for *Proteus* strain ECCN 23b, 0.535 for *Exiguobacterium* strain ECCN 21b, 0.431 for *Bacillus* strain ECCN 3b, 0.378for *Ancylobacter* strain ECCN 11b and 0.335 for *Exiguobacterium* strain ECCN 8b. In both cases, the temperature of 30 °C was the best in terms of the growth rate measured as the change in optical density at OD_{600} (1.863, CS/DCS and 1.767, WWTM).

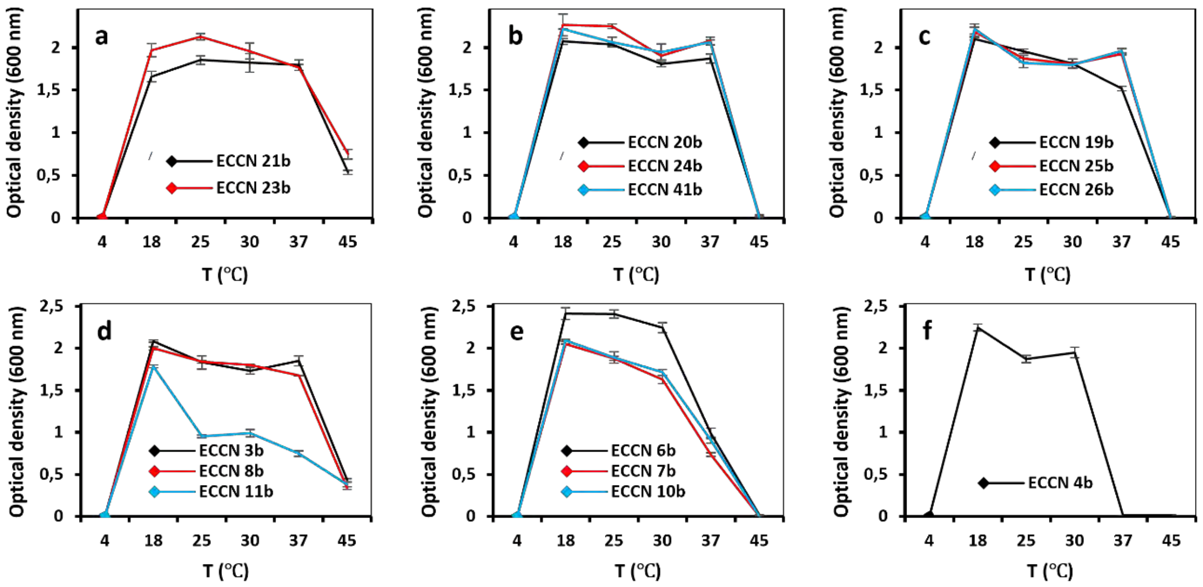


Figure 3. 6 Temperature stress tolerances of CS/DCS (a-c) and HRAOP (d-f) bacteria. Organisms were cultured in NB at temperatures ranging from 4 to 45 °C, 120 rpm for 3d. Culture density was measured at OD_{600}. Results are expressed as the mean and standard error (SE) of at least 3 replicates.

3.3.5 Carbonaceous Substrates

3.3.5.1 Carbohydrates

Twenty-two organisms including ten CS/DCS and 12 algae-based WWT isolated bacterial strains were screened for metabolic response activities regarding carbon substrate utilisation.

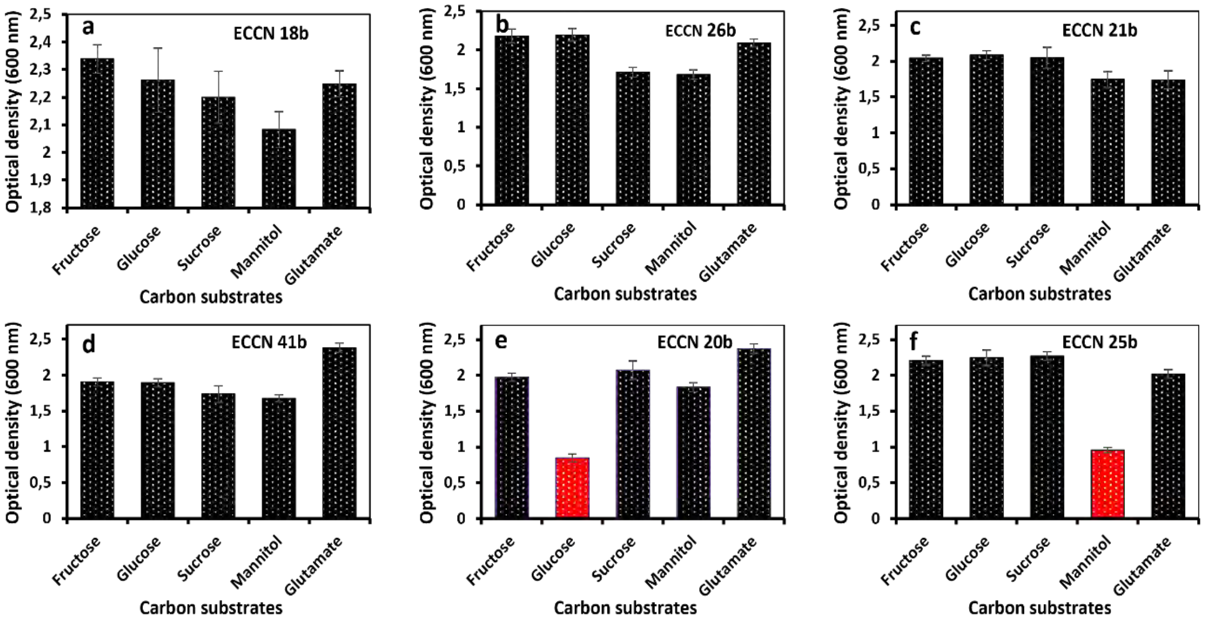


Figure 3. 7 Use of carbonaceous substrates by CS/DCS bacteria. Organisms were cultured in NB supplemented with different carbohydrates (25 g L^{-1}) at 30 °C, 120 rpm for 3 d. Culture density was measured at OD_{600}. Results are expressed as the mean and standard error (SE) of at least 3 replicates.

The results showed that 6 CS/DCS organisms (27.27%) effectively preferred all 5 carbon substrates, while 4 (18.18%) could use at least 4 carbon substrates (Figure 3.7e, f). Of the 6 organisms that could use both carbonaceous substrates, 4 isolates namely *Bacillus* strain ECCN 18b (2.226), *Bacillus* strain ECCN 26b (1.970), (*Exiguobacterium* strain ECCN 21b (1.931), and *Bacillus* strain ECCN 41b (1.918) were the best performed (Figure 3.7. a-d). Considering bacterial preferences on carbon substrates, sodium L-glutamate was the best carbon source (2.157), followed by fructose (2.095), sucrose (2.075), mannitol (1.822), and glucose (1.709).

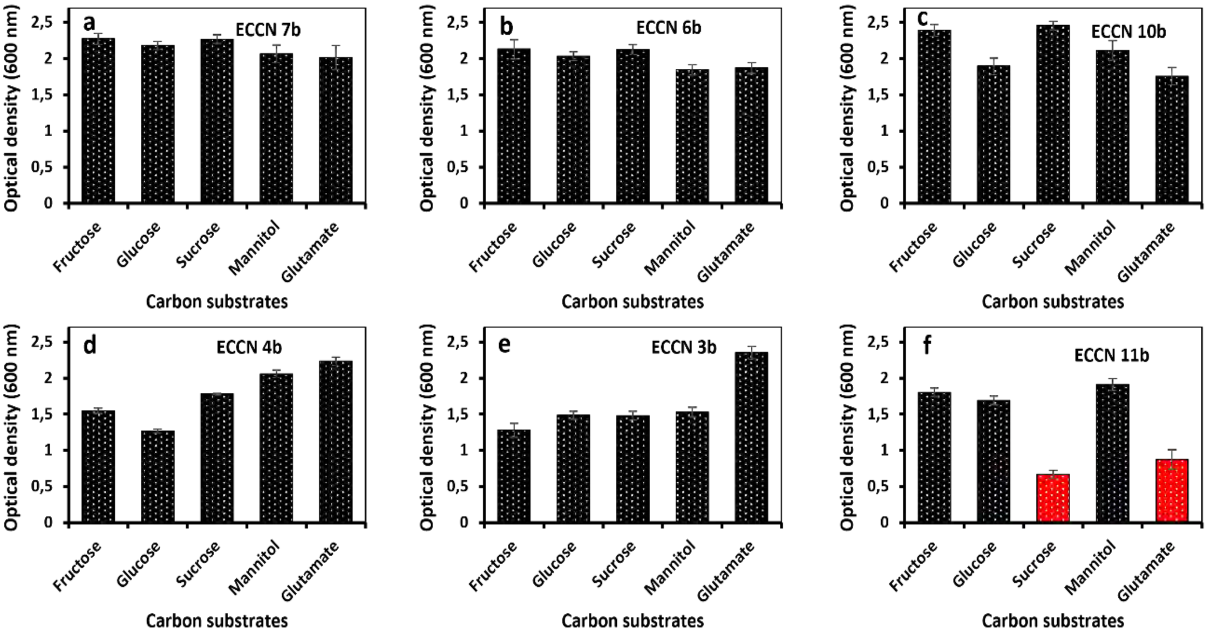


Figure 3. 8 Use of carbonaceous substrates by HRAOP bacteria. Organisms were cultured in NB supplemented with different carbohydrates (25 g L^{-1}) at 30 °C, 120 rpm for 3 d. Culture density was measured at OD_{600}. Results are expressed as the mean and standard error (SE) of at least 3 replicates.

The results of 12 bacterial strains generated from municipal wastewater treatment plants and screened for their ability to utilise different carbon sources were encouraging. Five isolates (41.67%) comprising *Enterobacter* strain ECCN 7b, *Arthrobacter* strain ECCN 6b, *Pseudomonas* strain ECCN 10b, *Aeromonas* strain ECCN 4b and *Bacillus* strain ECCN 3b were found to have multifunctional metabolic diversity responses by being able to use both carbonaceous substrates (Figure 3.8a-e) efficiently. Only *Ancylobacter,* strain (ECCN 11b) (8.33%), has used three sources of carbon (Figure 3.8 f). The changes in OD_{600} peaks for each of the 6 effective bacterial strains, isolates of WWT plants were 2,159 for *Enterobacter* (ECCN 7), 2,120 for *Pseudomonas* ECCN 10b)., 2,000 for *Arthrobacter* (ECCN 7b), 1,775 for *Aeromonas* (ECCN 4b), 1,623 for *Bacillus* (ECCN 3b), and 1,388 for *Ancylobacter* (ECCN

11b). Sodium L-glutamate, glucose, and sucrose were the preferred carbonaceous substrates over fructose and mannitol, with OD_{600} of 1.485, 1.326, and 1.306 each.

3.3.5.2 Coal Substrates

Based on the plate assay (Figure 3.9), the ability of bacteria to utilise coal as a carbonaceous source were rated as very good (+++) when the growth proliferation zones covered the entire plates as well as maximum strains pigmentation (Figure 3.9a, c-f and h-l), good (++) when it was weak (Figure 3.9b, g). In this case study, bacterial proliferation growth and pigmentation seemed more effective in coal substrate media compared to the controls.

Of the 22 bacterial isolates screened for the ability to utilise coal substrates, all 10 CS/DCS 100.00% (+++) were able to use both LRC and HRC as carbonaceous substrate versus 10 83.00% (+++) for HRAOP organisms as *Bacillus* sp., strain ECCN 1b and *Fictibacillus* sp., strain ECCN 2b have not shown any growth performances. These results showed how bacteria besides preferring common and known carbohydrates as carbon sources, might prefer other sources of carbon such as LRC and HRC.

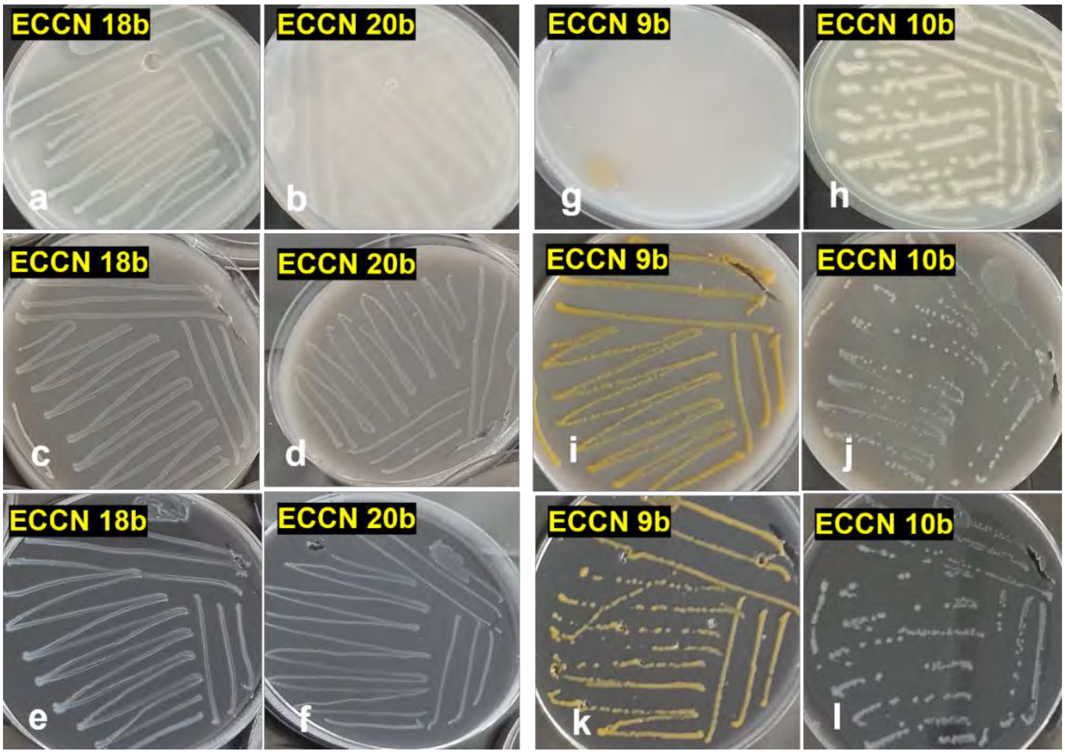


Figure 3. 9 Use of coals as carbonaceous substrates by CS/DCS (a-f) and HRAOP (g-l) bacteria CD bacteria. Organisms were cultured in YECA supplemented each with 10 g L^{-1} of LRC (c-d and i-j) and HRC (e-f and k-l) at 30 °C, 120 rpm for 5 d in comparison to the positive control (a-b, g,h). The bacterial isolates are identified as Bacillus sp., strain ECCN 18b, Proteus sp., strain ECCN 20b, Microbacterium sp., strain ECCN 9b, and Pseudomonas sp., strain ECCN 10b.

3.4 Summary

The results of the study confirmed the viability of all 22 ECCNs comprising ten CS and DCS isolates and twelve novel WWT bacteria had been recovered based on the colony morphological characteristics (shapes and colour) and purity. The ten bacterial isolates were generated from DCS/CS and identified as *Bacillus* sp. (ECCN 18b, ECCN 26b and ECCN 41b), *Citrobacter* sp. (ECCN 19b), *Proteus* spp. (ECCN 20b and ECCN 23b), *Exiguobacterium* sp. (ECCN 21b), *Microbacterium* sp. (ECCN 22b), *Serratia* sp. (ECCN 24b), and *Escherichia* sp. (ECCN 25b) could still show the same shape compared to some original strains. The viability and purity of the twelve novel algae-based WWT bacteria, *Bacillus* (ECCN 1b), *Fictibacillus* (ECCN 2b), *Bacillus* (ECCN 3b), *Aeromonas* (ECCN 4b), *Exiguobacterium* (ECCN 5b), *Arthrobacter* (ECCN 6b), *Enterobacter* (ECCN 7b), *Exiguobacterium* (ECCN 8b), *Microbacterium* (ECCN 9b), *Pseudomonas* (ECCN 10b), *Ancylobacter* (ECCN 11b), *Microbacterium* (ECCN 12b) were recovered as confirmed by morphological as well as the molecular properties.

The phylogenetic relatedness to PGPB of WWT microbial biocatalysts showed clusters with high bootstrap values with reference strains contained in the GenBank database. Only the cluster of *Pseudomonas* sp., strain ECCN 10b (MW672582) was outside the tree. It shared significant similarity (100%) with *Pseudomonas fluorescens* (CP015638) as a reference GenBank PGPB sequenced strain. *Proteus* sp., *Exiguobacterium* sp., *Proteus* sp., *Enterobacter* sp. and *Ancylobacter* showed high tolerance for saline and temperature stresses. However, *Serratia* sp., *Proteus* sp., *Escherichia* sp., *Bacillus* sp. and *Aeromonas* sp. were the most pH-tolerant bacteria. The NaCl concentration around 0.1 M (6-8 g L^{-1}) showed the best growth rate with a maximum OD_{600} of 1.748 for CS/DCSM and 1.744 for WWTM; however, the temperature of 30 °C was the best in terms of the growth rate measured as the change in optical density at OD_{600} (1.863, CS/DCS and 1.767, WWTM). Overall, the liquid screening bioassay revealed how 9 bacterial isolates (*Bacillus* sp., *Bacillus* sp., *Exiguobacterium* sp., *Bacillus* sp., *Enterobacter* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Aeromonas* sp. and *Bacillus* sp. used all carbon substrates and then, how Sodium L-glutamate, sucrose, and fructose ranked the best preferred carbonaceous substrates. Qualitative screening has shown how 100% CS/DCS and 83% WWT bacteria preferred other sources of carbon like LRC and HRC.

Chapter Four: Plant Growth-Promoting Characteristics of Selected Bacterial Isolates

4.1 Introduction

It is imperative to achieve the United Nations Sustainable Development Goals and help feed an exponentially growing global population. For this reason, the exploration and exploitation of microbial collections, mainly fungi and bacteria with biodegradable pollutant activities and biofertilization potential, have become a need (Whitman et al. 1998; Ryan et al. 2019; Coban et al. 2022; Sekhohola-Dlamini et al. 2022). Above all, it is necessary to study and understand the microbial interaction processes occurring in the soil before exploiting this potential to promote bioremediation or rehabilitation of degraded sites, plant growth, to promote sustainable agriculture (Kumar and Gopal, 2015; Van Breugel et al. 2019).

Since studies on the use of bacterial and fungal isolates as biofertilisers to promote plant growth development and yield were initiated at the laboratory level, there is a growing need to develop agroecological practices that can increase crop yields and produce healthy foods to advance food security and sustainability (Hayat et al. 2010; Odoh et al. 2020). Plant growth-promoting bacteria play an essential role in the soil by improving its physicochemical and biology. They facilitate water flow and nutrient availability for absorption. Used as biofertilizers, PGPB improves hormonal balance and plant growth, even under abiotic stress conditions (Abdelaal et al. 2021). Biofertilisation then becomes a biological pathway that can be used to meet current and future global food needs, as it plays an essential role in plant growth and development by providing organic matter and mineralised nutrients to the soil (Raimi et al. 2017; Odoh et al. 2020) in all conditions.

Plant growth-promoting bacteria as potential bio-stimulants offset reliance on fossil fuel-derived fertilisers and synthetic pesticides to help promote agricultural sustainability and increased crop yields (Lugtenberg and Kamilova 2009; Santoyo et al. 2016; Backer et al. 2018; Basu et al. 2021; de Los Santos-Villalobosa and Parra-Cota 2022). As rhizosphere organisms, PGPB comprise diverse microbial genera that mineralise nutrients (N, P, and K) and produce and/or modulate PGR (auxin, gibberellin, siderophore production, etc.), concentration and thus, impact on plant growth and development (Santoyo et al. 2016; Glick 2014; Gusmiaty et al. 2019). These biofertiliser organisms improve stress tolerance, influence several biochemical properties (Kang et al. 2019; Lata et al. 2018; Odoh et al. 2020), and stimulate rhizodeposition (Hallett et al. 2022). Mycological studies have shown their interest in exploring several fungal decomposition processes of organic matter that can improve soil nutrient cycling and associated biochemical processes (Naranjo-Ortiz and Gabald 2019; Odoh et al. 2020).

The present research evaluated several known PGP traits to assess the PGP activity of the 22 selected bacterial isolates described in Chapter 2 of this thesis. The parameters chosen included the production of indoles and specifically those with auxin-like activity such as indole-3-acetic acid (IAA), gibberellin (GA)-like activity, and factors thought to modify plant hormone action like increased 1-amino-cyclopropane carboxylic acid (ACC) deaminase activity and the production of siderophores which are iron-chelating molecules. The ability of bacterial isolates to mineralise essential plant nutrients N, P, and K was also evaluated. The data thus derived were used to rank the candidate bacteria in descending order of PGP activity for further use in selecting strains suitable for incorporation into a biofertiliser.

4.2 Material and Methods

4.2.1 Bacterial Isolates

All 22 bacterial strains were evaluated for their plant growth-promoting (PGP) and regulatory (PGR) activities. The ability of isolates to biomineralize essential plant nutrients including N, P, K, and humic acid (HA) from insoluble organic or inorganic sources (Zhou and Boyd 2016; Zhao et al. 2019; Masudi et al. 2023) was evaluated. However, the production of PGR, such as IAA, GA, ACC deaminase activity and siderophores (Schwyn and Neilands 1987; Penrose and Glick 2003; Sharma et al. 2018; Olawale et al. 2020; Titilawo et al. 2022) was performed.

4.2.2 Bio-Mineralisation Potential of Inorganic and Organic Rocks

4.2.2.1 Protein Hydrolysate from Peptone

Microbial ammonium production was assessed using peptone water (PW) as a culture medium and Nessler's reagent (NR) HgI_4K_2 as an indicator (Zhou and Boyd 2016; Zhao et al. 2019). Peptone water (HiMedia, M028) was prepared by dissolving in 1 L of distilled water, 10 g of peptone (Fluka, Sigma-Aldrich, USA), and 5 g of NaCl (Merck KGaA, Germany). However, NR (*HiMedia*, R010) was made by suspending in 100 mL of distilled water, 10 g of HgCl2 (univAR, RSA), 7 g of KI (Holpro L, RSA) and 16 g of NaOH (Merck, India). The inoculants were seeded into 40 mL of PW and incubated on a rotary shaker at 30°C and 120 rpm, 3 d. After incubation, 2 mL of each sample was pipetted and centrifuged at 3920 × g for 5 min to obtain supernatant, from which 1 mL was used to react with 0.1 ml of NR. The mixture was allowed to stand at room temperature for 5 min to obtain the full brown colour.

