Towards the development of a bio-fertiliser using mixed liquor from high rate algal oxidation ponds

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Abstract

Mixed liquor includes consortia of microalgae and bacteria produced in high rate algal oxidation ponds (HRAOPs). The consortia of microalgae and bacteria occur as flocs called microalgae-bacterial flocs (MaB-flocs). This study aimed to source bacteria from MaB-flocs generated in HRAOPs and, after isolation and identification, evaluate their potential as plant growth promoting (PGP) microorganisms. Twelve bacterial strains namely ECCN 1b, ECCN 2b, ECCN 3b, ECCN 4b, ECCN 5b, ECCN 6b, ECCN 7b, ECCN 8b, ECCN 9b, ECCN 10b, ECCN 11b, and ECCN 12b were successfully isolated and their molecular identity established using amplified 16S rRNA gene sequence analysis that was compared to sequences deposited in the NCBI gene database. Blast analysis identified these isolates at the genus level as *Bacillus* strain ECCN 1b, *Fictibacillus* strain ECCN 2b, *Bacillus* strain ECCN 3b, *Aeromonas* strain ECCN 4b, *Exiguobacterium* strain ECCN 5b, *Arthrobacte*r strain ECCN 6b, *Enterobacter* strain ECCN 7b, *Exiguobacterium* strain ECCN 8b, *Microbacterium* strain ECCN 9b, *Pseudomonas* ECCN strain 10b, *Ancylobacter* strain ECCN 11b and *Microbacterium* strain ECCN 12b. These isolates were able to grow in nutrient broth in a pH range between 6 and 10, with the best growth achieved at pH 8 to 9. The results on the use of carbon substrate revealed that 5 strains including *Arthrobacter* strain ECCN 6b, *Aeromonas* strain ECCN 4b, *Pseudomonas* strain ECCN 10b, *Enterobacter* strain ECCN 7b and *Bacillus* strain ECCN 3b were capable of using glucose, sucrose and mannitol. No faecal coliforms were found. However, of the 12 isolates screened for bio-fertilisation potential, *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* ECCN strain 7b, *Exiguobacterium* strain ECCN 8b and *Pseudomonas* strain ECCN 10b showed multifunctional plant growth promoting (PGP) potential. The potential for PGP included the production of ammonium-N, solubilisation of phosphate-P and potassium-K, oxidation of Mn and production of auxin, indole-3-acetic acid (IAA). Results are discussed in terms of the amount or concentration (mg L^{-1}) of plant essential nutrient and growth regulator produced by these isolated bacteria. Even so, further studies are needed to test and confirm the bio-fertiliser and plant growth promoting activity of these strains in pot trials and field experiments, or both.

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List of abbreviations

ACC	1-amino cyclopropane-1 carboxylate
ADP	Adenosine diphosphate
AFP	Advanced facultative pond
AIWPS	Algal integrated wastewater pond systems
ALM	Aleksandrow liquid medium
ANOVA	Analyses of variance
AOB	Ammonia-oxidizing bacteria
ASP	Algae settling pond
ATCC 111A	American Type Culture Collection agar
ATP	Adenosine triphosphate
BOD	Biochemical oxygen demand
DAFF	Department of Agriculture, Forestry and Fisheries of South Africa
EBRU	Environmental Biotechnology, Rhodes University
ECCNb	EBRU Culture Collection Bacterial
ePGPR	Extracellular plant growth promoting rhizobacteria
FAO	Food and Agriculture Organisation of the United Nations
FDA	Food and drug administration
GA	Gibberellic acid or Gibberellin
GPB	Glucose peptone broth
HRAOPs	High rate algal oxidation ponds
IAA	Indole-3 acetic acid
IAPS	Integrated algal pond system
IPD	In-pond digester
iPGPR	Intracellular plant growth promoting rhizobacteria
IYP	International Year of Pulses
KSMs	Potassium solubilising microorganisms
LBA	Luria-Bertani agar
MaB-flocs	Microalgae-bacterial flocs
MOB	Manganese oxidation broth
MPB	Mannitol peptone broth
NA	Nutrient agar

NB	Nutrient broth
Nd	No date
NOB	Nitrite-oxidising bacteria
NPK	Nitrogen, potassium and phosphorus
PGPR	Plant growth promoting rhizobacteria
PGR	Plant growth regulator
Pi	Inorganic phosphate
PVK	Pikovskayas
PW	Peptone water
rpm	Revolutions per minute
SAM	Solid Aleksandrow medium
SB	Splitter box
SPB	Sucrose peptone broth
UV	Ultraviolet
WRC	Water Research Commission
WWTW	Wastewater treatment works

Chapter 1: Literature review

1.1 Introduction

Food is a vital need for all and there is sufficient evidence that, due to substantial population growth, demand exceeds availability (Vejan et al. 2016). As a result of this global food shortage, the United Nations (UN) General Assembly has set 2016 as the year of the pulses (A/RES/68/231). This target was, to a larger extent, unmet and today it is likely that additional programmes will crop up in an attempt to ensure food security. This underscores the imperative and benefits of growing and producing more grain legumes per unit area, by developing more effective fertiliser technologies as an alternative to reducing environmental risks caused by the excessive use of chemical fertiliser (Hansen 1996; Tilman et al. 2002). To meet the growing demand for food while reducing the huge reliance on chemical fertilisers, it is necessary to explore other effective means and resources such as biomass generated by treatment of wastewater in a process amenable to resource recovery. One such bioprocess is the treatment of wastewater using integrated algal pond systems (IAPS). Outputs from this bioprocess, not only include biomass as a source of biofuel production (Hernandez et al. 2013), but also as feedstock for sustainable fertiliser or bio-fertiliser production (Xu et al. 2014; Young et al. 2017).