Quantification of NH_4^+ was performed by spectrophotometric analysis (Shimadzu UV-1280, Japan) at 430 nm (Cappuccino and Sherman 1992; Bumunang and Babalola 2014) followed by interpolation from a standard curve for NH_4Cl (25 mg L^{-1}) (Jeong et al. 2013) and data expressed in NH_4Cl equivalents.

4.2.2.2 Apatite and Zeolite Solubilisation

Qualitative solubilisation of apatite was assessed using Pikovskayas (PVK) plates and tri-calcium phosphate ($Ca_3(PO_4)_2$) as the sole source of insoluble phosphate (Pikovskaya 1948; Sharma et al. 2013). PVK agar (HiMedia, M520; Sharma et al. 2011) was prepared by suspending in 1 L of distilled water, 0.5 g of yeast extract (Merck, RSA), 10 g of glucose (Alpha, RSA), 5 g $Ca_3(PO_4)_2$ (HiMedia, India), 0.5 g $(NH_4)_2SO_4$ (Merck, Germany), 0.2 g KCl (Saarchem univAR, RSA), 0.1 g of $MgSO_4$ (Merck, RSA), 0.0001 g of $MnSO_4.H_2O$ (Saarchem, Merck, RSA), 0.0001 g of $FeSO_4.7H_2O$ (Holpro, RSA) and 15 g of agar (Biolab, Merck, RSA).

Zeolite solubilisation was investigated using solid Aleksandrow's medium (SAM), whereas potassium aluminosilicate ($AlKO_6Si_2$) (*HiMedia*, RSA) was the sole source of insoluble potassium (Saha et al. 2016). The medium preparation (*HiMedia* M1996) was carried out by suspending in 1 L of distilled water; 0.5 g $MgSO_4.7H_2O$ (Merck, RSA), 0.1 g $CaCO_3$ (Pal Chemicals), 2 g $AlKO_6Si_2$ (*HiMedia*, RSA), 5 g glucose (Alpha, RSA), 0.005 g $FeCl_3.6H_2O$ (Saarchem, RSA), 2 g $Ca_3(PO_4)_2$ (*HiMedia*, India) and 20 g agar (Biolab, Merck, RSA).

In both cases, the aliquot of cell suspension was placed at 3 equidistant positions on PVK/SAM, followed by incubation of the cultures at 30°C for 10 d. The solubilisation potential was then determined by observing the development of clear zones around active bacterial colonies (Baliah et al. 2016).

The PVK broth (*HiMedia*, M1719) and Aleksandrow liquid medium (ALM) (*HiMedia* M1997) were used for PO_4^{-3} phosphate and K solubilisation (Muthuraja and Muthukumar 2021). A bacterial inoculant was added to each 40 mL broth which was incubated in a rotary shaker at 30 °C, 120 rpm for 7 d. The colourimetric quantification of PO_4^{-3} was carried out at 690 nm after adding indicators to 5 mL of supernatant of each sample according to the protocol of P kits (Merck KGaA, 1.14848.0001, Germany). The K quantification protocol was performed using 2 mL of ALM supernatant. Following the protocol of the K kits, the resulting turbidity was measured at 690 nm (Merck KGaA, 1.14562.0001, Germany). The final concentration of solubilised PO_4^{-3} and K was determined by interpolating the results to standard curves prepared using K_2HPO_4 (Sigma-Aldrich, Japan) and KCl (Saarchem, Merck, RSA) (Titilawo et al. 2020) as equivalents.

4.2.2.3 Coal Bio-Mineralisation Potential

To study the degradation activity of coal by bacterial isolates, organisms were cultured on NA (31 g L^{-1}) (Biolab, Merck, RSA) containing LRC (1 g L^{-1}) as a substrate. Then, a 50 µL aliquot was placed at 2 equidistant positions on the agar (Figure 4.3). Fine sterile coal particles were sprinkled on the culture aliquots and incubated plates for 7-10 d at 30 °C. Microbial degradation of coal could be determined after observing the appearance of a brown halo taken to indicate the release of humic acid-like substances (Olawale et al. 2020). Controls were NA plates containing coal particles without fungal or bacterial inoculants.

4.2.3 Plant Growth-Promoting Activity

4.2.3.1 Indole Production

The bacterial potential in terms of indole production was studied using L-tryptophan (1g L^{-1}) (Merck, KGaA, RSA) and LRC (1g L^{-1}) as substrates in NB; however, Salkowski's reagent has been prepared as an indicator of IAA (Gordon and Weber 1951; Glickman and Dessaux 1995; Datta et al. 2015). Forty mL of NB was inoculated with 0.04 mL seed culture and incubated at 30°C and 120 rpm for 3 d. After incubation, 1 mL of the supernatant obtained by centrifugation at 3920 × g for 5 min (Costar, USA six-slot rotor) reacted with 1 drop (10 mM H_3PO_4) and 2 mL of Salkowski's reagent (1 mL, 0.5 M $FeCl_3$ into 50 mL 35% $HClO_4$). The mixture was allowed to stand at room temperature for about 15 min for maximum pink colour development. The amount of indole compounds was determined by spectrophotometry at 530 nm, followed by interpolation from a prepared standard curve of authentic IAA (100 mg L^{-1}) (Sigma-Aldrich, RSA) (Sarker and Al-Rashid 2013; Titilawo et al. 2020).

4.2.3.2 Gibberellin Production

The potential of isolated bacteria to produce gibberellic acid compounds (GA) was assessed using the standard method of Borrow et al (1955) and Holbrook et al (1961). Luria Bertani Broth (LBB) (HiMedia, M1245) was selected as the best culture medium. Zinc acetate, potassium ferricyanide and HCl (30%) as the best reagents (Kapoor et al. 2016; Gusmiaty et al. 2019). The LBB protocol consisted of dissolving in a litre of Milli-Q water, 10 g of tryptone (Sigma-Aldrich, Mexico), 5 g of yeast extract (Biolab, Merck, RSA) and 10 g of NaCl (Univar, Merck, RSA). The zinc acetate reagent composition was prepared by suspending 21.9 g of the salt in Milli-Q water containing 1 mL of glacial acetic acid. The solution was mixed; the final volume was made up to 1 L adding Milli-Q water. Potassium ferricyanide (Univar, Merck, RSA) solution was prepared by adding 10.6 g of $K_3Fe(CN)_6$ to 100 mL of Milli-Q water.

The seed culture was added to each 40 mL of LBB and incubated on a rotary shaker at 30°C and 120 rpm for 3d. After that, 15 mL of each freshly grown culture was centrifuged at 7741 × g for 10 min (Avanti centrifuge, JA 20 rotor, J-E, USA). To 5 mL of the supernatant, 0.4 mL of zinc acetate was added. The mixture was allowed to stand at room temperature for about 3 min, and 0.4 ml of potassium ferricyanide was added, followed by low-speed centrifugation of 121 x g, 15 min (JA 20 rotor, J-E, USA). A volume of 3 mL of HCl (30%) was added to each collected supernatant and incubated at 22°C for about 75 min (Sharma et al. 2018). The amount of GA produced by microbial biocatalysts was quantified as absorbance at 254 nm, followed by interpolation from a standard curve plotted from authentic GA (100 mg L^{-1}) (Sigma-Aldrich, China).

4.2.3.3 One-Aminocyclopropane-1-Carboxylic Acid Deaminase Activity

Organisms with some ranges of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity act as PGPR (Penrose and Glick 2003). They hydrolyse ACC to CH_3-H_2-CO-CO-OH (α-ketobutyrate) and NH_4^+, making it the only source of N (Penrose and Glick 2003; Yim et al. 2010). Qualitative evaluation of bacterial isolates for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity was performed on the minimal salt agar (SMA) supplemented with ACC (Dworkin and Foster 1958; Penrose and Glick 2003) as the sole nitrogen source.

The medium was prepared by suspending per litter of Milli-Q water 2 g of glucose (Merck, KGaA, Germany), 2 g of gluconic acid (Sigma-Aldrich, Germany), 4 g of KH_2PO_4 (Sigma-Aldrich, Japan), 6 g of Na_2HPO_4 (Merck, RSA), 0.2 g of $MgSO_4.7H_2O$ (Saarchem, RSA), 2 g of citric acid (Merck, RSA) and 15 g of agar (Biolab, Merck, RSA). However, the trace elements added were 10 mg of H_3BO_3 (Merck, RSA), 11.19 mg of $MnSO_4.H_2O$ (Merck, KGaA, Germany), 1 mg of $FeSO_4.7H_2O$ (AnalaR, England), 124.6 mg of $ZnSO_4.7H_2O$ (Saarchem, RSA), 78.22 mg of $CuSO_4.5H_2O$ (Saarchem, RSA), 10 mg of MoO_3 (Sigma-Aldrich, USA), and 3 mM (0.30 g) of ACC (Merck, USA).

4.2.3.4 Siderophore Production

Modified assay for the siderophore (SDR) activity was performed according to the protocol developed in 1987s by Schwyn and Neilands, which used chrome azurol sulfonate (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as biological indicators (Louden et al. 2011; Ghazy and El-Nahrawy 2021). The CAS agar protocol was carried out in three stages:

making the blue dye, mixing the solution, and preparing the CAS. To create the blue dye, three solutions were initially made. The first solution dissolved 0.12 g of CAS (Sigma-Aldrich, Japan) in 100 mL of Milli-Q water. The second consisted of suspending 0.003 g of $FeCl_3.6H_2O$ (Saarchem, RSA) in 100 mL of 10 mM HCl. Solution 3 required dissolving 0.15 g of HDTMA (Sigma-Aldrich, China) in 80 mL of Milli-Q water. To generate blue dye, solution 1 was mixed with 18 mL of solution 2 and then mixed with solution 3. This solution was autoclaved and stored in a plastic container.

The mixing solution was composed mainly of a set of stock solutions. The first stock solution, known as Minimal Medium 9 (MM9) salt, was prepared by dissolving 15 g KH_2PO_4 (Merk KGaA, Germany), 25 g NaCl (Merck, RSA), and 50 g NH_4Cl (Merk, RSA) in 500 mL of Milli-Q water. The second solution, glucose stock, was performed by measuring 20 g of glucose (Sigma-Aldrich, Macedonia) in 100 mL of Milli-Q water. About 150 ml of 4.17 M NaOH (Merk KGaA, Germany) was prepared as the third stock solution. In addition, 6 g of casein hydrolysate (CH) (Sigma-Aldrich, USA) was dissolved in 54 mL of Milli-Q water. Then, to remove all traces of iron, 3% 8-hydroxyquinoline (Sigma-Aldrich, China) in chloroform (equal volume) was added to the CH solution. The mixture was kept overnight in fume wood in a room for better chloroform decantation. Before use, the supernatant was pipetted, and filter sterilised.

Finally, CAS agar was prepared by suspending 100 mL of MM9 solution in 750 mL Milli-Q water. 32.24 g of PIPES (Sigma-Aldrick, USA) and 15 g of agar (Biolab, Merck, RSA) were added to the MM9 solution while pH was brought to 6.8 for complete dissolution of PIPES and then autoclaved. After autoclaving, the medium was left to cool to 50°C, and before pouring the plates, 30 mL of CH and 10 mL of glucose stock were pipetted into the MM9-PIPES solution. Slowly 100 mL of blue dye was pipetted down the glass wall and agitated sufficiently before being poured onto the plate. Bacterial isolates were cultured in YEMA agar (Himedia M715) for 3 d at 30°C to generate fresh colonies and, therefore, non-overlapping parallel streaks were made on triplicate CAS agar. The incubation process took a maximum of 3 d at 30°C; however, siderophore-producing type bacterial activity was determined by observing the development of an orange halo zone around active bacterial colonies (Baliah et al. 2016; Ghazy and El-Nahrawy 2021). Additionally, the diameter of the halo (cm) was measured, and the results were compiled as the mean and standard error (± SE).

4.3 Data and Statistical Analysis

All data were analysed using Excel (version 16, Microsoft Corporation, Redmond, WA). Where appropriate, the results were analysed by one-way analysis of variance (ANOVA), and significant differences between measures for the treatments were determined. Data are presented as the mean of at least three determinations ± standard error (SE). Principal component analysis was performed for the ranking of bacterial strains according to the potential for PGR and PGP activities using the Kruskal-Wallis H test ($P<0.050$) in Sigma Plot Version 11 (Systat Software Inc., San Jose, California, USA).

4.4 Results

4.4.1 Bio-Mineralisation

4.4.1.1 Protein Hydrolyse

Production was measured as NH_4Cl equivalents; however, the amount of NH_4^+ (mg L^{-1}) mineralised by 19 (86.36%) active bacteria varied between 4.72 and 9.78. All CS/DCS biocatalysts (100%) demonstrated NH_4^+ (mg L^{-1}) producing activity with the range between 4.72 and 7.79 and had an average ammoniacal nitrogen estimated at 6.24. Nine HRAOP were effective, with an average OD_{600} yield of 6.55.

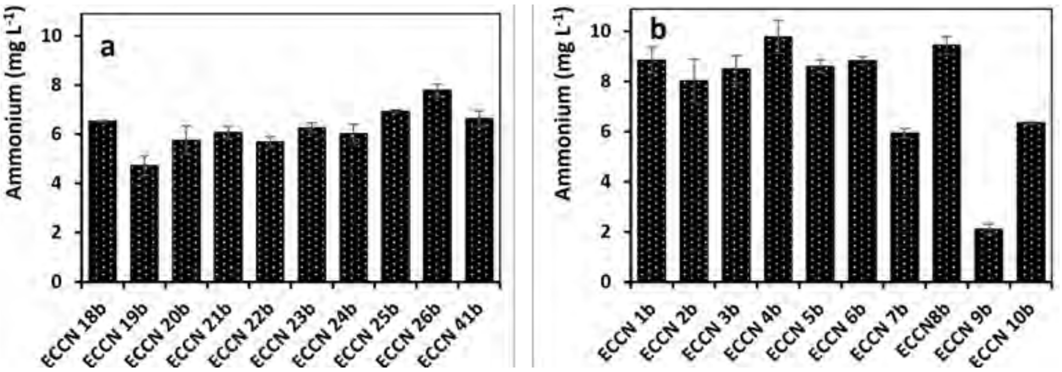


Figure 4. 1 Production of ammonium by CS/DCS (a) and HRAOP (b) bacterial biocatalysts. Organisms were seeded in PW and incubated at 30 °C for 3 d. Ammonium compounds were estimated at 430 nm as NH_4Cl equivalents. Results are expressed as the mean ± S.E indicating significant differences of at least 3 replicates ($p < 0.05$).

The top 7 strains capable of producing NH_4^+ (mg L^{-1}), included *Aeromonas* strain ECCN 1b (9.78), *Exiguobacterium* strain ECCN 8b (9.45), *Bacillus* strain ECCN 1b (8.85), *Arthrobacter* strain ECCN 6b (8.83), *Exiguobacterium* strain ECCN 5b (8.61) and *Fictibacillus* strain ECCN 2b 98.02), all of them were generated from WWT process.

4.4.1.2 Apatite and Zeolite Solubilisation

The plate assays were used as a preliminary screening, while the spectrophotometric analysis and absorbance data were used to quantify the activity. The solubilisation potential of tri-calcium phosphate ($Ca_3(PO_4)_2$) and potassium alumino silicate ($AlKO_6Si_2$) was confirmed by observing clear zones around active bacterial colonies on PVK agar or SAM after a maximum incubation of 10 d and at 30° C. Of the 22 bacteria screened for phosphate solubilisation, *Pseudomonas* sp., strain ECCN 10b alone was able to solubilise tri-calcium phosphate. This HRAOP bacterium developed the maximum clear zones around the colonies demonstrating phosphate solubilising activity (Figure 4.2b).

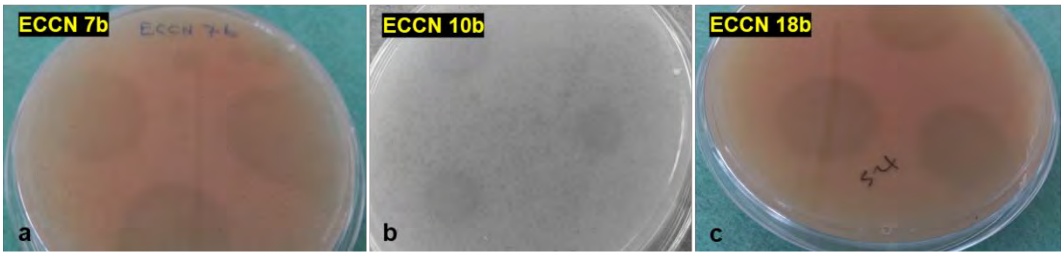


Figure 4. 2 Plate screening assays showing potassium (a, c) and phosphate (b) solubilisation halos by three bacterial isolates namely *Enterobacter* sp. (a), *Pseudomonas* sp. (b) and *Bacillus* sp. (c). Organisms were inoculated into PKV agar (b)/SAM (a, c) and (b) incubated at 30° C for a maximum of 10 d.

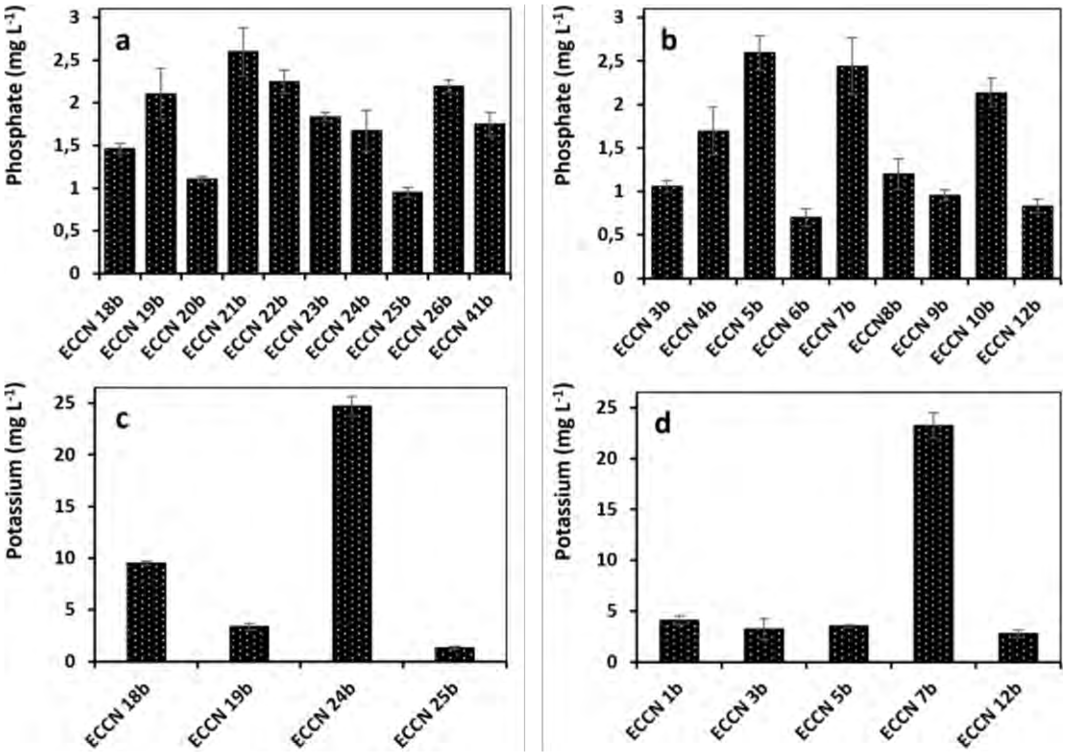


Figure 4. 3 Bacterial phosphate (a-b) and potassium (c-d) solubilisation potential. Organisms were inoculated into PKV broth and LAM and incubated at 30° C for 7 d. Soluble PO_4^{-3} and K^+ were estimated at 690 nm. Results are expressed as the mean ± S.E indicating significant differences of at least 3 replicates ($p < 0.05$).

It was found that *Enterobacter* strain ECCN 7b and *Bacillus* strain ECCN 18b were capable of solubilising $AlKO_6Si_2$ (Figure 4.2a, c). However, not all isolates showed sufficient clear zones in plaque assays for phosphate and potassium solubilisation. Therefore, a quantitative screening test was conducted in the liquid culture medium to determine the release of phosphate and potassium from insoluble inorganic sources. The spectrophotometric quantification of soluble PO_4^{-3} and K^+ was estimated at 690 nm using freshly cultured PVK and AM broths after 7 d incubation. Referring to Figure 4.3a-b data, 19 isolates were found to be phosphate-solubilising organisms with mean values ranging from 1.06 to 2.60 mg L^{-1}. However, the 2 most active PO_4^{-3} solubilising bacterial biocatalysts included *Exiguobacterium* ECCN 5b (2.59 mg L^{-1}) and *Exiguobacterium* ECCN 21b (2.60 mg L^{-1}). Other phosphate-solubilising competent organisms with mean soluble PO_4^{-3} values $<$ 2.11 mg L^{-1} were *Enterobacter* strain ECCN 7b, *Microbacterium* strains ECCN 22b, *Bacillus* strain ECCN 26b, *Pseudomonas* strain ECCN 10b, and *Citrobacter* strain ECCN 19b.

Potassium solubilisation results showed that *Serratia* strain ECCN 24b and *Enterobacter* strain ECCN 7b were the only strains demonstrating significant ability to solubilise $AlKO_6Si_2$ in LAM. The amount (mg L^{-1}) of solubilised K^+ is ranged from 1.31 to 24.73. However, other isolates including *Bacillus* strain ECCN 18b (9.51), *Bacillus* strain ECCN 1b (4.08), *Citrobacter* strain ECCN 19b, *Exiguobacterium* strain ECCN 5b (3.53), and *Aeromonas* strain ECCN 24b (3.24) showed potassium solubilisation activity to a much lesser extent (Figures 4.3c-d).

4.4.1.3 Coal Bio-Mineralisation Activity

As seen in Figure 4.4 (all data not displayed), the appearance of brown halos indicated bacterial degradation. The diameters of the brown halo zones varied from 8 to 21 mm. Therefore, most organisms showed bioremediation ability by degrading WC (Figure 4.4a, c).

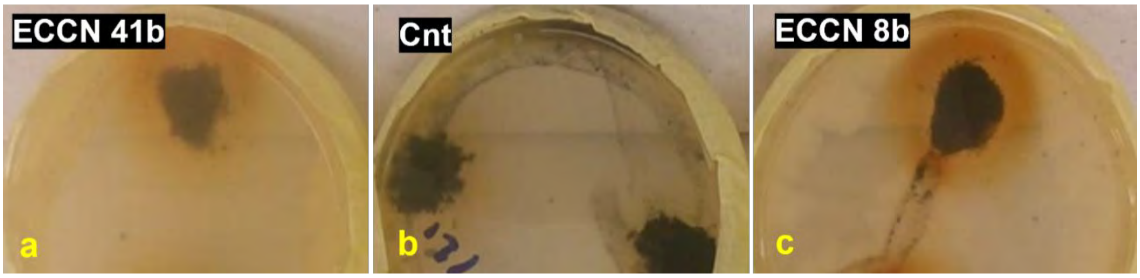


Figure 4. 4 Bacterial coal biodegradation activity. The appearance of brown halos indicated the biodegradation activity of Exiguobacterium sp., strain ECCN 8b and Bacillus sp., strain ECCN 41b.

The colour intensity of halo zones was higher when degradation was most significant. The HRAOP bacterial strains including *Bacillus* sp. (ECCN 3b), *Aeromonas* sp. (ECCN 4b), *Exiguobacterium* (ECCN 8b), *Pseudomonas* sp. (ECCN 10b), and A*ncylobacter* sp. (ECCN 11b), were also organisms capable of degrading coal. However, *Exiguobacterium* sp., strain ECCN 8b, and *Bacillus* sp., strain ECCN 41b, were selected most coal-degrading bacteria. No colour change was observed in the controls, indicating no spontaneous leaching of any latent or residual material into the coal as carbon substrates.

4.4.2 Plant Growth-Promoting Potential

4.4.2.1 Indole Production

Indole production potential, measured as indole-3-acetic acid (IAA) equivalents was performed 15 min after adding Salkowski's reagent to freshly cultured NB supernatant containing L-tryptophan (1 g L^{-1}). Pink colour development was more intense for isolates with indole and IAA-producing potential. Of the 22 bacterial strains tested for the ability to produce indoles/IAA, 6 (27.27%) isolates were effective microorganisms.

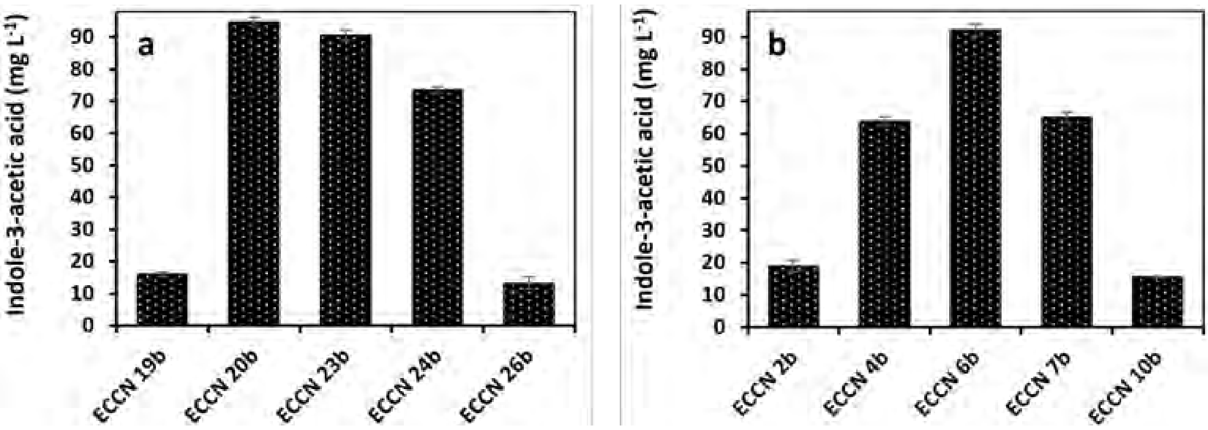


Figure 4. 5 Production of indole measured as IAA equivalents by CS/DCS (a) and HRAOP (b) bacteria. Organisms were seeded in NB supplemented with LT (1 g L^{-1}) and incubated at 30 °C for 3 d. Indole compounds were estimated at 530 nm as IAA equivalents. Results are expressed as the mean ± S.E indicating significant differences of at least 3 replicates ($p < 0.05$).

Among these potential organisms, 3 (50.00%) are known as DCS bacteria while the other 3 (50.00%) as algae based on WWT process bacteria. Based on the amount of IAA equivalents (mg L^{-1}) produced (Figure 4.5) these potential organisms are known as *Proteus* strains ECCN 20b (94,72), *Proteus* strain ECCN 23b (90,54), *Arthrobacter* strain ECCN 6b (92,07), *Serratia* strain ECCN 24b (73,52), *Enterobacter* strain ECCN 7b (65,12), and *Aeromonas* strain ECCN 4b (63,82). The analysis of variance (one-way ANOVA) established the significant difference between the bacteria considering the quantity (mg L^{-1}) of indole compounds produced ($P<0.05$).

4.4.2.2 Gibberellin Production

Gibberellin concentration measured as GA_3 equivalent was estimated in aliquots of LBB supernatant (HiMedia, M1245) after 3 d incubation at 30 °C and 120 rpm. Results showed that 4 bacterial strains (18.18%) belonging to the *Microbacterium* and *Ancylobacter* group were competitive (Figure 4.6). Among these competitive organisms, 3 isolated bacteria (75.00%), including the *Microbacterium* ECCN 9b strain, the *Ancylobacter* ECCN 11b strain and the *Microbacterium* ECCN 12b were generated from the WWT process; the other (25%), known as *Microbacterium* sp. ECCN 22b from coal mines. This further expressed the high potential of bacterial biocatalysts from HRAOP of IAPS environments in terms of plant growth regulating-like activity.

Considering the efficiency of GA production (mg L^{-1}) activities the bacterial isolates ranked first are *Ancylobacter* sp., ECCN 11b (31.07), followed by *Microbacterium* sp., ECCN 12b (29.81). *Microbacterium* sp., ECCN 22b (24.28) and *Microbacterium* sp., ECCN 9b (20.82).

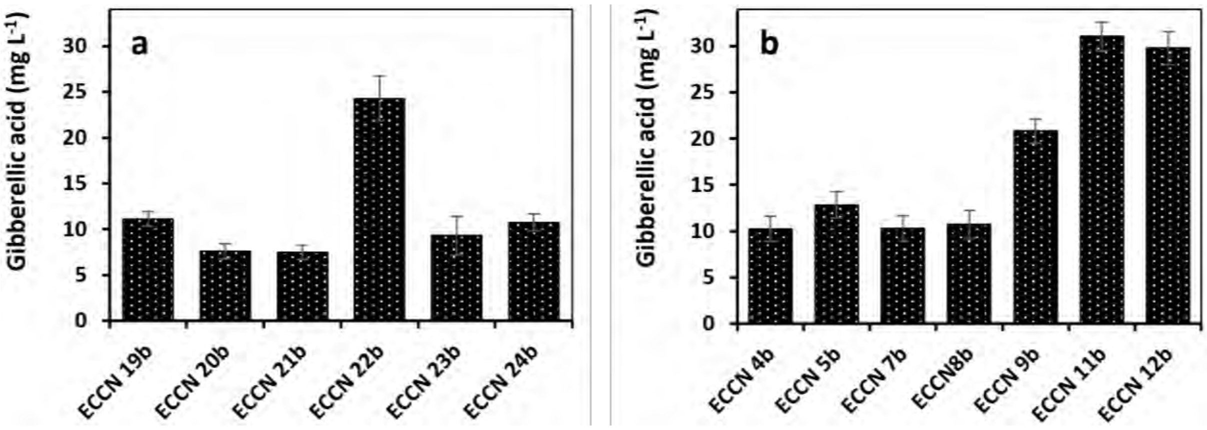


Figure 4. 6 Production of gibberellin measured as GA_3 equivalents by CS/DCS (a) and HRAOP (b) bacterial biocatalysts. Organisms were seeded in LB broth and incubated at 30 °C for 3 d. Gibberellic compounds were estimated at 254 nm as GA_3 equivalents. Results are expressed as the mean ± S.E indicating significant differences of at least 3 replicates ($p < 0.05$).

4.4.2.3 ACC Deaminase Activity

Some PGPB with ACC deaminase activity can grow on MSM agar supplemented with ACC and then act as biofertilisers due to their ability to use this compound as a nitrogen source.

Of the 22 bacterial strains tested, 9 (40.91%) showed ACC deaminase activity because they grew on MSM agar. They were then able to use ACC as the sole source of nitrogen to form colonies with a diameter varying between 1 mm (*Serratia* sp., strain ECCN 24b and *Enterobacter* sp. strain ECCN 7) to 2 mm (*Pseudomonas* sp. strain ECCN 10b and *Proteus* sp. strain ECCN 23b). Additionally, *Pseudomonas* sp. strain ECCN 10b (Figure 4.7b) was the most efficient organism. Among the 4 potential organisms, 50%, *Enterobacter* strain ECCN 7b and *Pseudomonas* strain ECCN 10b, are known HRAOP organisms. The two others including *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b (50%) as bacteria sourced from DCS.

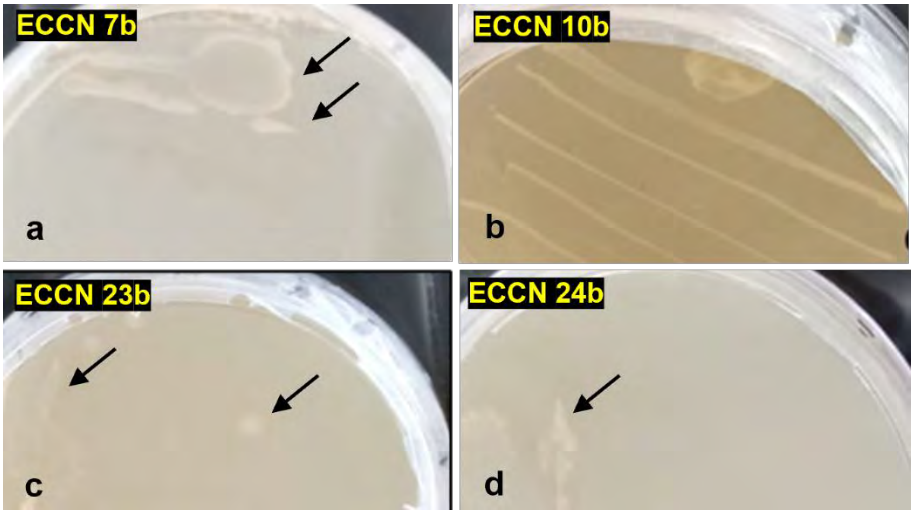


Figure 4. 7 Plate screening assays showing ACC deaminase activity of bacterial isolates. Enterobacter sp. (a), Pseudomonas sp. (b), Proteus sp. (c) and Serratia sp. (d). Organisms were inoculated into MSM supplemented with 3 mM ACC and incubated at 30° C for 3 to 5 d. The ability of culture to grow on MSM agar was considered as the deaminase ability of ACC.

4.4.2.4 Siderophores Production

For this purpose, 22 bacteria, including ten CS/DCS isolates and twelve treating biological wastewaters, were selected, and cultured on CAS blue agar for 3 d at 30°C; however, not all developed an orange halo zone around the bacterial colonies. Four bacterial isolates (18.18%) were found effective siderophore-producing biocatalysts as they exhibited each an orange halo around colonies (Figure 4.8). Two of these microorganisms comprising *Bacillus* sp., strain ECCN 18b and *Citrobacter* sp., strain ECCN 19b were isolated from CS/DCS. However, *Enterobacter* sp., strain ECCN 7b and *Pseudomonas* sp., strain ECCN 10b from the HRAOP. The diameter of the orange halo showed a significant difference ranging from 8.00 cm for *Enterobacter* sp., strain ECCN 7b, to 3.13 cm for *Citrobacter* sp., ECCN 19b.

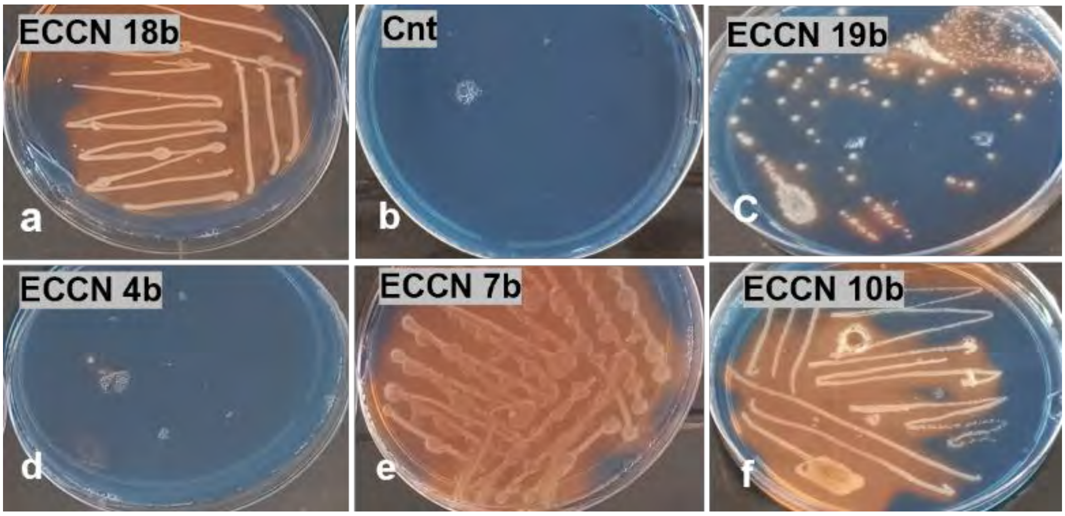


Figure 4. 8 The siderophore-producing potential of bacterial isolates on CAS blue agar bioassay. The appearance of orange halos indicated the production activity of *Bacillus* sp., strain ECCN 18b, *Citrobacter* sp., strain ECCN 19b, *Aeromonas* sp., strain ECCN 4b, *Enterobacter* sp., strain ECCN 7b and *Pseudomonas* sp., strain ECCN 10b. No colour change represented no production (ECCN 4b) or control (Cnt).

However, *Pseudomonas* sp., strain ECCN 10b, and *Bacillus* sp., strain ECCN 18b, were classified in the intermediate position because the diameter of the orange halo for each organism was 60.60 cm and 7.70 cm.

Table 4. 1 The potential of bacterial isolates to produce siderophore is indicated by the diameter of orange halos. *Enterobacter* sp., ECCN 7b, *Pseudomonas* sp., ECCN 10b, *Bacillus* sp., ECCN 18b and *Citrobacter* sp., ECCN 19 were found to have this potential.

Bacterial Siderophore-Producing Activity as Orange Halo Diameter (cm)					
Isolates		**ECCN 7b**	**ECCN 10b**	**ECCN 18b**	**ECCN 19b**
Parameter Analysed	Mean Values (MV)	8.00	6.60	7.70	3.13
	Standard Error (± SE)	0.49	0.26	0.59	0.84

Three bacterial strains known as *Enterobacter* sp., *Bacillus* sp., and *Pseudomonas* sp., were found to be the most effective siderophore producers and therefore were qualified as PGPB. These results showed how the organisms from polluted ecological sites coal mines, diesel-contaminated sites and biological wastewater may possess plant growth promotion-like activity.

4.4.3 Ranking Bacterial PGP-like Activity

The plant growth-promoting activity of the bacteria was assessed spectrophotometrically as the amounts (mgL^{-1}) of PGR (IAA and GA) and PEN (NH_4^+, $PO_4^{-3}$and K^+) produced by each bacterium. These produced or mineralized PGR and EPN were expressed as the means and standard errors of at least all 22 biological treatments (Tables 4.2 and 4.3).

Table 4. 2 Amount (mg L^{-1}) of regulators and nutrients produced/mineralised by isolated CS/DCS and HRAOP bacteria. The results are expressed as means ± S.E of at least 3 repetitions ($p < 0.05$).

Bacterial Strains	**Concentration (mg L^{-1}) of Regulators and Essential Plant Nutrients Produced/Mineralised**				
	PGR		**EPN**		
	IAA	**GA**	**NH_4^+**	**PO_4^{-3}**	**K^+**
CS/DGS Bacteria					
Bacillus sp. ECCN 18b	7.00	4.78	6.53	1.46	9.51
Citrobacter sp. ECCN 19b	15.98	11.13	4.72	2.11	3.36
Proteus sp. ECCN 20b	94.72	7.61	5.56	1.10	0.35
Exiguobacterium sp. ECCN 21b	6.33	7.48	6.07	2.60	0.28
Microbacterium sp. ECCN 22b	4.68	24.28	5.68	2.25	0.32
Proteus sp. ECCN 23b	90.54	9.31	6.24	1.84	0.18
Serratia sp. ECCN 24b	73.52	10.75	6.02	1.67	24.73
Escherichia sp. ECCN 25b	8.13	4.97	6.93	0.95	1.31
Bacillus sp. ECCN 26b	13.21	4.84	7.79	2.19	0.40
Bacillus sp. ECCN 41b	7.11	6.42	6.63	1.75	0.26
HRAOP Bacteria					
Bacillus sp. ECCN 1b	0.85	5.03	8.85	0.15	4.08
Fictibacillus sp. ECCN 2b	18.91	5.16	7.56	0.33	1.66
Bacillus sp. ECCN 3b	3.05	7.48	8.50	1.06	1.29
Aeromonas sp. ECCN 4b	63.82	10.26	9.78	1.69	1.25
Exiguobacterium sp. ECCN 5b	1.80	12.83	8.70	2.60	3.43
Arthrobacter sp. ECCN 6b	92.07	7.92	8.83	0.70	1.98
Enterobacter sp. ECCN 7b	65.12	10.31	5.95	2.44	22.25
Exiguobacterium sp. ECCN 8b	2.76	10.75	9.45	1.20	2.09
Microbacterium sp. ECCN 9b	0.71	20.91	2.12	0.96	1.30
Pseudomonas sp. ECCN 10b	15.55	6.16	6.34	2.14	1.50
Ancylobacter sp. ECCN 11b	2.06	31.06	0.92	0.49	0.60
Microbacterium sp. ECCN 12b	1.75	29.81	1.23	0.83	2.81

Six of the 22 bacteria screened produced IAA, four produced GA, eighteen produced mineralised NH_4^+, twelve solubilized P and two solubilized K. However, the one-way ANOVA over the ranks was performed using the continuous-level data in Table 4.3. The results, expressed as mean ± S E, showed significant differences ($p < 0.05$). The potential of CS/DCS bacteria for PGP activity on the ranks being: *Serratia* sp., strain ECCN 24b, *Proteus* sp., strain ECCN 20b, *Proteus* sp., strain ECCN 23b, *Citrobacter* sp., strain ECCN 19b, *Microbacterium* sp., strain ECCN 22b, *Bacillus* sp., strain ECCN 18b, *Bacillus* sp., strain ECCN 26b, *Exiguobacterium* sp., strain ECCN 21b, *Escherichia* sp., strain ECCN 25b and *Bacillus* sp., strain ECCN 41b. Thus, the algae-based WWT bacterial isolates were ranked as *Arthrobacter* sp., strain ECCN 6b, *Enterobacter* sp., strain ECCN 7b, *Aeromonas* sp., strain ECCN 4b, *Microbacterium* sp., strain ECCN 12b, *Ancylobacter* sp., strain ECCN 11b, *Fictibacillus* sp., strain ECCN 2b, *Pseudomonas* sp., strain ECCN 10b, *Exiguobacterium* sp., strain ECCN 5b, *Exiguobacterium* sp., strain ECCN 8b, *Microbacterium* sp., strain ECCN 9b, *Bacillus* sp., strain ECCN 3b and *Bacillus* sp., strain ECCN 1b (Table 3.6).

Table 4. 3 Ranking of bacterial strains according to PGP-like activities. Coal-degrading organisms were isolated either from coal mines (CM), coal slurry (CS) and slurries prepared from diesel-contaminated sites (DCS). However, the WWT bacteria were generated from the MaB-flocs in mixed liquor of the HRAOP of IAPS-supplied municipal sewage.

Bacterial strains	**Microbial Ranks in Terms of PGP-like Activities**				
	PGR		**PEN**		
	IAA	**GA**	**NH_4^+**	**PO_4^{-3}**	**K^+**
CS/DCS bacteria					
Serratia sp. ECCN 24b	+ +	+ -	+ +	+ +	+ +
Proteus sp. ECCN 20b	+ +	+ -	+ +	+ -	- -
Proteus sp. ECCN 23b	+ +	+ -	+ +	+ +	- -
Citrobacter sp. ECCN 19b	+ -	+ -	+ -	+ +	- -
Microbacterium sp. ECCN 22b	- -	+ +	+ +	+ +	- -
Bacillus sp. ECCN 18b	- -	- -	+ +	+ +	+ -
Bacillus sp. ECCN 26b	+ -	- -	+ +	+ +	- -
Exiguobacterium sp. ECCN 21b	- -	+ -	+ +	+ +	- -
Escherichia sp. ECCN 25b	- -	- -	+ +	+ -	- -
Bacillus sp. ECCN 41b	- -	- -	+ +	+ +	- -
HRAOP bacteria					
Arthrobacter sp. ECCN 6b	+ +	+ -	+ +	+ -	- -
Enterobacter sp. ECCN 7b	+ +	+ -	+ +	+ +	+ +
Aeromonas sp. ECCN 4b	+ +	+ -	+ +	+ +	- -
Microbacterium sp. ECCN 12b	- -	+ +	- -	+ -	- -
Ancylobacter sp. ECCN 11b	- -	+ +	- -	- -	- -
Fictibacillus sp. ECCN 2b	+ -	+ -	+ +	- -	- -
Pseudomonas sp. ECCN 10b	+ -	+ -	+ +	+ +	- -
Exiguobacterium sp. ECCN 5b	- -	+ -	+ +	+ +	- -
Exiguobacterium sp. ECCN 8b	- -	+ -	+ +	+ -	- -
Microbacterium sp. ECCN 9b	- -	+ +	+ -	+ -	- -
Bacillus sp. ECCN 3b	- -	+ -	+ +	+ -	- -
Bacillus sp. ECCN 1b	- -	+ -	+ +	- -	- -

Note: Results are from an ANOVA single factor on ranks (where, ++ means good, +- means intermediate; however, -- means lower activity) using Excel (version 16, Microsoft Corporation, Redmond, WA).