Integrated algal pond system (IAPS) is a passive wastewater treatment technology that uses anaerobic and aerobic biological processes to remediate liquid effluent from domestic, industrial and agricultural sources (Rose et al. 2007; Mambo et al. 2014a). It can be described as a cost-effective wastewater treatment technology that reduces the pollution potential of the treated effluent preventing the degradation of the same scarce sources (Rose et al. 2002). The anaerobic process includes an in-pond digester (IPD) and represents a primary treatment unit, while the aerobic processes occur in high rate algal oxidation ponds (HRAOPs) and are known as a secondary treatment unit. Aerobic biological processes exploit the mutual interaction between microalgae and bacteria (Figure 1.1) to generate water of a quality suitable for reuse and discharge to the environment (Craggs et al. 2014; Mambo et al. 2014a; Butler et al. 2017; Jimoh et al. 2019). In addition to the treatment of domestic sewage and production of water suitable for irrigation, IAPS can generate additional product streams, i.e. biogas and biomass. The biomass is a mixed liquor and it is composed of microalgae and bacteria produced in HRAOPs in the form of flocs known colloquially as microalgae-bacterial flocs (MaB-flocs). The MaB-flocs once produced can be used for many applications including the production of nutrient-rich fertilisers, animal feed materials, soil improvers, amino acids, vitamins, polysaccharides, extracellular polymeric substances (EPS), plant growth promoting rhizobacteria (PGPR) and as a starting material for the production of plant growth regulators (PGRs) (Aaronson et al. 1980; Borowitzka 1988; Rose et al. 2002; Wells 2005; Craggs et al. 2014; Xu et al. 2014; Butler et al. 2017; Kadmiri et al.2018).

The fertilising potential of solid or dry microalgae biomass from HRAOPs has already been explored (Mlambo 2014). It is useful to explore the potential of bacterial biomass generated in HRAOPs as bioactive flocs to produce biological catalysts that can be used to sustainable agricultural practices in plant growth promoting and regulating activities and then develop a bacterial bio-fertiliser. The term bio-fertiliser refers to the application of live microorganisms (bacteria, fungi and algae) to seeds, plant surfaces or soil in terms of colonisation of the rhizosphere to promote plant growth by increasing their ability to absorb nutrients and then to boost agricultural production (Vessey 2003). Bio-fertiliser has many potentials including atmospheric nitrogen (N_2) fixation, ammonification production, phosphate and potassium solubilisation and manganese oxidation. Also, they produce plant hormones, for example, indole-3 acetic acid (IAA), gibberellic acid (GA), 1-aminocyclopropane-carboxylic acid (ACC), etc. (Tanimoto et al. 2005; Akob et al. 2014; Bumunang and Babalola 2014; Baliah et al. 2016; Saha et al. 2016). Most bacterial bio-fertilisers used in sustainable agriculture are derived from soil, root nodules and rhizosphere (Bumunang and Babalola 2014; Islam et al. 2016; Etesami et al. 2017), but bacterial-based bio-fertiliser isolated from HRAOPs of IAPS, according to the literature at our disposal, has not yet been developed (Figure 1.1).