4.5 Summary

The work described in this chapter sought to evaluate the PGP characteristics of bacteria isolated from CS coal DCS and HRAOP of the IAPS. Additionally, the ability to tolerate and/or bio-degrade WC was viewed as desirable, given that humics/fulvics are among the reported products. Results were used to rank bacteria according to PGP potential based on strains mineralisation of N, P and K, a capacity to produce indole, auxins (measured as IAA equivalents) and gibberellins (measured GA_3 equivalents), deaminate ACC and siderophore. Based on the results, 19 bacterial strains (86.36%) out of 22 screened were confirmed NH_4^+-producing organisms. Phosphate solubilising activity was low in the solid screening. Only *Pseudomonas* strain ECCN 10b developed halos around bacterial colonies; however, it was more significant in PVK broth with up to 12 most active isolates. Potassium solubilising activity also appeared low in qualitative as well as quantitative screening. Only 2 bacterial strains namely *Enterobacter* strain ECCN 7b and *Bacillus* strain ECCN 18b obtained the solubilization of K^+ in the two test cases.

Coal degradation potential was significant in the strains of *Proteus* (ECCN 20b and ECCN 23b), *Exiguobacterium* (ECCN 21b) and *Serratia* (ECCN 24b). The study's demonstration of the biodegradation potential of bituminous coal by five bacteria isolated from an algae-based water treatment system including *Bacillus* (ECCN 3b), *Aeromonas* (ECCN 4b), *Exiguobacterium* (ECCN 8b), *Pseudomonas* (ECCN 10b) and *Ancylobacter* (ECCN 11b) was a novelty. The strains of bacteria isolated from CS and DCS, including *Bacillus* (ECCN 18b, ECCN 26b and ECCN 41b), *Citrobacter* (ECCN 19b), *Proteus* (ECCN 20b and ECCN 23b), *Exiguobacterium* (ECCN 21b), *Microbacterium* (ECCN 22b), *Serratia* (ECCN 24b), and *Escherichia* (ECCN 25b), appeared to have retained their coal degradation potential and bioactivity.

Bioassay results revealed that the bacterial isolates exude PGP-like substances into the culture medium. Six of the isolates, including *Proteus* spp. (ECCN 20b and ECCN 23b), *Serratia* sp. (ECCN 24b), *Aeromonas* sp. (ECCN 4b), *Arthrobacter* sp. (ECCN 6b) and *Enterobacter* sp. (ECCN 7b) produce indole compounds in NB-supplemented L-tryptophan. *Microbacterium* (ECCN 9b, ECCN 12b and ECCN 22b) and *Ancylobacter* (ECCN 11b) produced and exuded into the culture medium gibberellin-like compounds. In addition, 18.18% of the organisms demonstrated ACC deaminase activity which is known to attenuate the conversion of ACC to the plant hormone, ethylene. Further, iron chelator (i.e., siderophores) activity was also evident in four strains of *Bacillus* (ECCN 18b) and *Citrobacter* (ECCN 19b) originated from CS/DCS and *Enterobacter* (ECCN 7b) and *Pseudomonas* (ECCN 10b) from the HRAOP. Thus, the six competing bacterial strains in terms of auxin-like activity (*Aeromonas* sp., *Arthrobacter* sp., *Enterobacter* sp., *Proteus* sp., *Proteus* sp. and *Serratia* sp.) demonstrated multifunctional PGP activities. These were thus selected for further study to assess their biofertilisation potential individually and in consortium with or without waste coal as an alternative carbonaceous substrate.

Chapter Five: Towards a Bacterial Biofertiliser for Rehabilitation of Coal Mining-Disturbed and Degraded Land

5.1 Introduction

Biofertilisers are products containing PGP organisms with the potential to mineralise and mobilise nutrients from insoluble sources for absorption and assimilation by plants (Vessey 2003; Selvakumar et al. 2009). Biofertilisers can be formulated in gel or emulsion form (Malusá and Vassilev 2014). Just like the mixture of living and latent cells ensuring the fixation of N_2, the solubilisation of mineral P and K rocks, the oxidation of S and Mn, degradation of sedimentary rocks such coal and the production of PGR (IAA and GA). Bio-fertilising bacteria are also part of the microbial component of compost/manure which can accelerate soil biological processes to mobilise and increase plant nutrient availability and biocontrol from pathogens (Grayston and Germida 1991; Vessey 2003; Mishra et al. 2013; Zhang et al. 2015; Sekhohola-Dlamini et al. 2022).

Organisms benefit plants through direct and indirect mechanisms (Ahmad et al. 2014; Backer et al. 2018). Direct mechanisms include the production and mineralisation of nutrients such as N, P, K, S, Fe, and Mn from microbially decomposing organic matter or mineral rocks. These biologically produced nutrients are necessary for plant growth and development and do not require additional chemical input into the soil (Cerrato et al. 2010; Etesami et al. 2017; Titilawo et al. 2020; Basu et al. 2021). Plant-beneficial organisms can also provide carbohydrates in the form of organic polymers called extra-polymeric substances (EPS) (Vejan et al. 2016; Jimoh 2017; Backer et al. 2018) into the rhizosphere. These organic polymers provide a suitable soil environment by maintaining its moisture for microbial bioactivity and facilitating particle aggregation, the process enabling nutrient scavenging by the microbiota and plant root systems (Costa et al. 2018). Indirect mechanisms include the production of biopesticide including antibiotic agents or plant biocontrol. In the rhizosphere, biological control agents allow the host plant to fight against pathogenic bacteria, fungi, and viruses. These processes promote indirectly plant health, growth, and development; process resulting to increased crops yield (Chandler et al. 2011; Pathak et al. 2022).

It has been reported that strains of bacteria and fungi degrading xenobiotic compounds (coal pollutants) might not only be able to remediate or restore land polluted by coal mines but can also fertilise it (Van Breugel et al. 2019; Coban et al. 2022; Sekhohola-Dlamini et al. 2022). Efforts are then required to utilise cost-effective and environmentally friendly resources known as PGP/coal degrading organisms in the development of biofertilisers for restoration and horticulture practices promoting revegetation as well as sustainable agriculture (Sekhohola-Dlamini et al. 2022). The South African government has ordered mining companies to restore and rehabilitate mining land before mine closure to mitigate adverse environmental impacts and facilitate their transition for reuse (Land Rehabilitation Society of Southern Africa, Coaltech, Minerals Council of South Africa 2018). Doing research in biotechnology for the rehabilitation of land degraded by coal mining activities becomes an increased need.

Preliminary studies based on laboratory bioassays revealed that some of the bacterial isolates displayed an ability to produce auxin and gibberellin and mineralise essential plant nutrients such as N, P, and K. After detailed screening, strains *Aeromonas* sp. ECCN 4b, *Arthrobacter* sp. ECCN 6b, *Enterobacter* sp. ECCN 7b, *Proteus* sp. ECCN 20b, *Proteus* sp. ECCN 23b, and *Serratia* sp. ECCN 24b were used as a candidate species to develop a liquid bacterial biofertiliser to support the Fungcoal myco-phytoremediation and restoration strategy currently being implemented for post-coal mining. Bioassay protocols were established using low-rank coal as a substrate to evaluate the biofertiliser and bioremediation performance of competing organisms (*Enterobacter* sp. ECCN 7b, *Proteus* sp. ECCN 20b, and *Serratia* sp. ECCN 24b) singly or in consort. The choice of bioassay was based largely on the need to ensure the germination of grass species like barley (*Hordeum vulgare*), adventitious root and hypocotyl development, and overall vegetative growth response of selected *Fabaceae*, mung bean (*Vigna radiata*).

5.2 Material and Methods

5.2.1 Cultivation of Isolated Bacterial and Fungal Strains

5.2.1.1 Bacterial Culture

Proteus sp., strain ECCN 20b, *Proteus* sp., strain ECCN 23b, *Serratia* sp., strain ECCN 24b, *Aeromonas* sp., strain ECCN 4b, *Arthrobacter* sp., strain ECCN 6b and *Enterobacter* sp., strain ECCN 7b were used in this study. These six competing organisms were selected because of being most prominent in producing IAA and having demonstrated multifunctional activities promoting PG by increasing the availability of essential plant nutrients from organic (peptones) and inorganic (tri-calcium phosphate and potassium aluminosilicate) substrates. Bacterial strains were inoculated on NA and incubated for 3 d at 30 °C. Some pure agar plates were stored as solid seed cultures, while others were used to prepare liquid seed cultures. However, the concentration of the bacterial inoculum as colony-forming units (CFU) was maintained at approximately 10^9 mL^{-1} (Sieuwerts et al. 2008 and Sanders 2012). From each culture sample, a 10 mL aliquot was measured into a 10 mL test tube, labelled, and stored at 4 °C.

5.2.1.2 Fungal Culture

Fungal isolates consisted of three known and previously characterised coal degrading (CD) species viz, strains ECCN 84, ECCN 225 and ECCN 243. Sourced originally from Hope Colliery (ECCN 84) and Excelsior Coal Mine (ECCN 225 and ECCN 243) Witbank, Mpumalanga Province, South Africa (Igbinigie et al. 2008 and 2010), were resurrected from glycerol stocks stored at -80 °C following re-confirmation of their molecular identification as *Aspergillus* sp., strain ECCN 84, *Aspergillus* sp., strain 225 and *Penicillium* sp., strain ECCN 243.

Table 5. 1 Details of CD fungi sourced and isolated from coal mines (CM), identified, and enriched in a minimal salt medium with coal as the sole carbon source (Keshinro 2021).

Strains Number	Source	Microbial Blast Prediction	Identity (%)	GenBank Accession Number	Length (bp)	Microbial Culture Collection
ECCN 84	CM	*Aspergillus* sp.	100	MT239561	600	No deposit
ECCN225	CM	*Aspergillus* sp.	100	MT239564	595	No deposit
ECCN 243	CM	*Penicillium* sp.	100	MT239576	588	No deposit

From glycerol stock, fungal strains were grown in potato dextrose broth (PDB) (24 g L^{-1}) (Sigma-Aldrich, USA), supplemented with 10 g L^{-1} sodium L-glutamate (Merck, KGaA, USA) for 5 to 6 d at 30 °C with the agitation of 120 rpm.

To check the purity of the fungal strains, mycelial aliquots were grown for 6 d and at 30 °C on PDA (39 g L^{-1}) supplemented with 20 g L^{-1} of sodium L-glutamate (Merck, KGaA, USA). After confirmation of microbial purity, some plates were stored as stock cultures at 4 °C for further studies. The liquid seed culture was prepared by collecting pure fungal spores from plates and inoculating 40 mL of PDB. Cultures were incubated at 30 °C (120 rpm) for 3 to 6 d, then, 10 mL aliquots were stored at 4 °C in a 10 mL test tube.

Fungal characterisation included morphological assessment and SEM examination. As microorganisms in solid culture develop colonies whose shapes can help identify genera or even species (Sousa et al. 2013), morphological properties consisting of macroscopic (pigmentation, shape, margin, etc.) and microscopic views were investigated. Then photos were directly taken from fresh PDA plates. At the same time, glass slides containing fungal smears were brought to the Biotechnology Inovation Centre Department, Rhodes University for a microscopic view using an automated Thermo Scientific^{TM} Microscope (EVOSTM Auto 2, Invitrogen, USA).

However, the SEM analysis of fungal isolates was performed following the protocol of the Electron Microscopy Unit (EMU) of Rhodes University, using 1 mL pure fungal culture that was transferred to 2 mL microtubes (SSIBio, 1310-00, 23 USA) and centrifuged at 3920xg for 5 min. The resulting pellets were washed three times in phosphate-buffered saline (PBS) and then placed overnight in 2.5% glutaraldehyde fixative solution at 4°C. The samples were immersed twice in 0.1 M sodium phosphate buffer for 10 min, and the supernatant was discarded. Dehydration was carried out after washing the pallets with 30, 50, 70, 80, 90%, and 100% ethanol. The samples were suspended in absolute ethanol and coated with evaporated carbon (CO_2) using the critical point dryer for 2 h. With the dry point complete, the samples were mounted on stubs with double-sided adhesive tapes and gold-coated (Quorum Q15ORS, England). The SEM views were carried out on an analytical Tescan Vega 2 (Figure 2.3).

5.2.2 Screening Fungi for the Potential to Degrade Waste Coal

To investigate fungal bioactivity in terms of coal biodegradation on plate screening, strains were grown on NA (31 g L^{-1}) (Biolab, Merck, RSA) containing waste coal (WC) or low-rank coal (LRC) (1 g L^{-1}). After 4 d incubation, sterile LRC (~0.5 mm particle size) was scattered over the fungal mycelia and cultures were incubated for a further 7 to 9 d or until detection of colour change from almost dark black to brown grey which was taken as an indicator of coal biodegradation. Biodegradation was determined by the appearance of a brown halo taken to indicate the release of humic acid-like substances (Olawale et al. 2020). Controls were NA plates containing coal particles without fungal inoculants.

5.2.3 Bacteria/Bacteria Biotic Interactions

The bacteria-bacteria biotic interactions carried out in this chapter are described as cell-to-cell growth sensitivity or as the physical association (Zhang et al. 2020; Mandolini et al. 2021) using NA bioassay. Modified NA containing per litre of Milli-Q water, 5 g of peptone (uniLAB, Merch), 3 g of yeast extract (Biolab, Merck, R SA), 8 of NaCl (univAR, Merck) and 15 g of agar (Biolab, Merck) was prepared and autoclaved. Biotic interaction activities of the selected DCS (*Proteus* sp., ECCN 20b, *Proteus* sp., ECCN 23b and *Serratia* sp., ECCN 24b) and from HRAOP (*Aeromonas* sp., ECCN 4b, *Arthrobacter* sp., ECCN 6b and *Enterobacter* sp., ECCN 7b) were examined separately. To monitor cell-to-cell growth sensitivity, triplicate culture assays were performed on NA plates (Irabor and Mmbaga 2017). Bacterial plugs of 10 mm diameter from fresh solid seed cultures were placed at the centre of NA. A triplicate agar plate was prepared for DCS organisms as well as for the algae-based WWT bacteria. The cultures were incubated at 30°C for 3 d followed by assessing the biotic interaction activities that could be determined by observing the possible development of growth inhibition zones surrounding bacterial plugs.

5.2.4 Bacteria/Coal-Degradig *Aspergillus fischeri* strain ECCN 84 Interactions

Screening for biotic interaction activity was performed between selected and potential PGP bacteria namely *Proteus* sp., ECCN 20b, *Proteus* sp., ECCN 23b, *Serratia* sp., ECCN 24b, *Aeromonas* sp., ECCN 4b, *Arthrobacter* sp., ECCN 6b and *Enterobacter* sp., ECCN 7b against a most effective CD fungus, *Aspergillus fischeri* strain ECCN 84. Plate bioassay containing PDA (39 g L^{-1}) supplemented with 20 g of sodium L-glutamate (Merck, KGaA, USA) was used to carry out bacterio-fungal biotic interaction activities. Plates were prepared according to the number (six) of bacterial strains in triplicate. To test the bacterio-fungal biotic interaction, each plate was inoculated with approximately 50 µL of the selected freshly cultured bacterial suspension, and using a glass L-rod the sample was applied to the plate surface. Then, a fungal plug 10 mm in diameter (Toda et al. 2012) was taken from each pure and fresh fungal seed culture (*Aspergillus fischeri*) and transferred to the centre of the corresponding PDA plate. Plates were incubated at 30°C for up to 5 d. Biotic interaction activities could be determined by observing the possible development of growth inhibition zones around fungal plugs.

5.2.5 Bioassays Assessment of PGP Performance of the Novel Bacterial Biofertiliser

5.2.5.1 Barley Seed Germination Bioassay

As barley (*Hordeum vulgare* L.) seeds develop glumes at maturity, their germination capacity is almost reduced due to insufficient contact of ambient O_2 with the embryo. However, abscisic acid (ABA) and gibberellins (GA) interact with O_2 to regulate barley seed dormancy (Bradford et al. 2008). Therefore, the present research used the culture filtrate of the CD bacterium (*Microbacterium* sp., ECCN 22b) as well as the municipal WWT organisms (*Microbacterium* sp., strain ECCN 9b, *Ancylobacter* sp., strain ECCN 11b and *Microbacterium* sp., strain ECCN 12b) to assess the germination sensitivity on barley seeds. The four organisms mentioned above demonstrated an ability to produce gibberellins during the *in vitro* biological tests.

Luria-Bertani broth (*HiMedia*, M1245) containing per litre of Milli-Q water, 10 g of tryptone (Sigma-Aldrich, Mexico), 5 g of yeast extract (Biolab, Merck, RSA), and 10 g of NaCl (Univar, Merck, RSA) was prepared and autoclaved. Two controls included uninoculated LBB as a negative control (Cnt-) as well as commercial GA (50 mg L^{-1}) (Sigma-Aldrich, China) as a positive control (Cnt+). The cultures were then inoculated and incubated at 30 °C, 120 rpm for 3 d. The 45 mL volume of each freshly grown culture (including controls) was centrifuged at 18900xg for 10 min to obtain the cell-free supernatant used for germination sensitivity tests. Barley (*Hordeum vulgare* L.) seeds (Sunnyside Garden Centre, RSA) were washed twice with Milli-Q water before being immersed in 1 % NaOCl (Jik, RSA) for 20 min and rinsed four times. About 50 barley seeds were sown per Whatman paper (Cutiva, China) Petri dish containing 20 mL fresh supernatant. Plates were kept in the dark at 20 °C; seed germination was assessed daily for up to 4 d, and the results were recorded.

5.2.5.2 Mung Bean Adventitious Rooting Bioassay

Mung bean (*Vigna radiata* (L.) R. Wilczek) seeds were surface sterilised by immersion in 80% ethanol for 2 min, then washed and sown in cotton soaked in distilled water in open Petri dishes and incubated in the dark for 2 d at 20 °C. Sprouted plants were transferred to laboratory light and growth continued under ambient conditions for a minimum of 7 d or until hypocotyls were about 5.5 cm long. Supernatants were diluted up to the gradient concentrations of 10^{-2}, 10^{-3} and 10^{-4}. However, the 10^{-3} dilution (0.039 to 0.057 mg L^{-1}, bacteria) and (0.098 mg L^{-1}, authentic IAA), were considered better for adventitious rooting tests due to the number of roots obtained. Hypocotyls from 10 d old seedlings of similar size were cut to 5 cm and placed in test tubes containing 12.5 mL (10^{-3}) of test solution comprising bacteria-free supernatant (18,900 × g for 10 min) from cultures of *Proteus* sp., ECCN 20b, *Proteus* sp., ECCN 23, *Serratia* sp., ECCN 24b, *Aeromonas* sp., ECCN 4b, *Arthrobacter* sp., ECCN 6b and *Enterobacter* sp., ECCN 7b grown in NB supplemented with L-tryptophan (1 g L^{-1}). The uninoculated broth was used as the negative control (Cont-) and NB containing approximately 98 mg L^{-1} of authentic IAA as the positive control (Cont+).

Explants were incubated in laboratory light under ambient conditions for 10 d. Hypocotyls were assessed daily for adventitious root (AR) formation and the number was recorded. When specified, and after a 3 h acclimation period, each hypocotyl's initial fresh mass (FM_{t0}) was determined. The final fresh mass (FM_{t10}) was determined after 10 d. Then, the hypocotyls were oven-dried at 50 °C to constant weight, and the final dry mass (DM_{t10}) was determined.

5.2.6 Effect of Liquid Biofertiliser on Growth of Mung Bean Seedlings

The liquid biofertiliser used in this study was developed from a modified yeast mannitol (YEMA) broth. The medium composition per litre of Milli-Q water was 1 g of yeast extract

(Biolab, Merck, RSA), 10 g of mannitol, 0.5 g of K_2HPO_4 (Sigma-Aldrich, USA), 0 .2 g of $MgSO_4$ (Unilab, Saarchem, RSA), 0.1 g of NaCl (Sigma-Aldrich, RSA), 1 g of $CaCO_3$ (Pal Chemicals), and 1 g of waste coal (WC). The cultures were then seeded using individual or consortium inoculants from the cultures of *Proteus* sp., ECCN 20b, *Serratia* sp., ECCN 24b and *Enterobacter* sp., ECCN 7b for a total of 8 treatments. These 8 treatments comprised three individual cultures of *Enterobacter* sp ECCN 7b (1), *Proteus* sp. ECCN 20b (2), *Serratia* sp. ECCN 24b (3) and five combined cultures of *Enterobacter* + *Proteus* (4), *Enterobacter* + *Serratia* (5), *Proteus* + *Serratia* (6), *Serratia* + *Enterobacter* (6), *Enterobacter* + *Proteus* + *Serratia* (7) terminated by the Control (8).

However, some bioassays assessing the regulators and nutrients in each liquid bacterial biofertiliser (IAA, NH_4^+, PO_4^{-3}, K^+, and siderophores) were carried out. In addition, the antibiotic resistance (ANT.R) activity using two antibiotics, rifampicin and tetracycline was performed from single and mostly consortium cultures.

Cultures were then incubated at 30 °C, 120 rpm for up to 5 d and after that, 30 mL of each freshly grown culture (including controls) was centrifuged at 18900xg for 10 min to obtain the cell-free supernatant. The supernatant was diluted to the gradient concentration of $10^{-2,}$ which was better for performing a liquid bacterial biofertiliser formulation test due to the expected results obtained.

The vegetative growth response of *Vigna radiata* (L.) to bacterial biofertiliser using hypocotyls from 12-d-old seedlings of 6 to 8 cm long was investigated. Initially, four parameters including hypocotyl's initial fresh mass (HFM_{t0}), root initial fresh mass (RFM_{t0}), hypocotyl initial height (HH_{t0}) and root initial height (RH_{t0}) were measured. However, three mung bean seedlings were equidistantly placed in the plastic specimen bottle containing each 55 mL of YEMA liquid biofertiliser and transferred to laboratory light to continue their development under ambient temperature and for a 10-d incubation period. Then, hypocotyl's final fresh mass (HFM_{t10}), root final fresh mass (RFM_{t10}), hypocotyl final height (HH_{t10}) and root final height (RH_{t10}) were measured. Finally. the hypocotyls were oven-dried at 50 °C to constant weight, and the final dry mass (DM_{t10}) was recorded.

5.3 Data and Statistical Analysis

All data were analysed using Excel (version 16, Microsoft Corporation, Redmond, WA). Where appropriate, the results were analysed by one-way analysis of variance (ANOVA), and significant differences between measures for the treatments were determined. Data are presented as the mean of at least three determinations ± standard error (SE). Principal component analysis was performed for the ranking of bacterial biofertiliser potential activities using the Kruskal-Wallis H test ($P<0.050$) in Sigma Plot Version 11 (Systat Software Inc., San Jose, California, USA).

5.4 Results

5.4.1 Viability of Most Performed Microbial Culture Collections

Ten bacteria and three fungi were selected as they performed coal degradation (CD) and displayed multiple PGP-likes activities. Bacterial strains included the isolates *Aeromonas* sp. ECCN 4b, *Arthrobacter* sp. ECCN 6b *Enterobacter* sp. ECCN 7b, *Microbacterium* sp. ECCN 9b, *Ancylobacter* sp. ECCN 11b, *Microbacterium* sp. ECCN 12b, *Proteus* sp. ECCN 20b, *Microbacterium* sp. ECCN 22b, *Proteus* sp. ECCN 23b and *Serratia* sp. ECCN 24b (Figure 4.1). While fungal isolates were identified as *Aspergillus* sp. ECCN 84, *Aspergillus* sp. ECCN 225 and *Penicillium* sp. ECCN 243 (Igbinigie et al. 2008; Keshinro 2021) (Figure 5.1).

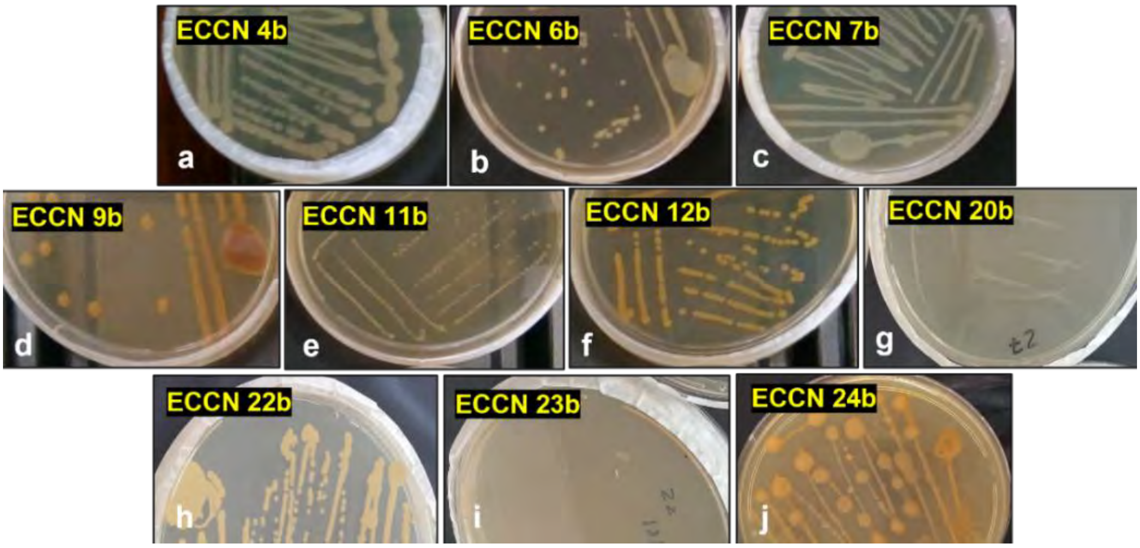


Figure 5. 1 Confirmation of bacterial strains purity and colony morphology.

The verification and maintenance of strain purity were performed after sub-culturing bacteria from fresh NB to NA at 30 °C and 1 to 2 d incubation and fungi from PDB to PDA for 5 d. Morphological analysis was carried out in comparison to the images of the original strains and the purity of all organisms was confirmed as presented in Figures 5.1 and 5.2.

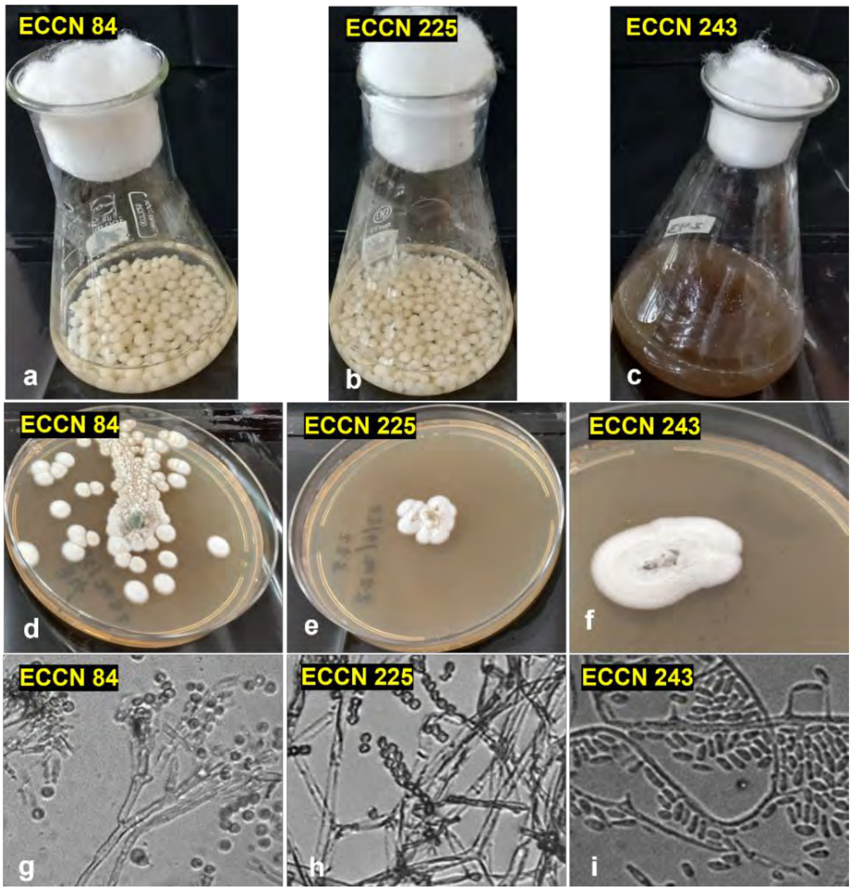


Figure 5. 2 Cultivation of fungal strains and morphology in PDB (a-c) and PDA (d-f). Microscopic view (g-i) was carried out using an automated microscope (EVOS^{TM} Auto 2, Invitrogen, USA). However, ECCN 84 (a, d, and g) and ECCN 225 (b, e, and h) were identified as *Aspergillus* spp., while ECCN 243 (c, f, and i) as *Penicillium* sp.

The SEM results showed the presence of conidia developed in two fungal strains, *Aspergillus* sp., strain ECCN 84 (A) and *Penicillium* sp., strain ECCN 243 (C) (Figure 5.3).

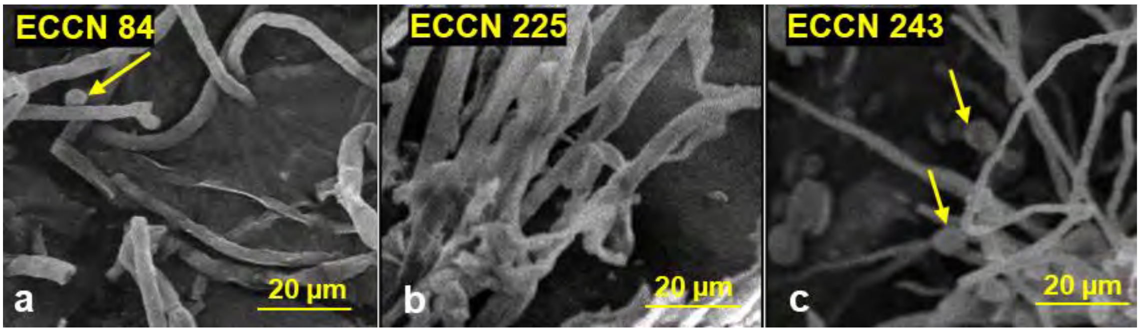


Figure 5. 3 Scanning electron micrographs showing mycelial and conidia morphology of fungal strains identified as *Aspergillus* sp., strain ECCN 84 (A), *Aspergillus* sp., strain ECCN 225 (B), and *Penicillium* sp., strain CCN 243 (C) visualised using an analytical Vega Tescan SEM (LMU -TPD 011, Germany).

5.4.2 Fungal Coal-Degradation Activities

As seen in Figure 5.4 (all data not displayed), colour changes from black to grey-brown were considered positive for the ability of fungi to degrade WC (Figure 5.4a). Therefore, *Aspergillus* sp., strain ECCN 84 was the best-performing fungus for biodegradation of WC. However, the colour intensity was higher when degradation was greater. No colour change was observed in the controls, indicating no spontaneous leaching of any latent or residual material such as humic/fulvic acid into the coal as carbon substrates (Figure 5.4b).

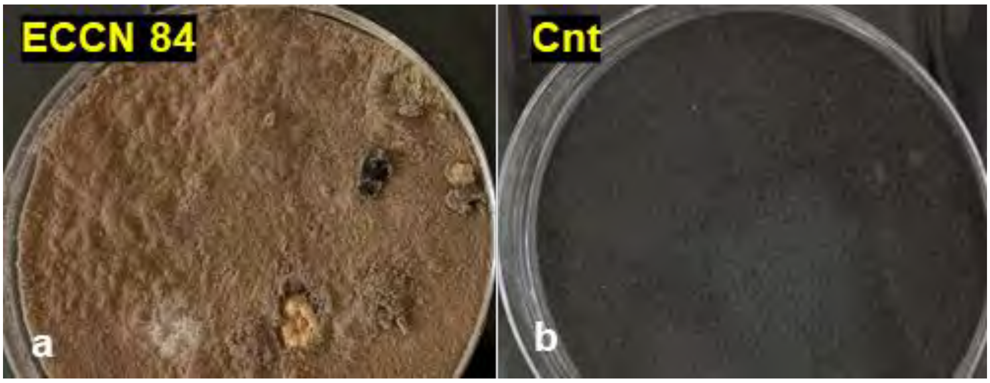


Figure 5. 4 Fungal **c**oal degradation activity. The colour changes of the coal substrate from dark black (Cnt) to brown grey (a) showed fungal coal bioconversion. However, ECCN 84 represented the fungal strain *Aspergillus* fischeri. Whereas Cnt was the control treatment or untreated (b).

5.4.3 Biotic Interactions Bacteria-Bacteria

The in *vitro* bacterial biotic interaction activity was determined using a cell-to-cell growth sensitivity approach. The biotic interactions of bacteria generated from biologically treated wastewater and those from DCS were carried out separately (Figure 5.5).

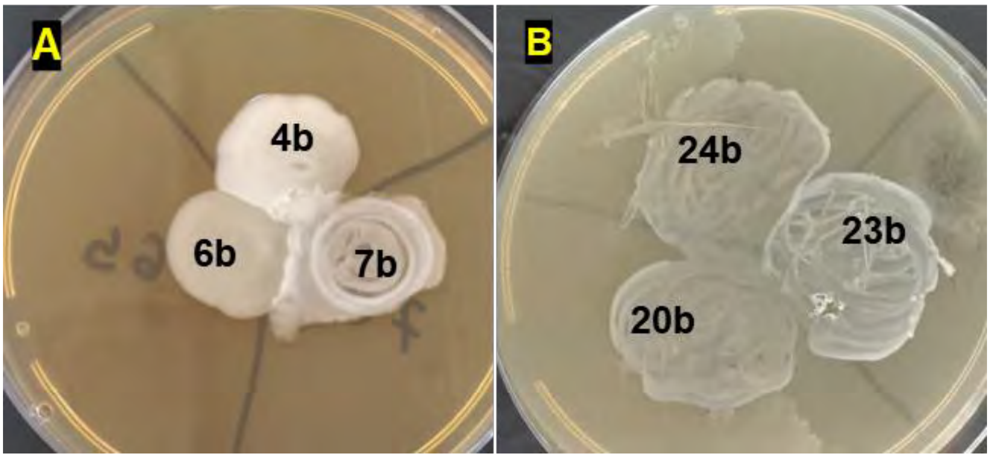


Figure 5. 5 Biotic antagonism activities bacteria-bacteria originated from HRAOP (A) and DCS (B) and grown on NA for 3 d and at 30 °C. Organisms were identified as *Aeromonas* sp. ECCN 4b (4b), *Arthrobacter* sp. ECCN 6b (6b), *Enterobacter* sp. ECCN 7b (7c), *Proteus* sp. ECCN 20b (20b). *Proteus* sp. ECCN 23b (23b) and *Serratia* sp. ECCN 24b (24b).

The microbial antagonistic activities could be determined after observing the development of growth inhibition zones surrounding the bacterial plugs. According to the results in Figure 5.5, no antagonistic activity between bacteria could be determined. The organisms showed mutual acceptance by growing together without producing inhibition zones around bacterial plugs. The bacterial growth plugs increased from 10 mm to 12 mm (A) and 14 mm (B) after 3 d of incubation. The cell-to-cell growth sensitivity was then found to be positive. As symbiotically active, these organisms appear amenable to being combined or cultured in microbial consortia for biofertiliser formulation.

5.4.4 Biotic Interactions Bacteria-Fungus *Aspergillus fischeri*, Strain ECCN 84

Biotic stress tolerance between bacterial and the fungal strain *Aspergillus fischeri*, strain ECCN 84 was assessed by monitoring the cell-to-cell growth sensitivity on PDA plates incubated at 30°C for 5 d.

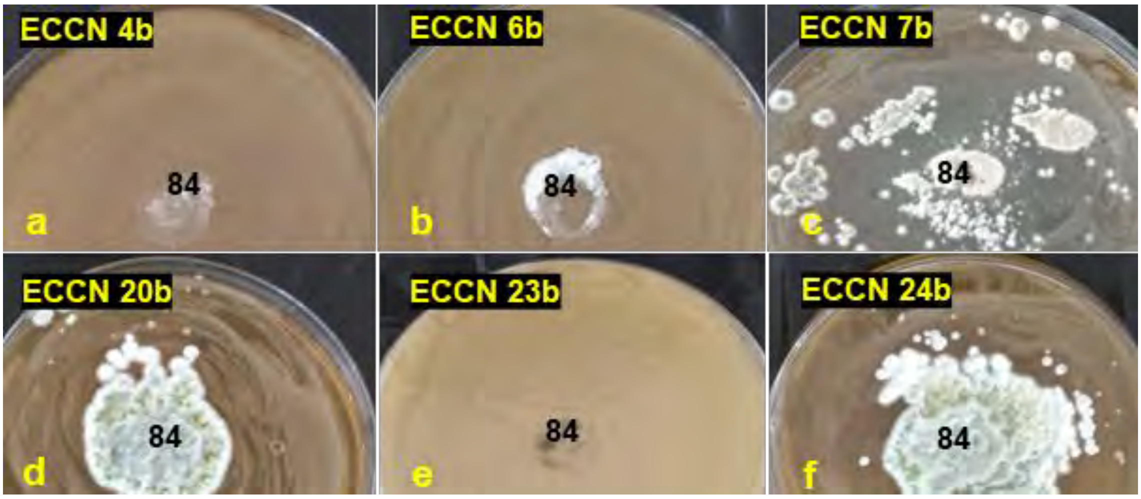


Figure 5. 6 Interspecific biotic antagonism activities between bacteria *Aeromonas* sp. ECCN 4b, *Arthrobacter* sp. ECCN 6b, *Enterobacter* sp. ECCN 7b, *Proteus* sp. ECCN 20b. *Proteus* sp. ECCN 23b and *Serratia* sp. ECCN 24b and the fungus *Aspergillus fishery* (84). Organisms were cultured on PDA for 5 d at 30 °C. Inhibition of fungal growth observed (a-b, e) was used to estimate biotic antagonistic activity.

The bacterio-fungal biotic antagonism was established based on the development of a growth inhibition zone around the bacterial plugs. This study used inhibition of fungal growth (84) by bacterial cultures (ECCN 4b, ECCN 6b and ECCN 23b). Among the six organisms examined, three strains of *Enterobacter* sp. ECCN 7b, *Proteus* sp. ECCN 20b and *Serratia* sp. ECCN 24b did not show antagonistic biotic activity (Figure 5.6c-d, f). These bacteria could be used to develop a mutualistic or symbiotic relationship with *Aspergillus fischeri* allowing the diameter of the fungal plug to reach 15 mm or more in bacterial culture environments. On the other hand, *Aeromonas* sp., strain ECCN 4b and *Arthrobacter* sp., strain ECCN 6b expressed inhibitory activity against *Aspergillus fischeri*, strain ECCN 84. Finally, *Proteus* sp., strain ECCN 23b, demonstrated total inhibitory activity by completely suppressing fungal growth (Figure 5.6e).

5.4.5 Plant Growth Promoting Performances

5.4.5.1 Barley Seed Germination Test

Four potential GA-producing bacteria including *Microbacterium* sp., ECCN 9b, *Ancylobacter* sp., ECCN 11b, *Microbacterium* sp., ECCN 12b, and *Microbacterium* sp., ECCN 22b were evaluated in terms of induction/acceleration of barley (*Hordeum vulgare* L.) seed germination. Fresh LBB culture supernatants were used as GA sources. Uninoculated LBB was prepared as Cnt- while LBB containing commercial GA (50 mg L-1) as Cnt+.

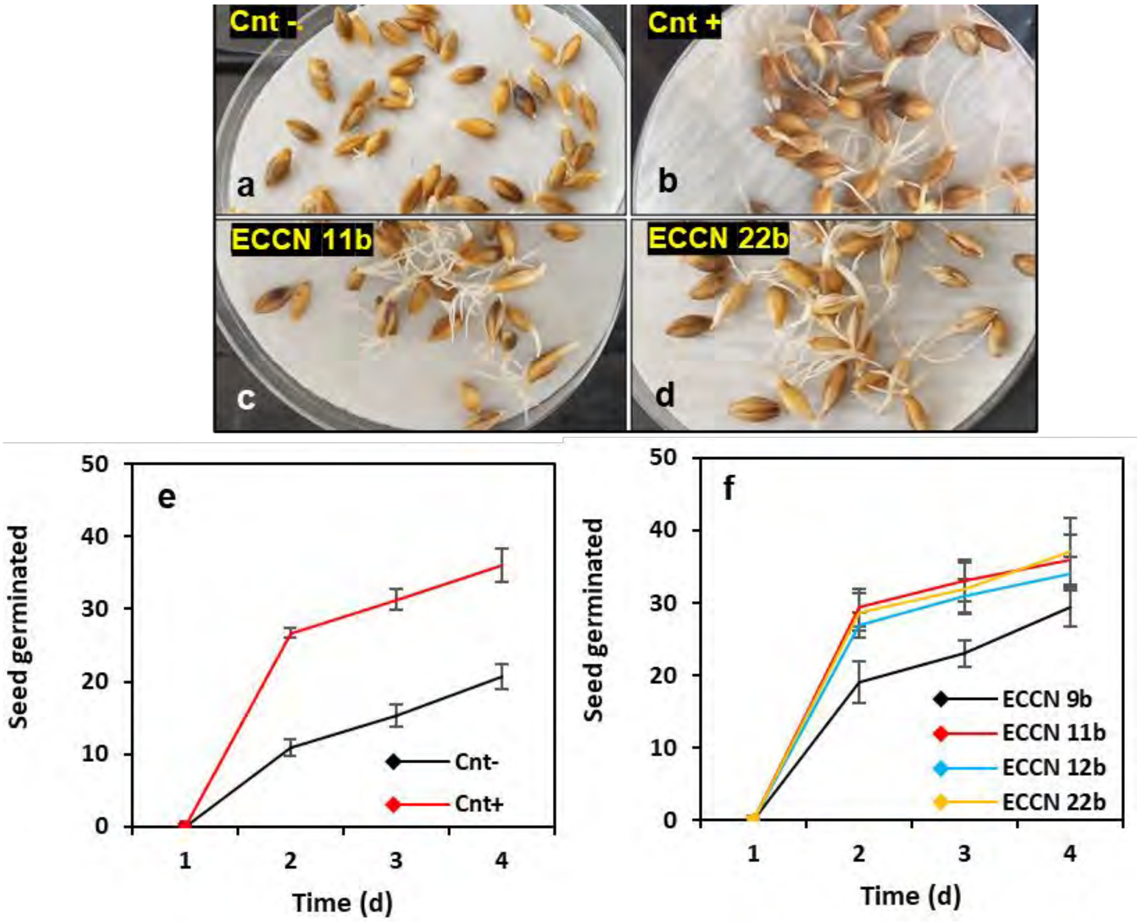


Figure 5. 7 Assay showing the impact of bacterial GA on barley seed germination. The organisms were cultured on LBB for 5 d at 30 °C and 120 rpm. The cell-free supernatants were used as the sources of GA. The seed germination rates were significantly different ($p < 0.05$) compared to the Cnt- treatments. Results are expressed as the mean ± S.E of bacterial filtrate treatments.

The results (Figure 5.7) showed how all strains could induce the germination of barley seeds. However, the three most effective bacteria included *Microbacterium* sp. ECCN 22b, with a GR equivalent to 74.00%, *Ancylobacter* sp. ECCN 11b (72.00%) and *Microbacterium* sp. ECCN 12b (68.00%) while the GR of Cnt+ was 72.00% and that of Cnt-, 42.00%. There was no significant difference between Cnt+ and all bacterial treatments ($p>0.05$).

5.4.5.2 Mung Beans Adventitious Rooting Formation

All six PGPB produced an amount of IAA between 63.82 and 69.72 mg L^{-1}. Some indole compounds could even be observed as pink or red colour changes in the culture of *Enterobacter* sp. ECCN 7b (7b), *Proteus* sp. ECCN 20b (20b), *Proteus* sp. ECCN 23b (23b) and *Serratia* sp. ECCN 24b (24b) (Figure 5.8).

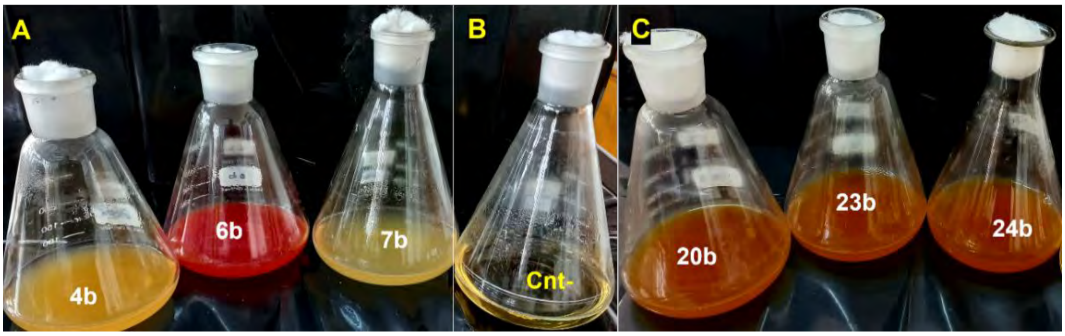


Figure 5. 8 Images showing the algae-based WWT bacterial strains promoting plant growth and producing IAA (a) followed by WC- degrading bacteria (c) joined by the Cnt- (b). The organisms were cultured on NB containing L-tryptophan (1 g L^{-1}). *Aeromonas* sp. ECCN 4b (4b), *Arthrobacter* sp. ECCN 6b (6b), *Enterobacter* sp. ECCN 7b (7b), *Proteus* sp. ECCN 20b (20b), *Proteus* sp. ECCN 23b (23b) and *Serratia* sp. ECCN 24b (24b).