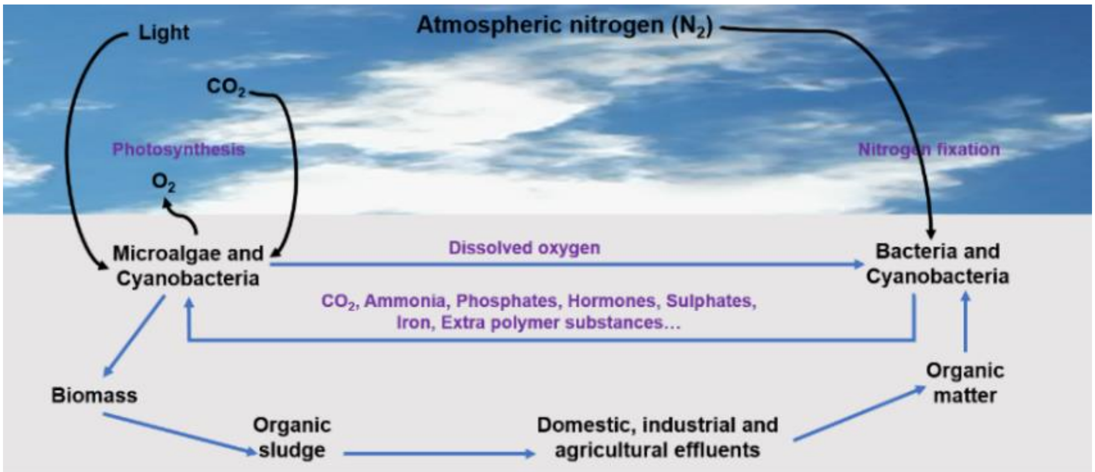


Figure 1. 1: Mechanisms of symbiotic interaction between aerobic bacteria and photosynthetic microalgae in high rate algal oxidation ponds (HRAOPs) (modified from Oswald et al. 1955; Ramanan et al. 2016).

As illustrated in Figure 1.1, it is important to understand more about the beneficial nutrient exchanges between microalgae and bacteria in HRAOPs of IAPS in terms of transferring this potential in soil environments as beneficial nutrient exchanges between bacteria and rhizosphere, and as a source of plant growth promotion for sustainable agricultural practices (Xu et al. 2014; Ramanan et al. 2016).

This project focuses exclusively on the use of biomass in order to develop a PGPR bio-fertiliser from MaB-flocs generated in HRAOP of IAPS. To explore the potential of the biological wastewater treatment process, such as producing bacterial bio-fertiliser from HRAOP, Water Research Commission (WRC) has championed IAPS as a wastewater treatment technology for implementation by local municipalities. The technology developed at Rhodes University aims to provide smaller rural municipalities with a means to address constraints related to the water-energy- nexus using MaB-flocs. In collaboration with the WRC, EBRU has designed a commercial-scale IAPS for sustainable organic and bio-fertiliser production at the Barberton wastewater treatment work (WWTW) in Mpumalanga and the treatment system is in the pre-construction phase.

Finally, soil bio-fertilisation using appropriate bacteria is now a common and attractive way to improve sustainable agriculture (Kifle et al. 2016). The biotechnological framework of this study is the use of bacterial strains isolated from HRAOP as candidates for the preparation of a bio-fertiliser intended to improve agricultural yields.

1.2 Integrated algal pond system as a wastewater treatment technology

1.2.1 Historical information

Integrated algae pond systems (IAPS) have about 70 years of historical information and derived from the Oswald's algal integrated wastewater pond systems (AIWPS®), developed in the 1950s (Rose et al. 2007; Mambo et al. 2014a). The technology combines the use of anaerobic and aerobic bioprocesses for the treatment of domestic wastewater (Mambo et al. 2014a) and consists mainly of a primary facultative pond (PFP), high rate algal oxidation ponds (HRAOPs), algal settling ponds (ASPs) and maturation ponds (MPs). Since the 1950s, AIWPS® have been the subject of numerous studies around the world. Subsequently, in 1990, the Water Research Commission (WRC) initiated the application of the IAPS protocol to a range of wastewater treatment in South Africa (Rose et al. 2007). The WRC funded the construction of the typical IAPS research facility at Rhodes University at the field station of the Institute for Environmental Biotechnology (EBRU). This typical IAPS was a version of the AIWPS® designed by Oswald and adapted to the conditions of the local municipality (Mambo et al. 2014a; Cowan et al. 2016). This technology transfer initiative was undertaken as an engineering foundation for the development of the IAPS at EBRU and as a municipal wastewater treatment platform (Mambo et al. 2014b). From this initiative, several applications have emerged and subjected to salinity, water sanitation and sustainability (Rose et al. 2007).

1.2.2 Role of IAPS in wastewater treatment

Integrated algal pond system (IAPS) technology combines the use of anaerobic and aerobic bioprocesses to treat wastewater (Mambo et al. 2014a). The typical IAPS research facility located at the field station of EBRU includes:

1. An advanced facultative pond (AFP) as a primary processing unit. It is an anaerobic bioprocess of wastewater treatment that includes an anaerobic digester (AD). The AD partially reduces the organic load of municipal wastewater by fermentation and removes suspended solids by sedimentation processes (Mambo et al. 2014a; Craggs et al. 2014).
2. The high rate algal oxidation ponds (HRAOPs) known as aerobic bioprocesses. The systems have been developed to facilitate the treatment of effluents from the primary treatment unit. HRAOPs have been designed to further remove dissolved and biodegradable organic matter, to disinfect and generate the valuable microalgae and bacterial biomass as flocs (Rawat et al. 2016; Mambo et al. 2014a; Jimoh and Cowan 2017).
3. The algal settling ponds (ASPs), used as tertiary wastewater treatment consist of harvesting or removing microalgal biomass from treated wastewater prior to discharge into public water bodies (Rose et al. 2007; Mambo et al. 2014a). However, if the treated wastewater is to be used for irrigation, it must be settled and stored sufficiently to meet or achieve specific discharge standards in conformity with the bacterial Mean Probable Number (MPN) $<10^3$ mL^{-1}, required for irrigation water (Rose et al. 2007).