The percentage germination of mung bean seeds was 79.63% and the hypocotyls used as explants were healthy and robust, of a good size of around 5.5 cm (Figure 4.9b.

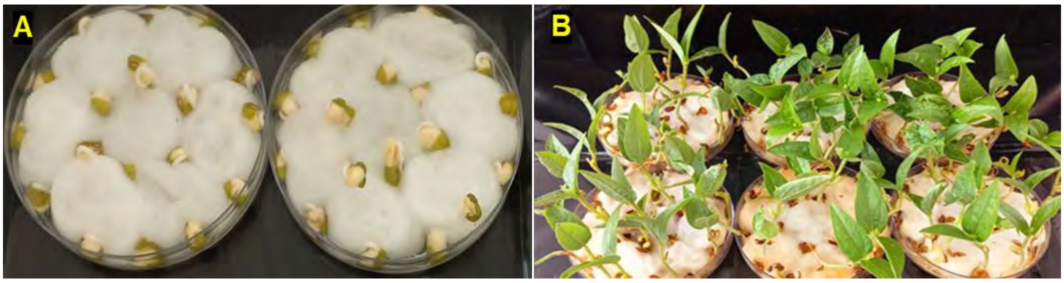


Figure 5. 9 Plate assay (a) showing the germination of mung bean seeds (b). Seeds were sown in cotton soaked in distilled water in open Petri dishes and incubated in the dark for 2 d at 20 °C (a). Sprouted plants were transferred to laboratory light, and growth continued under ambient conditions for a minimum of 7 d or until hypocotyls were about 5.5 cm long (b).

Indole production increased linearly for cultures of the six top-ranked strains viz., *Aeromonas* ECCN 4b, *Arthrobacter* ECCN 6b, *Enterobacter* ECCN 7b, *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b in response to increasing concentrations of L-tryptophan. However, the resulting culture filtrates (dilution 10^{-3}) stimulated adventitious root formation by mung bean hypocotyls compared to Cnt- and Cnt+ (Figure 5.9).

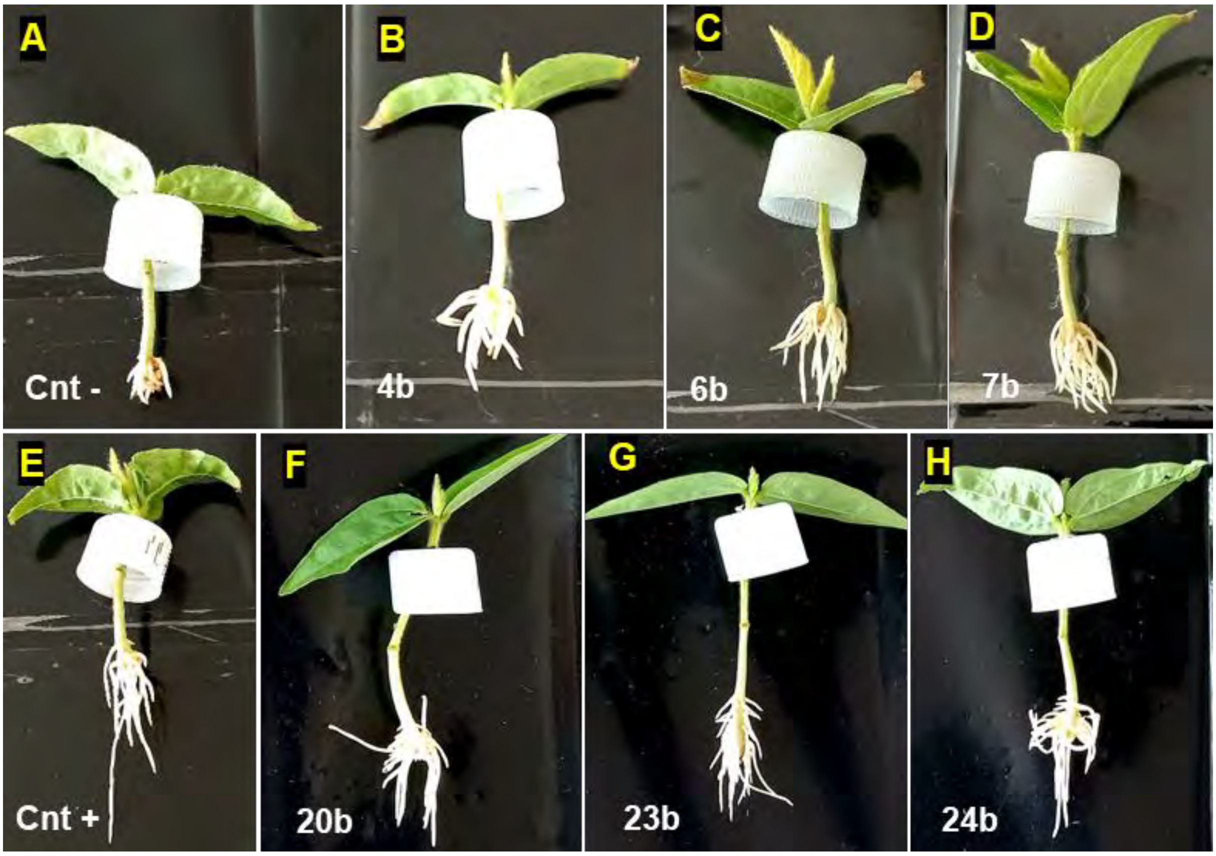


Figure 5. 10 Adventitious root formation in mung bean hypocotyls by cell-free filtrates of *Aeromonas* ECCN 4b (4b), *Arthrobacter* ECCN 6b (6b), *Enterobacter* ECCN 7b (7b), *Proteus* ECCN 20b (20b), *Proteus* ECCN 23b (23b) and *Serratia* ECCN 24b (24b) grown in the medium L-tryptophan-supplemented Roots formed in mung bean hypocotyls treated either with IAA (Cnt+) or cell-free bacterial filtrate (b-d, f-h) were significantly different ($p<0.05$) compared to the control, Cnt- (a).

The time course of adventitious roots and the fresh dry mass obtained are illustrated in Figures 5.10 and 5.11. There was no significant difference ($p>0.05$) between the positive control (IAA-treated hypocotyls) and those treated with cell-free filtrate from L-tryptophan-supplemented cultures of *Aeromonas* ECCN 4b, *Enterobacter* ECCN 7b, *Arthrobacter* ECCN 6b, *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b. Interestingly and as shown in Figure 5.11, d-f, the dry mass of the adventitious roots formed was almost identical, with no discernible difference in the detected mass ($p>0.05$) but significantly different in comparison to the negative controls ($p<0.05$).

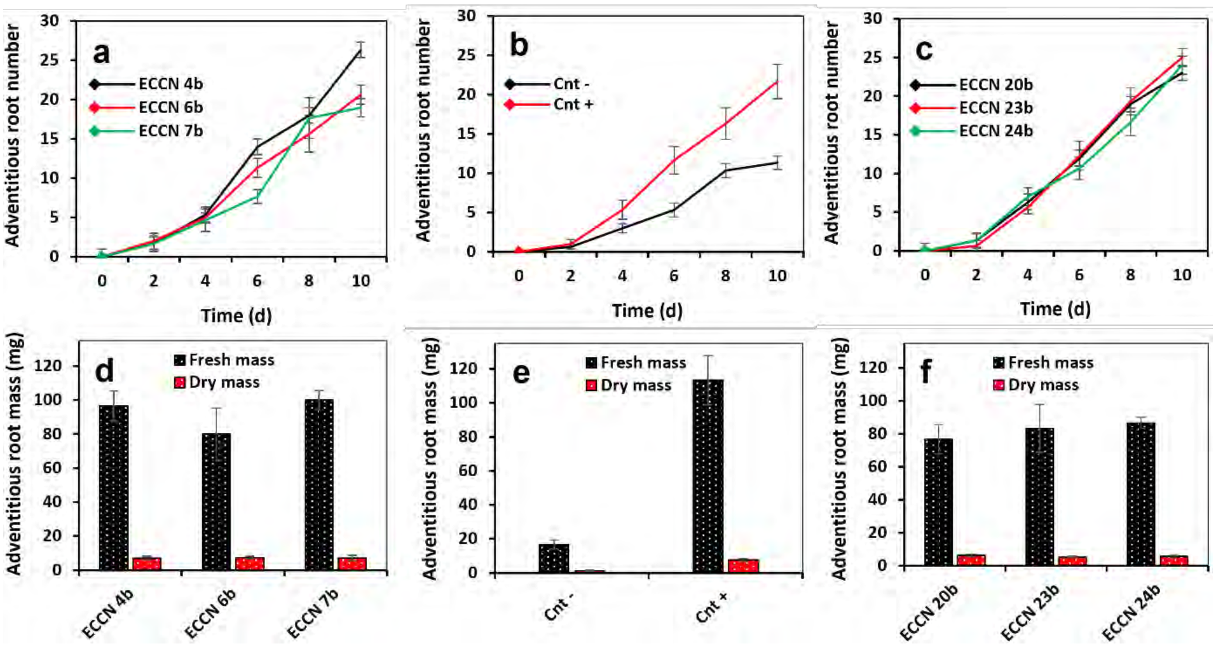


Figure 5. 11 Number of adventitious root numbers per day (a-c). Biomass FM_{t0} and the DM_{t10} of mung bean (*Vigna radiata* L.*)* (d-f). The daily adventitious root production was assessed every 2 d for a period of 10 d.

To rule out the probability that the results in Figure 5.11 were due to differences in the physiological status of the mung bean explants, FM_{t0}, FM_{t10} and DM_{t10} were determined, and the results are shown in Figure 5.12. Statistical analysis and one-way ANOVA revealed no significant difference in either FM_{t0} or DM_{t10} ($p>0.05$), confirming the uniformity of the mung bean explants used in the bioassay.

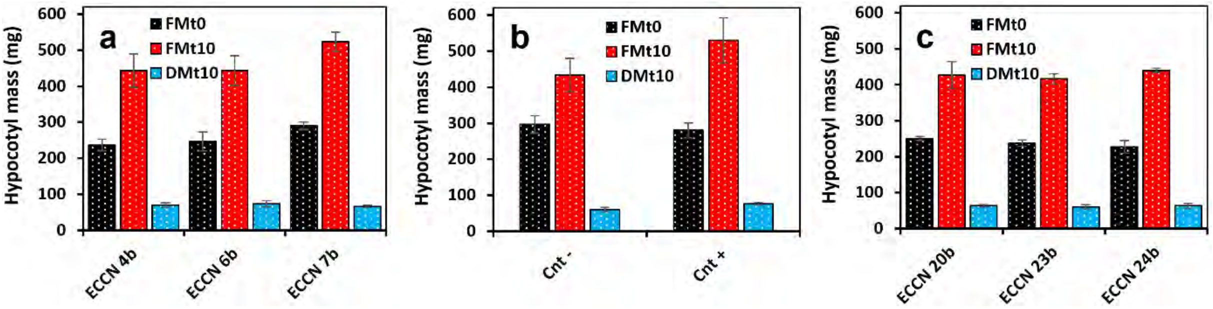


Figure 5. 12 Fresh and dry mass of mung bean hypocotyls treated either with IAA or with culture filtrates from L-tryptophan-supplemented medium of *Aeromonas* ECCN 4b, *Arthrobacter* ECCN 6b, *Enterobacter* ECCN 7b, *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b. The FM_{t0} was determined at the beginning of the experiment, while FM_{t10} and DM_{t10} were measured after a 10-d incubation period.

5.4.6 Mung Bean Vegetative Growth Response

Enterobacter sp. ECCN 7b, *Proteus* sp. ECCN 20b and *Serratia* sp. ECCN 24b were then selected as the best for the preparation of bacterial biofertilisers. The liquid bacterial biofertiliser from modified YEMA broth accounted for 8 treatments including three individual cultures of *Enterobacter* sp. ECCN 7b (7b), *Proteus* sp. ECCN 20b (20b), *Serratia* sp. ECCN

24b (24b) and four combined cultures of *Enterobacter* + *Proteus* (7b+20b), *Enterobacter* + *Serratia* (7b+24b), *Proteus* + *Serratia* (20b+24b), *Enterobacter* + *Proteus* + *Serratia* (7b+20b+24b) terminated by the Cnt- as illustrated in the following Table 5.2.

Table 5. 2 Culture treatment for bacterial biofertiliser preparation from selected organisms sourced either from algae-based WWT bacterium *Enterobacter* sp., strain ECCN 7b (7b), and from CM organisms identified as *Proteus* sp., strain ECCN 20b (20b) and *Serratia* sp., strain ECCN 24b (24b). Organisms were grown in YEMA broth supplemented with waste coal (1g L^{-1}) for 5 d at 30 °C and 120 rpm.

Liquid Bacterial Biofertiliser Treatment Formulation							
Single Cultures			**Consortia/Combined Cultures**				**Control**
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
7b	20b	24b	7b + 20b	7b + 24b	20b + 24b	7b + 20b + 24b	Cnt-

The mung bean seeds were germinated in sand so the root system could not be damaged during the transplantation process (Figure 5.13).

Figure 5. 13 Mung bean seeds (a) germination in silicate sands containing Milli-Q water in open plastic plates. The incubation was carried out in the dark for 2 d at 20 °C (a). Sprouted plants were transferred to laboratory light and growth continued under ambient conditions for 10 d or until hypocotyls were about 6 cm long (b).

Referring to Table 5.3 and Figure 5.14 it was observed that the biofertiliser nutrient content seemed to maintain good records in the combined cultures compared to the individual cultures. Indole and ammonium were sufficiently produced by the consortia number 5, *Enterobacter* strain ECCN 7b and *Proteus* strain ECCN 20b (7b+20b), number 6 *Proteus* strain ECCN 20b with *Serratia* strain ECCN 24b (20b+24b), and number 7 *Enterobacter* strain ECCN 7b, *Proteus* strain ECCN 20b and *Serratia* stain ECCN 24b (7b+20b+24b). The average amount of IAA equivalent produced without L-tryptophan ranged from 8.65 to 9.04 mg L^{-1} compared to single cultures, 3.65 to 8.46 mg L^{-1} was summarised in Table 5.3.

Table 5. 3 The growth regulator and nutrient activity of the bacterial biofertiliser developed using cultures of *Enterobacter* sp. ECCN 7b, *Proteus* sp. ECCN 20b, and *Serratia* sp. ECCN 24b. These organisms were grown in YEMA broth supplemented with waste coal (1g L^{-1}) for 5 d at 30 °C and 120 rpm.

Biofertiliser Compounds Production and Microbial Antibiotic Resistance Activity								
		Single Cultures			**Consortia/Combined Cultures**			
		1	**2**	**3**	**4**	**5**	**6**	**7**
PGP/ANT.R		7b	20b	24b	7b+20b	7b+24b	20b+24b	7b+20b+24b
IAA	mg L^{-1}	**3.65**	**7.95**	**8.46**	**3.01**	**9.04**	**8.72**	**8.65**
NH_4^+	mg L^{-1}	**8.44**	**13.56**	**14.50**	**2.98**	**8.34**	**13.24**	**7.58**
PO_4^{-3}	mg L^{-1}	**8.49**	**0.05**	**0.04**	**17.73**	**14.45**	**0.07**	**5.79**
K^+	mm	**7.00**	**6.67**	**6.00**	**9.00**	**9.33**	**7.33**	**9.33**
SDR	mm	**81.67**	**0.00**	**0.00**	**73.33**	**29.33**	**0.00**	**84.33**
ANT.R	R (mm)	**7.33**	**8,33**	**8.67**	**7.67**	**8.33**	**10.00**	**10.67**
	T (mm)	**18.33**	**7.33**	**6.33**	**7.33**	**8.00**	**17.00**	**15.00**

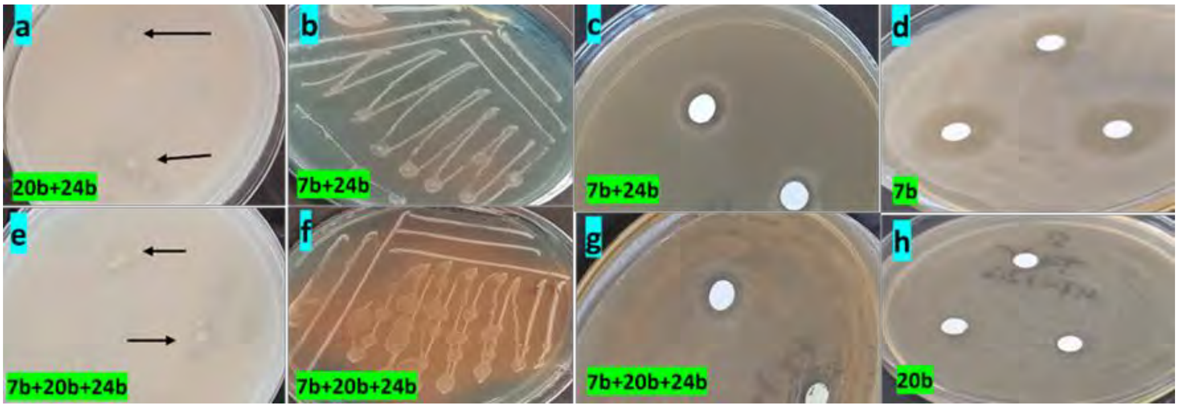


Figure 5. 14 Plate assays showing PGP and bacterial antibiotic resistance (ANT.R) activity to rifampicin (c and g) as well as tetracycline (d and h) using YEMA agar. Solid Aleksandrow medium (SAM) was using K solubilisation assays (a and e), while CAS blue agar for SDR (b and f) activity.

The effective results were observed for siderophores (SDR) activity except for the consortium culture 6 which appears to possess any SDR activity as no appearance of orange halos was observed (Figure 5.14b and f). Consortium cultures 6 and 7 did not show any antibiotic resistance (ANT.R) activity. The biofertiliser dilution 10^{-2} was considered better for vegetative growth response tests of mung beans (*Vigna radiata* L.) plants as it produced the expected results. Shoot and root development (Figure 5.15) were expressed as plant biomass production.

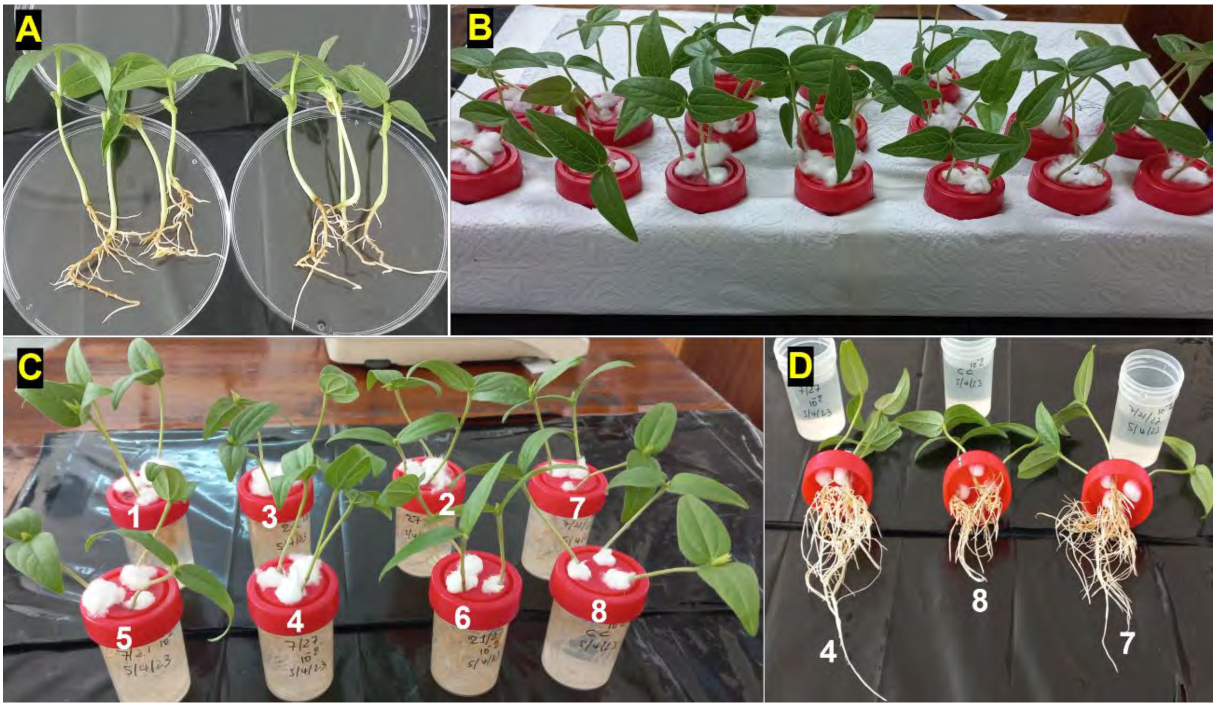


Figure 5. 15 Vegetative growth response of mung bean hypocotyls to a liquid biofertiliser produced by single (c1-3) and combined (c4-7) microbial cultures of *Enterobacter* ECCN 7b (c1), *Proteus* ECCN 20b (c2) and *Serratia* ECCN 24b (c3). Organisms were grown in L-tryptophan and WC-supplemented YEMA broth. The overall plant biomass formation of mung bean was significantly different ($p<0.05$) compared to the Cnt- (d8).

Interestingly, as shown in Figures 5.16a and b, consortia are ranked first in both cases. In the root development test, the average mass production was evaluated as 466.67 mg for the combined culture of 7b+20b+24b (*Enterobacter* ECCN 7b, *Proteus* ECCN 20b and *Serratia* ECCN 24b) followed by 7b +24b, 460.00 mg then single culture of *Serratia* ECCN 24b 453.33 mg.