The IAPS for treatment of wastewater was developed to preserve the benefits (simplicity, cost-effectiveness, no use of chemicals, disinfection and reliability) and to mitigate the disadvantages (poor effluent quality, high land use and odour potential) (Oswald et al. 1995; Mambo et al. 2014a). The ability of biological wastewater treatment technology has been studied and the result showed that IAPS can treat wastewater to an acceptable quality within a fifth of the time of other lagoon systems while using 50% less surface (Young et al. 2017). The research done at two of Australia's largest sewage treatment plants demonstrated the positive outcome achieved for large-scale algal biofuel and energy production (Batten et al. 2013). Therefore, the MaB-flocs biomass generated in the wastewater treatment was examined for its bio-fertiliser potential in the cultivation of rice, tea, wheat and barley grains. These studies showed also a positive outcome (Ben Rebah et al. 2007; Rose et al. 2007; Xu et al. 2014; Odgerel and Tserendulam 2016; Chatterjee 2017).

1.2.3 Microalgae-bacterial formation in HRAOPs

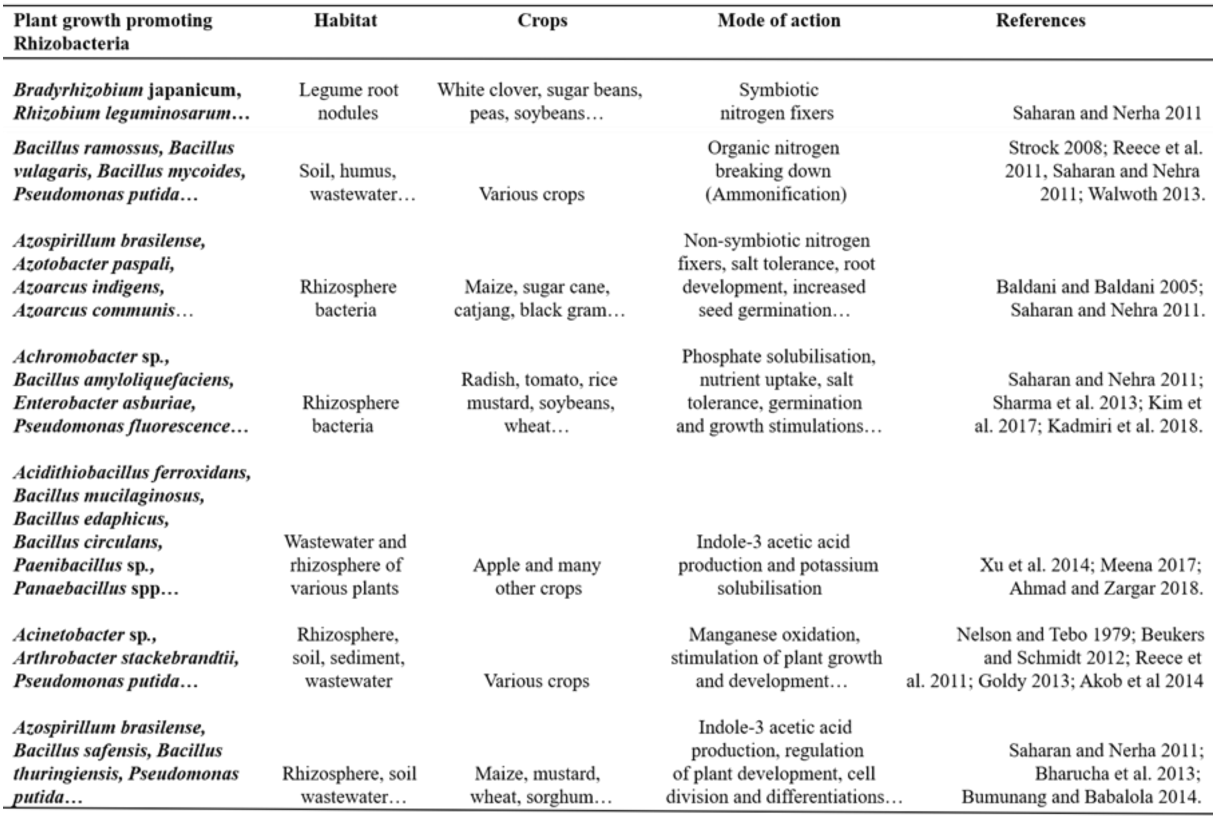
The partially treated effluents from the primary treatment unit (AFD) flow into the HRAOPs to generate, in this second treatment unit, the biomass of microalgae and bacteria as sludge. This sludge composed of MaB-flocs or mixed liquor suspended solids (MLSS) (Cowan et al. 2016) is dominated by microalgae and always appears green. When exposed to the open air, the sludge (MaB-flocs) can be contaminated (Chisti 2016). Therefore, the disinfection potential of this system is important.