According to the results shown in Figure 5.16a, FMt10 and DMt10 root masses were significantly different ($p < 0.05$) compared to bacterial biofertiliser filtrates and Cnt treatments. However, as significant differences were observed between treatments, it is entirely probable that YEMA positively affected plant biomass production liquid biofertiliser. Finally, plant biomass production results showed the top bacterial biofertilisers ranked as 7b+20b (840.00 mg), 7b+20b+24b (830.00 mg) and 7b +24b (823.33

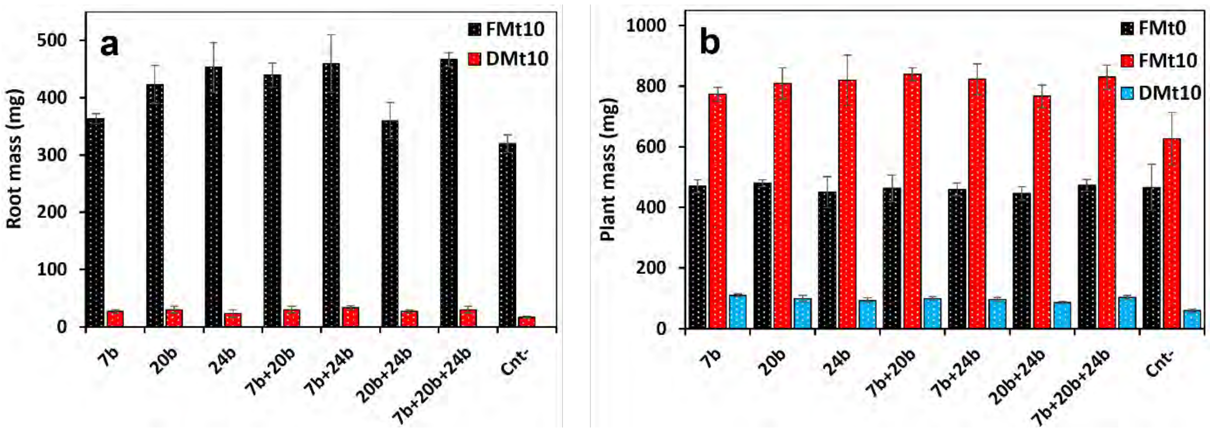


Figure 5. 16 Vegetative growth response of mung bean to liquid bacterial biofertiliser from single or consortia cultures of *Enterobacter* ECCN 7b, *Proteus* ECCN 20b, *Serratia* ECCN24b. The growth is expressed as root (a) and plant (b) biomasses. The FM$_{t0}$ was determined at the start of the experiment while FM$_{t10}$ and DM$_{t10}$ after a 10-d incubation period.

5.5 Summary

In this chapter, the results of bioassays to produce bacterial biofertiliser intended to support the Fungcoal phytoremediation strategy established that:

The fungal strain *Aspergillus fischeri* ECCN 84 still maintains the biological activity linked to the degradation/solubilisation of WC. However, three organisms among the six selected and known as *Enterobacter* sp., strain ECCN 7b, *Proteus* sp., strain ECCN 20b and *Serratia* sp., ECCN 24b showed no biotic antagonism against *A. fischeri* ECCN 84. The bacterial culture filtrate of *Microbacterium* sp., ECCN 9b, *Ancylobacter* sp., ECCN 11b, *Microbacterium* sp., ECCN 12 and *Microbacterium* sp., ECCN 22b contained PGR compounds that acted as authentic and commercial GA by inducing barley seeds (*Hordeum vulgare* L.). Additionally, three selected and potential PGPB, namely *Enterobacter* sp., strain ECCN 7b, *Proteus* sp., strain ECCN 20b and *Serratia* sp., ECCN 24b had produced indole compounds that activated adventitious root formation of mung bean (*Vigna radiata* L.) hypocotyls. Finally, no antibiotic resistance was observed from consortium cultures. They produced better biofertilisers and resulted in greater root and plant biomass of mung bean explants.

Chapter Six: General Discussion and Conclusion

Coal mining activities exist in many countries, including Australia, the United States, the United Kingdom, Mongolia, India, China, and South Africa (Measham et al. 2013), as the primary sources of economic growth and job creation. In South Africa, coal mining provides around 70% of the country's energy, and about 90% of its electricity is generated from coal-fired power stations. In addition to electricity generation by Eskom, Sasol's coal conversion offers approximately 50% of the country's liquid fuel needs (Jeffrey et al. 2015). Together, these activities are responsible for effects that deteriorate the structure of environmental ecosystems and the biochemical properties of soils and soil microbiomes (Laisani and Jegede 2019). The South African government currently tasks mining companies to responsibly rehabilitate mined land upon cessation of mining activities and before mine closure to mitigate any adverse environmental impacts facilitate the transition for land reuse and build and develop a circular economy system in coal companies (Zhao et al. 2011; Land Rehabilitation Society of Southern Africa, Coaltech, Minerals Council of South Africa 2018). Therefore, efforts that make use of cost-effective biological resources to restore soil physicochemical, biochemical, and microbiological properties to promote sustainable revegetation are necessary and urgently required (Land Rehabilitation Society of Southern Africa, Coaltech, Minerals Council of South Africa 2018; Cowan et al. 2016; Sekhohola-Dlamini et al. 2022).

6.1 Rehabilitation of Degraded Land: Towards a Responsible Strategy

The depletion of natural resources, including land degradation, is one of the critical problems facing humans and the entire biosphere. Land degradation has been identified as the most severe source of environmental destruction in South Africa and many countries worldwide (Mesene 2018). Several actions, such as natural disasters, overexploitation of energy reservoirs, industrialisation, agriculture, mining and urbanisation, cause land degradation, which results in the loss of biological activities of the soil and its productive capacity. Known as a global challenge, land degradation directly or indirectly affects current and future ecological products and services, causing numerous environmental and food disruptions, including climate change, food insecurity, and rising food prices (Emadodin and Bork 2012; Azubuike et al. 2016; Global Environment Facility 2022). Due to the varied nature and type of pollutants and the associated land degradation, the need to find better remediation and rehabilitation strategies coupled with the reduced potential for carbon sequestration by polluted soil environments has become a pressing issue (Higgins et al. 2018; Bandyopadhyay and Maiti 2022). To achieve effective and sustainable rehabilitation, environmentally friendly technologies that are passive and adapt to local realities must be developed, transferred, and adopted. Therefore, local natural resources must be prioritised and potentially used sustainably through appropriate technology implementation (Pandey 2001; Mesene 2018), and via a circular economy, before or during the planning, execution, and closure of surface and deep-level coal mines (Pavloudakis et al. 2023). Research has shown that only the principles of the circular economy can contribute to the success of a successful mine closure project. It maximises the recovery of waste materials discarded while preparing rehabilitated mining land by maintaining biomass cycling for formal reuse to support sustainable development (Zhao et al. 2011; Haas et al. 2023; Pavloudakis et al. 2023).

Studies carried out in the EBRU laboratories have shown that microbial biocatalysts, including bacteria (*Achromobacter* sp., *Arthrobacter* sp., *Azotobacter* sp., *Azospirillum* sp., *Bacillus* sp., *Pseudomonas* sp., *Serratia* sp.) and fungi (*Aspergillus* sp. previously known as *Neosartorya fischeri* and *Penicillium* sp.) have the potential to bio-solubilise, bio-convert and bio-degrade coal discard including materials such as BC, LRC or CW, coal fines and others coal residues with the resultant value-added products offering new reuse potential (Sekhohola and Cowan 2017; Olawale et al. 2020). Typical products include soil amendments such as humic and fulvic acids and humic-rich technosols that can be used to replace topsoil. These technosols contain essential nutrients for plant growth and development to support agroecological practices for increased production of healthy food crops. In addition, they become beneficial as a component in bioremediation strategies for restoration of specific polluted environments from coalfields (Gray and Smith 2005; Igbinigie et al. 2008; Olawale et al. 2020; Akimbekov et al. 2022; Masudi et al. 2023). One potential caveat remained; most of these rehabilitation protocols still depend on the use of fossil fuel-derived fertiliser to initiate and ‘sustain’ soil stabilisation through re-vegetation.

6.2 Sustainable Bacterial Biofertiliser to Support Fungcoal Phytoremediation Strategy

As stated in Chapter One, the present study aimed to develop a bespoke bacterial biofertiliser to support the Fungcoal phytoremediation bioprocess for rehabilitating mining-disturbed and degraded land. This process as summarised by Sekhohola-Dlamini et al (2022) is based on the mutualistic interaction between C4 grasses such as *Cynodon dactylon*, *Eragrostis tef*, *Pennisetum clandestinum*, among others root exudate, a suite of arbuscular mycorrhizal fungi (AMF), and at least one coal-degrading fungus, such as the isolates, *Aspergillus* strain ECCN 84 also referred to as *Neosartorya fischeri* strain ECCN 84.

To arrive at a functional biofertiliser, several sources of bacteria were used to find candidate strains with PGR and biofertiliser activity. These organisms included, 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) known to degrade hydrocarbon in polluted soil, waste and weathered coal, and which were later shown to possess plant growth-promoting (PGP) activity (Olawale et al. 2020; Titilawo et al.2020). The second was a group of aerobic pollutant-degrading bacteria (*Bacillus* strain ECCN 1b, *Fictibacillus* strain ECCN 2b, *Bacillus* strain ECCN 3b, *Aeromonas* strain ECCN 4b, *Exiguobacterium* strain ECCN 5b, *Arthrobacter* strain ECCN 6b, *Enterobacter* strain ECCN 7b, *Exiguobacterium* strain ECCN 8b, *Microbacterium* strain ECCN 9b, *Pseudomonas* strain ECCN 10b, *Ancylobacter* strain ECCN 11b, and Microbacterium strain ECCN 12b) isolated from HRAOP of an integrated algae-based WWT process known as IAPS. The third source was a group of newly isolated bacteria from various coals from the Emalahleni Coalfields. No known PGP bacteria have been separated from HRAOP or mined and waste coal. This study reported the biochemical and molecular characterisation of the bacterial isolates with potential for use in the construction of a biofertiliser and the efforts to establish an appropriate consortium capable of replacing fossil fuel-derived and chemically synthesised fertilisers and plant growth regulators.

To achieve this, the specific objectives were to 1) establish viability and bioactivity of the biocatalysts, 2) to screen all organisms for ability to utilise carbonaceous substrates and withstand abiotic stress, 3) to set up bespoke bioassays for assessment of biofertiliser potential, 4) formulate a biofertiliser containing the most active PGPB.

6.2.1 Role of Microbial Catalysts in Bioremediation

As previously stated, bacteria and fungi are intimately involved in various bioremediation processes related to water and sanitation, nutrient cycling, and pollutant removal and/or stabilisation. They impact the environment's physicochemical properties, chemistry, and structure (da Silva et al. 2023; Sekhohola and Cowan 2017; Olawale et al., 2020). Some of the organisms utilised in the present study including *Achromobacter* sp., *Arthrobacter* sp., *Azotobacter* sp., *Azospirillum* sp., *Bacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Aspergillus* sp. (*Neosartorya fischeri*) and *Penicillium* sp., which have been shown to degrade carbonaceous pollutants including LRC and bituminous coals (BC). Furthermore, they demonstrated the ability to solubilise and mineralise potassium alumino silicate and tricalcium phosphate, used in this research as the sole sources of inorganic plant nutrients K and P. The same bacteria showed their ability to produce plant growth regulating substances (IAA, GA, etc.) (Titilawo et al. 2020; Masudi et al. 2023). These biological processes are essential for the bioremediation of specific polluted environments such as coalfields (Gray and Smith 2005; Igbinigie et al. 2008; Olawale et al. 2020; Akimbekov et al. 2022).

Previous studies conducted in EBRU laboratories described bacterial strains isolated from DCS and CS that exhibited coal biodegradation potential as individuals or in consortium (Edeki 2015; Olawale et al. 2020). However, no reported scientific studies have been carried out to study the potential of these organisms in promoting plant growth. According to the biological tests carried out in the present study, these organisms were not alone in performing coal bio-solubilisation. Some of these coal-degrading bacteria were confirmed as plant growth promoting and isolates from HRAOP including *Bacillus* sp. strain ECCN 3b, *Aeromonas* sp., ECCN 4b, *Exiguobacterium* sp., ECCN 8b, *Pseudomonas* sp., strain ECCN 10b, *Microbacterium* strain ECCN 9b, *Microbacterium* strain ECCN 12b, and *Ancylobacter* sp., strain ECCN 11b were able to degrade and solubilise WC or BC. These results showed that like the previously isolated and characterised coal-degrading strains reported by Olewale et al. (2020), these novel isolates could be used in soil bioremediation, especially in degraded land caused by coal mining.

Biodegradation potential, particularly concerning hydrocarbon pollutants, is due to the ability of bacteria and fungi to produce ligninolytic enzymes, which are biological mediators that degrade lignin polymers known as one of the main components of low-rank coal (Mönkemann et al. 1997; Pawlik et al. 2016). The LRC has high lignin content, so its biological degradation is likely to produce humic acids (HA), making this coal type valuable as a source of soil carbon amendment and organic fertilisers (Giannouli et al. 2009; Titilawo et al. 2020). Verification of the organisms' viability before, during, and after research work was essential (Díaz Rodríguez et al. 2021).

A metagenomic analysis of LRC samples showed eight phyla: Firmicutes, Proteobacteria, Bacteriodata, Actinobacteriota, Planctomycetota, Gemmatimonadota and Verrucomicrobiota. However, Firmicutes and Proteobacteria were most abundant, with higher relative abundances of 59.46% and 33.01%. Studies have highlighted how microbial biocatalysts such as bacteria and fungi could be used for post-mining restoration (Lors et al. 2010; Sekhohola and Cowan 2017; Olawale et al. 2020; Akimbekov et al. 2022). According to Strąpoć et al (2011), LRC contains more bioavailable compounds and macerals richer in heteroatoms than HRC and attracts soil microbiota such as Firmicutes (*Bacillus* spp.), Actinobacteriota (*Microbacterium* spp.), Proteobacteria, etc. Studies have shown the benefits of microbial diversity in coal-affected environments and bacteria belonging to the phylum Proteobacteria, can be biological indicators of the potential biodegradation of coal (Lors et al. 2010; Akimbekov et al. 2022).

These organisms with multiple physiological properties can produce biological compounds that can degrade LRC and generate HS via alkalis, biocatalysts and chelates. Organisms producing chelators and alkaline substances attack the macromolecular matrix of the coal and dissolve it into humic substances (Fakoussa and Hofrichter 1999). Additionally, as endophytic, and epiphytic bacteria are abundant in the LRC environment, coal typically comes from plant materials and has inherent plant interaction capabilities (Ezeokoli et al. 2020; Akimbekov et al. 2021; Li et al. 2021; Wagner 2021). Finally, the abundance of Actinobacteria and Firmicutes in coal-polluted soils can easily depolymerise the coal matrix to mobilise plant nutrients such as N, P and K and regulate compounds such as auxins (Sekhohola et al. 2013; Romanowska et al. 2015; Akimbekov et al. 2021; Titilawo et al. 2020).

6.2.2 Impact of Environment on Performance of Biocatalysts

Bacteria have evolved specific substrate utilisation profiles that allow them to grow in various environments and develop resistance to abiotic or biotic stress. They will need to assimilate organic substrates to derive carbon and energy for growth and biomass production (Mekonnen et al. 2019). The present study focused on seven carbon substrates, including fructose, mannitol, sucrose, glucose, sodium L-glutamate, low-rank coal (LRC) and high-rank coal (HRC) or bituminous coal (BC) to assess bacterial carbon utilisation efficiently patterns. About 40.90% of the isolates successfully showed an ability to utilise all substrates. However, some bacterial isolates assimilated other carbon sources, including sodium L-glutamate, fructose, sucrose, and waste coal.

Studies have shown that some microorganisms use different sugars and other available nutrient sources to produce ATP and other metabolites necessary to maintain biological activities related to growth and reproduction (Covert and Moran 2001; Abbaszadeh-Dahaji et al. 2012; Ali et al. 2016; Bren et al. 2016; Mekonnen et al. 2019). Sodium L-glutamate was the preferred carbon source, followed by glucose and sucrose for most of the isolates. Coal was 100% utilised by previously isolated hydrocarbon-degrading bacteria, as all organisms could grow well on agar plates supplemented with LRC and HRC, compared to 83.00% for HRAOP organisms. This observation showed the possibility of using some of these organisms in biofertiliser construction for bioremediation, as they can use recalcitrant materials such as LRC/BC as a carbon source.

As all isolates used in the present study were confirmed to share phylogeny with PGPB (Titilawo et al. 2020; Masudi et al. 2023), they appear to have evolved metabolic behaviour that allowed them to tolerate prevailing conditions and produce enzymes and other molecules and extracellular polysaccharides, making them competent biological indicator organisms (Muglia et al. 2007; Gupta et al. 2012; Gopalakrishnan et al. 2015; Pandey et al. 2015; Wang et al. 2018; Le et al. .2020). Abiotic stress tolerance studies showed that different strains responded differently to a change in conditions such as temperature, salinity and pH confirming previous reports (FDA 2001; Gupta et al. 2012).

Le et al. (2020) demonstrated that some bacteria used for aquaculture wastewater treatment can tolerate high salinity up to 30.00 g L^{-1}. However, according to Egamberdieva et al (2019) and Sharma et al (2021), approximately 20% of irrigated land worldwide is affected by salinity. Salt-tolerant organisms used as biofertilisers could therefore overcome the adverse effects of high salinity to benefit plant growth and yield and help overcome disease resistance. Literature states that, although temperature could be a limiting factor for microbial growth, mesophilic or thermophilic bacteria can tolerate higher temperature environments of up to 45°C or 80°C (Pandey et al. 2015) typical of what might occur at the surface of a waste coal dump. Thermophilic bacteria may possess higher metabolism, physically and chemically stable enzymes, and lower growth but higher product yields (Haki and Rakshit 2003) and be ideally suited to the harsh conditions that prevail on exposed coal and waste coal surfaces. It would be beneficial therefore to use these organisms to develop biofertilisers for land remediation/rehabilitation strategies post coal mining.

However, as the relationships between microbial survival mechanisms and plant growth promotion are not fully understood (Msimbira and Smith 2020), further research on biological catalysts for incorporation into biofertilisers should be encouraged. This study also revealed the effectiveness of organisms from waste or polluted environments in tolerating extreme environments. These potentials can be useful in practices related to recycling and bioremediation strategies for degraded environments.

6.2.3 Plant Growth Promotion Potential of Biocatalysts

Nitrogen, phosphate, and potassium provide essential nutrients for plant growth and development (Reece et al. 2011; Goldy 2013; Parmar and Sindhu 2013). Inappropriate use of these nutrients applied as mineral-based fertilisers can cause environmental risks (Parmar and Sindhu 2013). Also, any changes or bioremediation strategies that rely on fertilisers derived from fossil fuels will not be sustainable.

Ammonium is one of two forms of nitrogen that plants can absorb. Ammonifying or nitrogen-fixing bacteria produce NH_3 from the decomposition/hydrolysis of organic nitrogen (proteins) or atmospheric N_2 fixation. The produced NH_3 can be reduced to NH_4^+, a nutrient for plants and other soil organisms (Reece et al. 2011).

The ability of 19 (86.36%) isolates to decompose/hydrolyse organic nitrogen from peptone to NH_4^+ showed the ammonification potential of ECCN bacteria. All DCS/CS biocatalysts demonstrated NH_4^+ production activity with an average ammoniacal nitrogen rate estimated at 6.24. Previous studies have observed that some bacteria carry out ammonification in any ecosystem and that if the amount of NH_4^+ produced was more than their own metabolic needs, the surplus was excreted into the environment and could, therefore, be used by plants and other microorganisms as essential nutrients (Strock 2008; Reece et al. 2011).

Of the 22 bacteria screened for phosphate solubilisation, *Pseudomonas* sp., strain ECCN 10b alone was able to solubilise $Ca_3(PO_4)_2$ as mineral phosphate. This isolate, *Pseudomonas* sp., strain ECCN 10b developed maximum clear zones around colonies demonstrating P-solubilising activity. *Enterobacter* strain ECCN 7b and *Bacillus* strain ECCN 18b were also capable of solubilising $AlKO_6Si_2$ selected as the sole source of mineral potassium. Potassium solubilisation results showed that *Serratia* strain ECCN 24b and *Enterobacter* strain ECCN 7b were the only strains demonstrating significant ability to solubilise $AlKO_6Si_2$.

While bacteria solubilise and mobilise macronutrients such as phosphate to make it available for plant uptake (De-Bashan and Bashan 2004; Chen et al. 2003), Parmar and Sindhu (2013) showed that K solubilisation activity depends on the type of insoluble inorganic K used and was higher in Aleksandrow broth containing K_2SO_4 than in broths amended with $AlK(SO_4)_2$ or mica powder.

Many recent studies suggest that indigenous bacterial isolates from coal environments including coal tailings, coal mine soils and coal tailings water, have greater potential to solubilise coal than exogenous microbial communities (Akimbekov et al. 2022).

Therefore, generating five coal biodegrading bacteria, *Bacillus* sp. ECCN 3b, *Aeromonas* sp. ECCN 4b, *Exiguobacterium* sp. ECCN 8b, *Pseudomonas* sp. ECCN 10b and *Ancylobacte*r sp. ECCN 11) from HRAOP of the IAPS is a novelty of the present study.

The ability to produce indole compounds of the auxin class (IAA) is widespread among microorganisms, including bacteria in soils, fresh and marine waters, as well as plant and animal hosts (Patten et al. 2013). Bharucha et al (2013) reported that many environmental bacteria use L-tryptophan to produce IAA. So, it was not surprising to isolate IAA-producing microorganisms from DCS/CS and MaB-flocs generated in HRAOP of IAPS for domestic wastewater treatment. As PGR, IAA may be involved in many microbial-plant symbiotic interactions in diverse ecological ecosystems (Fuentes et al. 2016; Titilawo et al. 2020; Masudi et al. 2023).

Among the GA-producing bacteria, three organisms (75.00%), including *Microbacterium* sp., strain ECCN 9b, *Ancylobacter* sp., strain ECCN 11b, and *Microbacterium* sp., strain ECCN 12b, were generated from HRAOP; while *Microbacterium* sp., strain ECCN 22b, isolated from DCS. This further expresses the high potential of bacterial biocatalysts from HRAOP environments of IAPS in plant growth regulatory activity.

Previous research has shown that *Rhizobium* sp. produces gibberellin-type substances (Atzornet et al.1988) in relation to nodulation, and later, the production of gibberellins by many bacterial genera such as *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., *Azospirillum* sp., and *Sphingomonas* sp. was confirmed (Probanza et al. 2002; Kapoor et al. 2016: Salazar-Cerezo et al. 2018). These GA-producing organisms improved plant health under stressful conditions. The GA-producing *Pseudomonas putida* culture filtrate enhanced the growth of soybean plants under salt and water stress (Kang et al. 2014b). Strains of *Sphingomonas* sp. producing GA-induced tolerance of tomato crops to salt stress (Halo et al. 2015). Microorganisms possess natural but beneficial capabilities that could be exploited by biotechnological applications to help improve plant health and development under environmental conditions to mitigate the detrimental effects of biotic and abiotic stress (Salazar-Cerezo et al. 2018) and without reliance on fossil fuel-derived fertilisers.