The microalgae-bacterial formation in HRAOPs is achieved by mixing and turbulent flow facilitated by a paddlewheel that allows atmospheric oxygen, carbon dioxide and light to enter the MLSS so the biomass productivity can be enhanced (Cowan et al. 2016). Microalgae convert solar irradiance to chemical energy through photosynthesis and in doing so, generate oxygen which is used directly by aerobic bacteria. In return, bacteria oxidise recalcitrant organic matter into its basic constituents, which increases the nutrient loading of the flocs (Cowan et al. 2016; Chisti 2016). This microalgae and bacteria abstract nutrients from wastewater in the presence of oxygen and sunlight to generate suspended solids known as MaB-flocs (Cowan et al. 2016). MaB-flocs typically consist of a consortium of microalgae, cyanobacteria and bacteria, and may include a few rotifers, ciliates and precipitates (Van Den Hende et al. 2011). The growing interest of microalgae as mentioned above makes them a target for scientific studies in many fields such as energy and bio-fertiliser production as well as in other industrial applications (Ben Rebah et al. 2007; Al Darzins et al. 2010; Xu et al. 2014; Jimoh et al. 2019).

1.3 Plant growth promoting rhizobacterial bio-fertiliser

Plant growth promoting rhizobacteria (PGPR) include all prokaryotic microorganisms known to have beneficial effects on plant growth and development by providing nutrients through a variety of biological processes, such as N_2 fixation, solubilisation and oxidation of substances into the soil and rhizosphere (Vejan et al. 2016). These microorganisms include the group of extracellular plant growth promoting rhizobacteria (ePGPBR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Figueiredo et al. 2011; Ahemad and Kibret 2014). The microorganisms belonging to ePGPR live in the rhizosphere, on the rhizoplane while those belonging to iPGPR are located inside root cells, usually in specialised structures called root nodules (Figueiredo et al. 2011; Ahemad and Kibret 2014). The example of some ePGPR includes the genera *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas and Serratia* while certain of the iPGPR are *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium* and *Rhizobium*, all the family *Rhizobiaceae* (Vessey 2003; Ahemad and Kibret 2014). Table 1.1 illustrates some of the rhizobacteria isolates used in the development of sustainable bio-fertiliser to improve agricultural practices that stimulate crop production while controlling and protecting soil ecosystems.

Table 1. 1: Examples of some plant growth promoting rhizobacteria used in soil improvement, nutrient uptake, seed germination and crop growth stimulation (modified from Vejan et al. 2016 and Majeed et al. 2018).



Plant growth promoting Rhizobacteria	Habitat	Crops	Mode of action	References
Bradyrhizobium **japanicum,** ***Rhizobium leguminosarum…***	Legume root nodules	White clover, sugar beans, peas, soybeans…	Symbiotic nitrogen fixers	Saharan and Nerha 2011
Bacillus ramossus, Bacillus vulagaris, Bacillus mycoides, Pseudomonas putida…	Soil, humus, wastewater…	Various crops	Organic nitrogen breaking down (Ammonification)	Strock 2008; Reece et al. 2011, Saharan and Nehra 2011; Walwoth 2013.
Azospirillum brasilense, Azotobacter paspali, Azoarcus indigens, Azoarcus communis*…*	Rhizosphere bacteria	Maize, sugar cane, catjang, black gram…	Non-symbiotic nitrogen fixers, salt tolerance, root development, increased seed germination…	Baldani and Baldani 2005; Saharan and Nehra 2011.
Achromobacter **sp.,** ***Bacillus amyloliquefaciens, Enterobacter asburiae, Pseudomonas fluorescence…***	Rhizosphere bacteria	Radish, tomato, rice mustard, soybeans, wheat…	Phosphate solubilisation, nutrient uptake, salt tolerance, germination and growth stimulations…	Saharan and Nehra 2011; Sharma et al. 2013; Kim et al. 2017; Kadmiri et al. 2018.
Acidithiobacillus ferroxidans, Bacillus mucilaginosus, Bacillus edaphicus, Bacillus circulans, Paenibacillus **sp.,** ***Panaebacillus*** **spp…**	Wastewater and rhizosphere of various plants	Apple and many other crops	Indole-3 acetic acid production and potassium solubilisation	Xu et al. 2014; Meena 2017; Ahmad and Zargar 2018.
Acinetobacter **sp.,** ***Arthrobacter stackebrandtii, Pseudomonas putida…***	Rhizosphere, soil, sediment, wastewater	Various crops	Manganese oxidation, stimulation of plant growth and development…	Nelson and Tebo 1979; Beukers and Schmidt 2012; Reece et al. 2011; Goldy 2013; Akob et al 2014
Azospirillum brasilense, Bacillus safensis, Bacillus thuringiensis, Pseudomonas putida…	Rhizosphere, soil wastewater…	Maize, mustard, wheat, sorghum…	Indole-3 acetic acid production, regulation of plant development, cell division and differentiations…	Saharan and Nerha 2011; Bharucha et al. 2013; Bumunang and Babalola 2014.

1.3.1 Rhizosphere

The rhizosphere is the root-soil zone that interfaces with microbial activity, creating a confined nutrient pool in which essential macro and micronutrients are extracted (Burdman et al. 2000). In this zone, the roots secrete compounds (exudates) capable of binding soil particles, absorbing water and promoting the growth of beneficial microorganisms known to stimulate plant growth (Kloepper et al. 1989; Burdman et al. 2000). The interacting components that exist in the rhizosphere include soil, rhizoplane and roots. Soil is the habitat structure that gives life to roots and microbiota. Rhizoplane is the outer surface of the roots, including strongly adherent soil particles and debris, while roots are components of the plant systems. However, many microorganisms, such as endophytic bacteria can colonise root surfaces and tissues (Barea et al. 2005; Ahemad and Kibret 2014). Microbial colonisation of rhizoplane and root tissues is known as root colonisation, while root-influenced soil colonisation is known as rhizosphere colonisation (Kloepper et al. 1989; Barea et al. 2005; Ahemad and Kibret 2014).

1.3.2 Bacterial bio-fertiliser

The bacterial bio-fertiliser includes all prepared fertiliser containing live bacteria that can be used to inoculate seeds, plant surfaces and/or soil. These bacteria colonise the rhizosphere or the interior of the root system, stimulating plant growth by increasing the availability of nutrients to host plants (Malusá and Vassilev 2014; Souza et al. 2015). The bio-fertiliser can be prepared in liquid or solid form. It can also be a mixture of living latent cells promoting the fixation of N_2, the plant solubilisation of P and K, the oxidation of Mn, or microorganisms used for composting. These bacteria used as bio-fertiliser accelerate soil processes, increasing the availability of nutrients that can then be easily absorbed and assimilated by host plants (Vessey 2003; Mishra et al. 2013; Vejan et al. 2016).

Bacterial bio-fertiliser also stimulate plant growth by mobilisation of nutrients, production of plant growth regulators, protection of plants from phytopathogenic microorganisms, benefits that can improve soil composition and bioremediation (Hayat et al. 2010; Rajkumar et al. 2010; Braud et al. 2009; Ahemad and Kibret 2014). The bacteria used to prepare a bio-fertiliser must have the following characteristics: (i) the ability to colonise the surface of the roots; (ii) the ability to survive, to multiply and to compete with other microbiota in the soil, at least for a time sufficient to express their growth promotion activities and (iii) the ability to promote plant growth (Kloepper 1994; Ahemad and Kibret 2014).

1.3.3 Symbiotic rhizobacteria

Symbiotic rhizobacteria play an important role in regulating the dynamics of matter in the soil ecosystem (Duarah et al. 2011). They provide host plants with nitrogen resources derived from the atmosphere by N_2 fixation (Figure 1.2). These mutual exchanges of nutrients promote the ability of both organisms to strengthen their symbiotic relationship. This beneficial effect is called "partner fidelity feedback" and considered as a stabilising factor in this symbiotic relationship (Sachs et al. 2004; Friesen 2012; Fujita et al. 2014).

1.3.3.1 Nitrogen cycle

The nitrogen cycle is a major biogeochemical transformation of nitrogen into its several oxidation states. It is essential for nitrogen recycling and depends on the activities of various bacteria, archaea and fungi (Bernhard 2011). The nitrogen cycle appears in the soil and aquatic environment, as well as in the HRAOPs of IAPS (Walworth 2013; Ramanan et al. 2016).