Organisms that exert 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity act as PGPR (Penrose and Glick 2003). Activity linked to ACC deaminase was observed in four bacteria capable of growing on MSA and identified as *Enterobacter* ECCN 7b, *Pseudomonas*

ECCN 10b, *Proteus* ECCN 23b, and Serratia ECCN 24b. *Pseudomonas* sp. strain ECCN 10b performed best.

Isolates of *Aneurinibacillus*, *Arthrobacter* sp., *Achromobacter* sp., *Bacillus* sp., *Brevibacterium* sp., *Burkholderia* sp., *Citrobacter* sp., *Enterobacter* sp., *Leclercia* sp., *Micrococcus* sp., *Ochrobactrum* sp., *Parastrephia* sp., *Pseudomonas* sp., *Ralstoni* sp., and *Serratia* sp., all carry out ACC deaminization (Barnawal et al. 2014; Acuña et al. 2019; Orozco-Mosqueda et al. 2020). Additionally, the enzymatic activity of ACC deaminase leads to the production of ammonia as a nitrogen source, reduces plant stress-induced ethylene synthesis (Grichko and Glick 2001; Penrose and Glick 2003; Orozco-Mosqueda et al. 2020). This aids in nutrient absorption as well as plant health, growth, and development (Zarei et al. 2020).

Bacillus sp. ECCN 18b and *Citrobacter* sp. ECCN 19b were CS/DCS bacteria, while *Enterobacter* sp. ECCN 7b and *Pseudomonas* sp. ECCN 10b HRAOP isolates. Studies have shown that plants require iron (Fe) as a micronutrient for growth and development. However, Fe mobilisation may be reduced in dry and alkaline soils, becoming unavailable to plants (Ahmed and Holmstrom 2014). Siderophore molecules facilitate the availability of Fe for plant uptake as a nutrient after complexation with Fe(III) and its reduction to Fe(II) (Marschner 1986; Wang et al. 2014; Ahmed et al. Holmstrom 2014). The contribution of siderophore-producing organisms to PGP has manifested itself in several ways; in particular, *Pseudomonas fluorescens* enhanced Fe absorption in some broadleaf grasses and weeds. *Bacillus subtilis* in pepper showed significant suppression of Fusarium wilt caused by *Fusarium oxysporum* (Shirley et al. 2011; Yu et al. 2011; Santoyo et al. 2012). Inoculants of *Penicillium citrinum*, *Aspergillus niger*, and *Trichoderma harzianum* increased the growth parameter of annual leguminous chickpea, *Cicer arietinum* (Yadav et al. 2011).

6.2.4 Towards a bespoke biofertiliser for bioremediation using Fungcoal

Previous studies recommended that researchers first re-evaluate the viability and biological/metabolic activities of organisms as well as their ability to reproduce (Cangelosi and Meschke 2014; Braissant et al. 2020) before exploiting them in new or other scientific fields related to microbiology or biotechnology.

The capacity of *Aspergillus fischeri* ECCN84 to degrade coal is due to the production of the ligninolytic enzyme laccase (Igbinigie et al. 2008; 2010 Sekhohola et al. 2013; 2017). Furthermore, it was observed that fungal interactions with coal particles led to their degradation and bioconversion into a mixture of macromolecules, which are mainly humic acids (Olawale et 2020; Sekhohola et al. 2017; Sekhohola-Dlamini et al. 2022). There is then a link between the degradation of WC, the rehabilitation of degraded soils, and the promotion of plant growth (Titilawo et al. 2020; Sekhohola-Dlamini et al. 2022).

One aspect that must be considered is the interaction between the coal-degrading catalysts of Fungcoal and any microorganism incorporated into a biofertiliser to be used in this rehabilitation process. Thus, a series of biotic interaction studies was initiated.

The results of biotic interactions between bacteria showed no antagonistic activity. The organisms developed mutualistic interactions by growing together without producing inhibition zones around bacterial plugs. The sensitivity between growing cells was found to be positive and active. Three of these strains, *Enterobacter* sp. ECCN 7b, *Proteus* sp. ECCN 20b and *Serratia* sp. ECCN 24b, did not show antagonistic activities for biotic interactions with the fungus *Aspergillus fischeri* ECCN 84. Interestingly, research by Zhang et al (2020) and Zhou et al (2022) pointed out that antagonistic activity between microorganisms was due to the production of toxic metabolites and antibiotic compounds or competition for nutrients. The results of the present study revealed that these mutualistic bacterial strains appear likely to be combined into consortia to formulate a bacterial biofertiliser to support Fungcoal as a phytoremediation strategy.

Four bacterial strains induced the germination of barley (*Hordeum vulgare* L.) seeds. This is due to the likely production of gibberellic compounds. Among these microorganisms, *Microbacterium* sp. ECCN 22b, *Ancylobacter* sp. ECCN 11b and *Microbacterium* sp. ECCN 12b were the most effective. Studies have highlighted that many bacterial strains have demonstrated the potential to produce gibberellins and to promote plant growth and development (Bastian et al. 1998; Bottini et al. 2004; Joo et al. 2004). However, no scientific reports have been provided to study the potential of DCS/CS and HRAOP microorganisms regarding the production of gibberellic compounds and their impact on plant seed germination.

Further experiments showed the positive effect of bacterial indole compounds in the production of adventitious roots. Indole production increased linearly for cultures of the six top-ranked strains, namely *Aeromonas* ECCN 4b, *Arthrobacter* ECCN 6b, *Enterobacter* ECCN 7b, *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b. And, the resulting culture filtrates stimulated adventitious root formation in mung bean (*Vigna radiata* L.) explants/hypocotyls.

Aeromonas sp., *Enterobacter* sp., *Proteus* sp., and *Serratia* sp. are Gram-negative bacteria, while *Arthrobacter* sp. is Gram-positive. They are all found in fresh and brackish waters around the world. Considering that some of these organisms include pathogenic strains, they mainly contain strains associated with the oxidative degradation of polymeric compounds and play critical roles in the biodegradation of agrochemical pollutants (Westerberg et al. 2000; Camargo et al. 2003). Additionally, several strains of *Arthrobacter* appear to possess PGP characteristics. Some recent examples include *Arthrobacter* sp. UKPF54-2 (Shen et al. 2019), *Arthrobacter wenxiniae* sp. nov. (Sun et al. 2022), *Arthrobacter* sp. GN70 (Chhetri et al. 2022) and *Arthrobacter nicotinovorans* JI39 (Jiang et al. 2022).

Furthermore, *Arthrobacter* spp. have been isolated from the rhizosphere and shown to promote growth of legumes (Aviles-Garcia et al. 2016; Mekonnen and Kibret 2021) and rice plants (Chhetri et al. 2022). Like *Arthrobacter*, species of other soil bacteria, *Enterobacter*, *Proteus* and *Serratia* are known to have a wide range of PGP characteristics including nitrogen fixation, degradation of recalcitrant matter such as coal, phosphorus mineralisation, production of antibiotics, siderophores, chitinase, ACC-deaminase and release hydrolytic enzymes that, together with extra-cellular polysaccharides, enhance soil porosity (Jha et al. 2011, Olawale et al. 2020; Titilawo et al. 2020). Of relevance to the present study, as emphasised by these authors, is the ability of *Enterobacter* spp. to increase root growth, root fresh weight, and promote the release of organic acids into the rhizosphere. Together, this information suggests enhanced development of plant root systems by isolates of the genus *Enterobacter* like ECCN 7b which, in the present study, along with the isolates *Aeromonas* ECCN 4b, *Arthrobacter* ECCN 6b, *Proteus* ECCN 20b/ECCN 23b and *Serratia* ECCN 24b promoted adventitious root formation in mung bean hypocotyls.

All PGPB that produce IAA have been reported to promote adventitious root formation (Mayak et al. 1999). Thus, it is presumed that bacteria produce IAA in parallel with the secretion of the enzyme ACC deaminase, plant-derived ethylene (De Klerk and Hanecakova 2008), root-produced galactoglucomannan oligosaccharides, H_2O_2 and salicylic acid (Kollárová et al. 2005; Li et al. 2009; Yang et al. 2013) which act in concert as signalling agents to drive adventitious root formation.

The vegetative growth response of test plants to a bacterial biofertiliser showed the differential efficacy of consortium cultures compared to single isolate cultures. Furthermore, no antibiotic resistance activity was observed for consortium cultures formulated by combining the effective

PGPB, *Enterobacter* ECCN 7b, *Proteus* ECCN 20b, and *Serratia* ECCN 24b. Ranking regarding root biomass production was 1) *Enterobacter, Proteus* and *Serratia*, 2) *Enterobacter* and *Serratia,* and 3) *Serratia* single culture. Plant biomass production ranking prioritised the three consortium cultures as follows 1) *Enterobacter* and *Proteus*, 2) *Enterobacter*, *Proteus,* and *Serratia* and, 3) *Enterobacter* and *Serratia* as the most effective.

Mutualistic plant-microbe interactions use variable biological processes to colonize root systems and enhance growth (Vílchez et al. 2015). Some may be effective as individuals or as consortia. The success of beneficial microbes in improving plant growth, food quantity and quality depends on adequate plant-microbial interactions that improve nutrient uptake and stress tolerance (Du Jardin 2015; Vílchez et al. 2015; Molina-Romero et al. 2021). However, when used individually or in consortia, they should be harmless and safe for soil microbiota, host plants and animals (Vílchez et al. 2015; Molina-Romero et al. 2021). Approximately 99% of microbiological interactions occur as consortia in any active ecological system and have been used for environmental remediation, food production, and human health. However, with the development of biotechnology, many biosynthetic pathways with different functions have been introduced and explored in microbial consortia (Shong et al., 2012; Tanouchi et al. 2012; Ortiz-Marquez et al. 2013). Multiple inoculations stimulated more plant growth and increased crop yield compared to a single inoculation (Sivaramaiah et al. 2007; Souza et al. 2015; Chaudhary and Sindhu 2016).

Considering these results, *Enterobacter* sp., strain ECCN 7b, *Proteus* sp., strain ECCN 20b and *Serratia* sp., strain ECCN 24b, were selected as the best candidates for further development of a bacterial biofertiliser intended to support Fungcoal phytoremediation strategy. This will mitigate the dependence on chemical-based fertilisers and promote sustainable practices for rehabilitating land degraded by coal mining.

6.3 Conclusion

The present study demonstrated the bacterial biofertiliser potential of 22 bacterial isolates from diesel-contaminated sites (DCS), coal slurry (CS), and an algae-based wastewater treatment system. Specifically, strain viability, purity, maintenance of biological activity, and molecular characteristics were carried out to support the classification of these bacteria as plant growth promoters. A bacterial biofertiliser was developed to provide PGR (IAA and GA), essential plant nutrients (N, P, K), and enzymes (ACC and siderophores) necessary for plant growth and development. Single and paired isolates were more effective when assembled as a consortium (at least three strains) cultures. They comprised the most competitive strains including *Enterobacter* sp. strain ECCN 7b, *Proteus* sp. strain ECCN 20b and Serratia sp. ECCN *strain* 24b.

Gram-negative bacteria from *Enterobacteriaceae* have been shown to have a perfect mutualistic relationship with the fungus *Aspergillus fischeri* strain ECCN 84 and proficient for PGP characteristics using the biochemical parameters described in this study. Bacteria with PGP characteristics could help solve contemporary challenges related to the water-energy-food nexus and food security. The production of cost-effective biofertilisers from DCS and HRAOP and their reuse as a rehabilitation strategy can reduce dependence on chemical fertilisers and the current expensive rehabilitation strategies. This could make DCS and HRAOPs more attractive to local authorities, especially if additional revenue streams are likely. Further studies are therefore underway to investigate the PGP activity of these isolates as consortia under field conditions.

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Appendices

Section A. Standard Curves

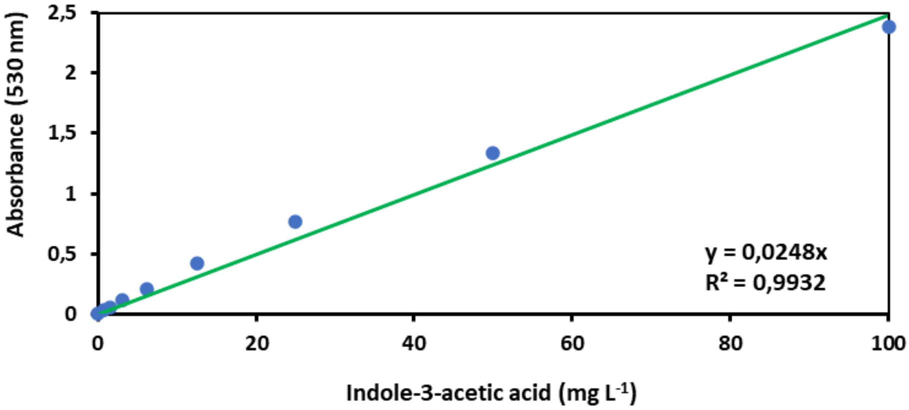

Figure A.1 The increasing concentrations of IAA at a wavelength of 530 nm using pure IAA. The standard curve purchased from Microsoft Excel, 2016 was used to determine the unknown concentration of IAA, auxin in L-tryptophan NB.

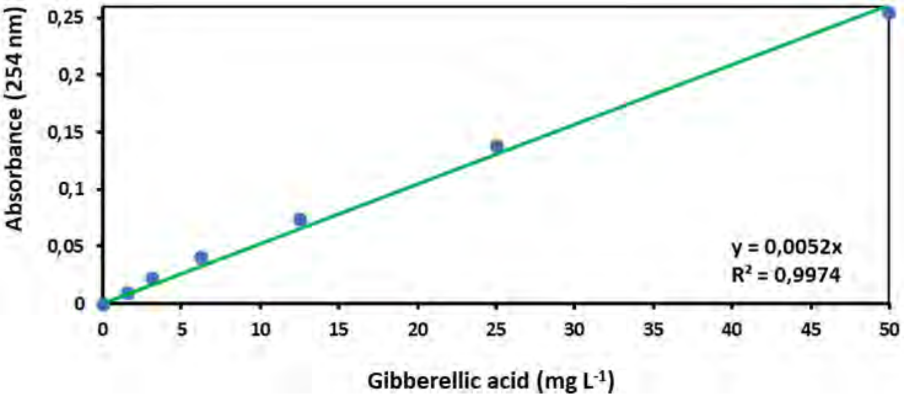

Figure A.2 The increasing concentrations of GA at a wavelength of 254 nm using pure authentic/commercial GA. The standard curve purchased from Microsoft Excel, 2016 was used to determine the unknown concentration of IAA in PDB-glutamate without and with L-tryptophan.

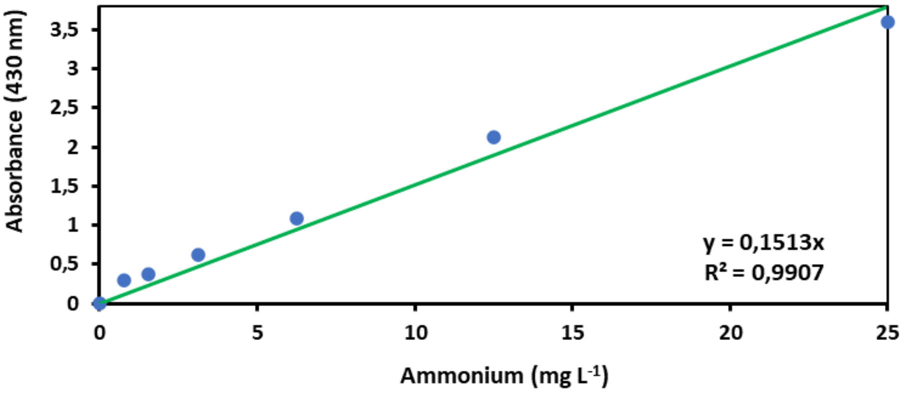

Figure A.3 The increasing concentrations of NH_4^+ at a wavelength of 430 nm using NH_4Cl. The standard curve purchased from Microsoft Excel, 2016 was used to determine the unknown concentration of NH_4^+ in PW.

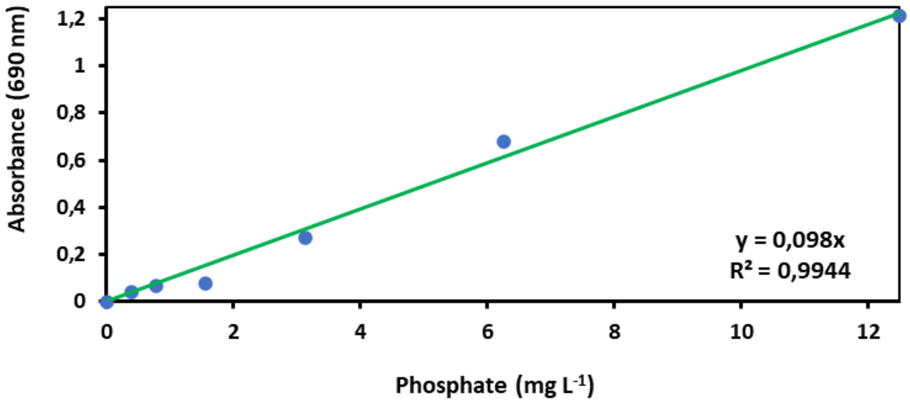

Figure A.4 The increasing concentrations of PO_4^{-3} at a wavelength of 690 nm using K_2HPO_4. The standard curve purchased from Microsoft Excel, 2016 was used to determine the unknown concentration of PO_4^{-3} in PVB containing $Ca_3(PO_4)_2$) as the sole source of inorganic phosphate.

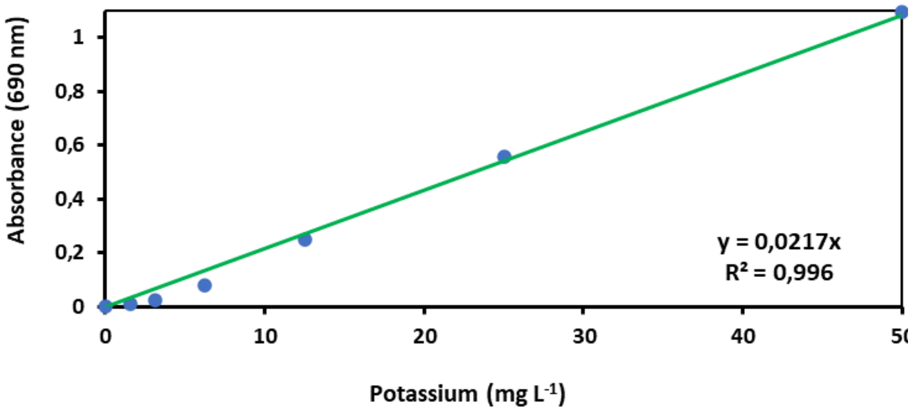

Figure A.5 The increasing concentrations of K^+ at a wavelength of 690 nm using KCl. The standard curve purchased from Microsoft Excel, 2016 was used to determine the unknown concentration of K^+ in AB containing $AlKO_6Si_2$ as the soil source of inorganic potassium.

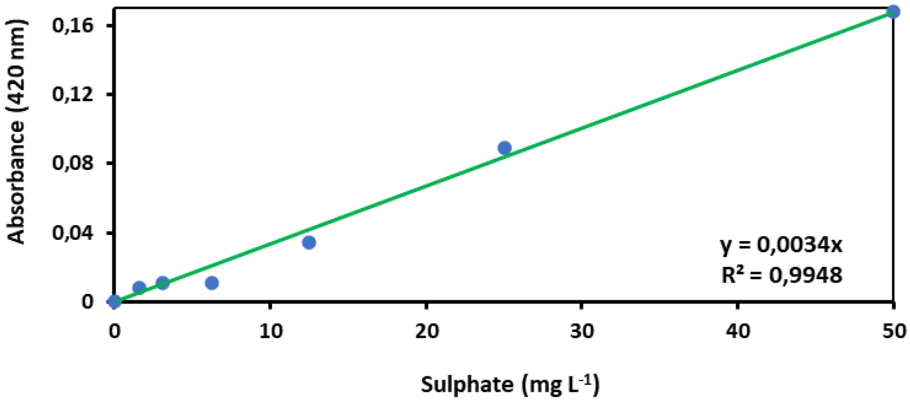

Figure A.6 The increasing concentrations of SO_4^{-2} at a wavelength of 420 nm using Na_2SO_4. The standard curve purchased from Microsoft Excel, 2016 was used to determine the unknown concentration of SO_4^{-2} in coal filtrate.

Section B. Some Cases of Single-Way Analysis of Variance (ANOVA) With or Without Significant Differences Between All Bacterial Treatments.

B.1 Abiotic Stress Tolerances

Table B.1.1 pH Stress Tolerances

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.00	11.00	0.00	5.72	0.00	2.72
Within Groups	0.00	12.00	0.00			
Total	0.00	23.00				

Table B.1.2 Salt Stress Tolerances

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	21.43	8.00	2.68	5.18	0.00	2.00
Within Groups	74.40	144.00	0.52			
Total	95.83	152.00				

Table B.1.3 Temperature Stress Tolerances

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.00	9.00	0.00	0.93	0.52	2.39
Within Groups	0.00	20.00	0.00			
Total	0.00	29.00				

B.2 Plant Growth Promoting Potential

Table B.2.1 Ammonium Production

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	10.54	9.00	1.17	5.81	0.01	3.02
Within Groups	2.02	10.00	0.20			
Total	12.55	19.00				

Table B.2.2 Phosphate Solubilisation

ANOVA SINGLE FACTOR						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	13.69	11.00	1.24	12.16	0.00	2.72
Within Groups	1.23	12.00	0.10			
Total	14.92	23.00				

Table B.2.3 Potassium Solubilisation

ANOVA SINGLE FACTOR						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	758.88	11.00	68.99	117.32	0.00	2.72
Within Groups	7.06	12.00	0.59			
Total	765.93	23.00				

Table B.2.4 Indole-3-Acetic Acid Production

ANOVA SINGLE FACTOR						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	22013.18	11.00	2001.20	850.87	0.00	2.72
Within Groups	28.22	12.00	2.35			
Total	22041.40	23.00				

Table B.2.5 Gibberellic Acid Production

ANOVA SINGLE FACTOR						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	909.32	9.00	101.04	19.29	0.00	2.39
Within Groups	104.76	20.00	5.24			
Total	1014.08	29.00				