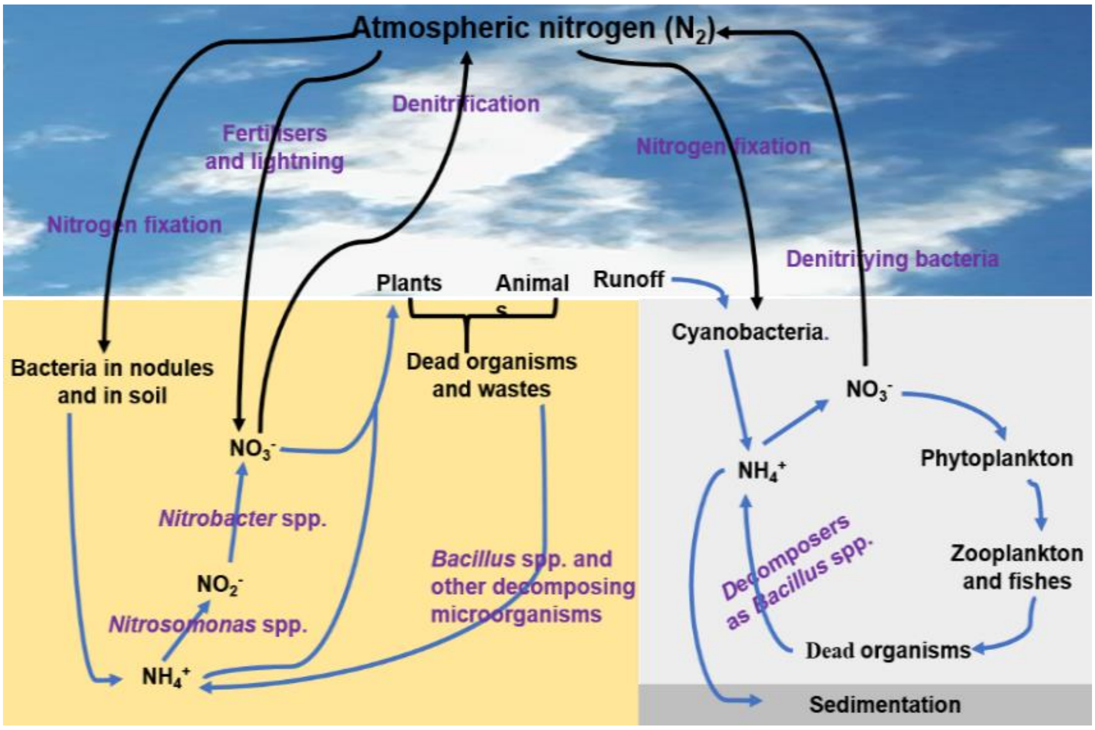


Figure 1. 2: Diagram showing the nitrogen cycle (modified from Walworth 2013).

1.3.3.2 Nitrogen fertilisation

Nitrogen is the 5th most abundant element in the universe and accounts for about 79% of the Earth's atmospheric gas (Los Alamos National Laboratory 2016). As an important gaseous component of the Earth's atmosphere, N_2 does not benefit plants due to the formation of molecules with strong triple bonds between atoms. These triple bonds require a large amount of energy for their dissipation (Reece et al. 2011; Bernhard 2010). Although lightning produces certain amounts of NO_3^- and NH_4^+, which are carried into the soil by rainwater, most of the soil nitrogen comes from the activity of prokaryotic microorganisms such as bacteria and cyanobacteria (Reece et al. 2011). Plants absorb nitrogen in the ionic forms of NH_4^+ and NO_3^- which are produced during ammonification and nitrification. These NH_4^+ and NO_3^- absorbed, are assimilated by cells for the biological synthesis of amino acids and proteins, vitamins, nucleic acids and other molecules essential for the metabolisms. The processes involved in the biogeochemical cycle of nitrogen leading to nitro-fertilisation include N_2 fixation, ammonification, nitrification, assimilation and denitrification as described by Walworth (2013) and Ramanan et al (2016). These 5 processes occur in the soil and the aquatic environments as well as in the HRAOPs of IAPS, as detailed below.

1.3.3.3 Nitrogen fixation

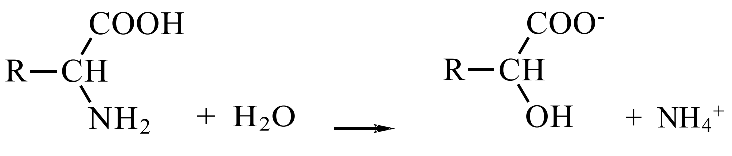
Structurally, the fixation of atmospheric N_2 depends on different bacterial activity and is achieved via the molybdenum cofactor requiring enzyme nitrogenase synthesised in all diazotrophic cells (Bishop and Jorerger 1990; Ahemad and Kibret 2014). The conversion of atmospheric N_2 to NH_3 is a complex and multi-step process (Equation 1) that requires energy from ATP (Reece et al. 2011).

$$N_2 + 8e^- + 8H^+ + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi \qquad (1)$$

Rhizobacteria involved in N_2 fixation utilise a carbon source which is provided by high carbohydrate intake from decaying material and root secretions or from vascular tissues of the roots of host plants (Reece et al. 2011; Berg et al. 2012).

1.3.3.4 Ammonification

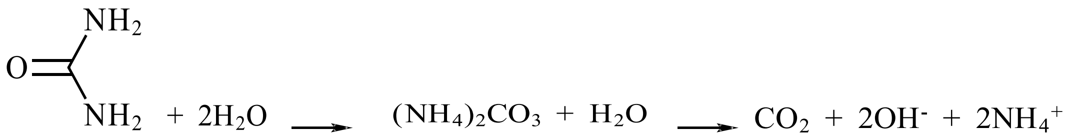
Ammonia (NH_3) and ammonium (NH_4^+) are synthesised by breaking down proteins and other organic nitrogen substances found in humus by decomposing bacteria such as *Bacillus ramosus, Bacillus vulgaris,* and *Bacillus mycoide*s (Strock 2008; Reece et al. 2011) as summarised in Equations 2 and 3 below.



$$R{-}CH(COOH)(NH_2) + H_2O \longrightarrow R{-}CH(COO^-)(OH) + NH_4^+$$

(From soil organic matter)

(2)



$$O=C(NH_2)_2 + 2H_2O \longrightarrow (NH_4)_2CO_3 + H_2O \longrightarrow CO_2 + 2OH^- + 2NH_4^+$$

(From urea) (3)

Ammonium (NH_4^+) is one of two forms of nitrogen that plants can absorb. Ammonifying bacteria, which are mainly decomposers release NH_3. At the same time, N_2-fixing microorganisms also convert atmospheric N_2 to NH_3. In both cases, the NH_3 produced can capture a proton (H^+) in the soil or aquatic environments to form NH_4^+ (Reece et al. 2011).

1.3.3.5. Nitrification

Nitrification involves the conversion of NH_4^+ or NH_3 to nitrite (NO_2^-) or nitrate (NO_3^-). It is the oxidation process that takes place in 2 steps: The first step is the conversion of NH_4^+ or NH_3 to NO_2^- (Equation 4) by *Nitrosomonas* as ammonia-oxidising bacteria (AOB). The second and final step includes the conversion of NO_2^- to NO_3^- (Equation 5) via nitrite-oxidising bacteria (NOB), usually *Nitrobacter* species (Forså et al. 2016).

$$2NH_4^+ \; / \; 2NH_3 + 3O_2 \longrightarrow 2NO_2^- + 2H_2O + 4H^+ \; / \; 2H^+ \qquad (4)$$

$$2NO_2^- \; / \; NO_2^- + O_2 \; / \; H_2O \longrightarrow 2NO_3^- \; / \; NO_3^- + 2H^+ + 2e^- \qquad (5)$$

1.3.3.6 Assimilation

Ammonium and nitrate resulting from ammonification and nitrification are taken up and used by plant cells and other microorganisms such as bacteria, archaea and fungi as essential mineral nutrients (Strock 2008). These NH_4^+ and NO_3^- are converted by plants and microorganisms for the biological synthesis of nucleotides, amino acids, and other vital substances involved in cellular metabolisms (Reece et al. 2011).

1.3.3.7 Denitrification

The biological reduction process that is known as denitrification (equations 6 and 7) consists of breaking down NO_3^- or NH_4^+ and other forms of nitrogen, thereby releasing the harmless N_2 into the atmosphere to complete the biogeochemical cycle (Reece et al. 2011). Denitrifying bacteria include species of the genera *Paracoccus*, *Pseudomonas*, *Achromobacter*, and *Bacillus* (Rose et al. 2007; Bernhard 2010; Reece et al. 2011). The denitrification process takes place in 4 steps and produces the intermediate forms of nitrogen as described in Equation 7.

$$2NO_3^- + 10e^- + 12H^+ \longrightarrow N_2 + 6H_2O \text{ or } NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O \qquad (6)$$

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2 \quad (7)$$

1.3.4 Free-living nitrogen-fixing bacteria

The free-living N_2-fixing microorganisms are diverse and widely distributed among bacterial, algal and fungal taxa. About 80% of the biological N_2 fixation is achieved by rhizobacteria in symbiotic forms (Peoples et al. 1995; Orr et al. 2011). However, under certain conditions, some bacteria that are free-living microorganisms in the soil and the aquatic environments, as well as in the HRAOP of IAPS (*Azospirillum, Azotobacter, Clostridia, and Pseudomonas*) can fix significant amounts of N_2 estimated approximately to 60 kg per hectare per year (Kahindi et al. 1997; Bürgmann et al. 2004; Orr et al. 2011; Walworth 2013; Ramanan et al. 2016). However, few blue-green algae such as *Nostoc, Oscillatoria, Plectoneme* and *Rhodospirillum,* and fungi such as *Saccharomyces* and *Rhodotorula* can also fix atmospheric N_2 in the non-symbiotic form (Berman et al. 1985; Knoth et al. 2013). These bacteria get energy from decomposing materials and root secretions and, in return, fix nitrogen needed by the plant. The fixed nitrogen is then taken up by the plant root system and transported to other parts of the plant to be used to form tissues in terms of boosting plant growth and development (Reece et al. 2011; Berg et al. 2012; Aczel 2019).

1.3.5 Phosphate-solubilising bacteria

Phosphorus (P) represents about 0.2% of the dry weight of the plant and is known to contribute to metabolism and plant growth processes by regulating protein synthesis, growth of new tissues, division of cells and roots development (Widawati and Suliasih 2006; Tajer 2016; Gizaw et al. 2017). The large amounts of insoluble phosphate in soil and wastewater are one of the main causes of eutrophication, which negatively affects many water resources (Weigelhofer et al. 2018). Bacteria have the potential to solubilise and mobilise phosphorus from water or soil making it available for plants uptake (De-Bashan and Bashan 2004). Certain bacteria, algae, and fungi can solubilise both fertiliser and bound phosphorus in the soil (Khan et al. 2007; Gizaw et al. 2017). Some groups of bacteria and fungi identified as phosphate-solubilising microorganisms include the taxonomic genera of *Penicillium, Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium and Serratia*. These bacteria and fungi have been reported to enhance plant growth and development (Kloepper et al. 1989; Sundara et al. 2002; Saharan and Nehra 2011; Gizaw et al. 2017).

Research has shown that the use of a bacterium known as *Kocuria turfanensis* isolated from rhizospheric soil can solubilise phosphate, produce indole-3-acetic acid, ammonium and siderophores. (Goswami et al. 2007; Vejan et al. 2016).

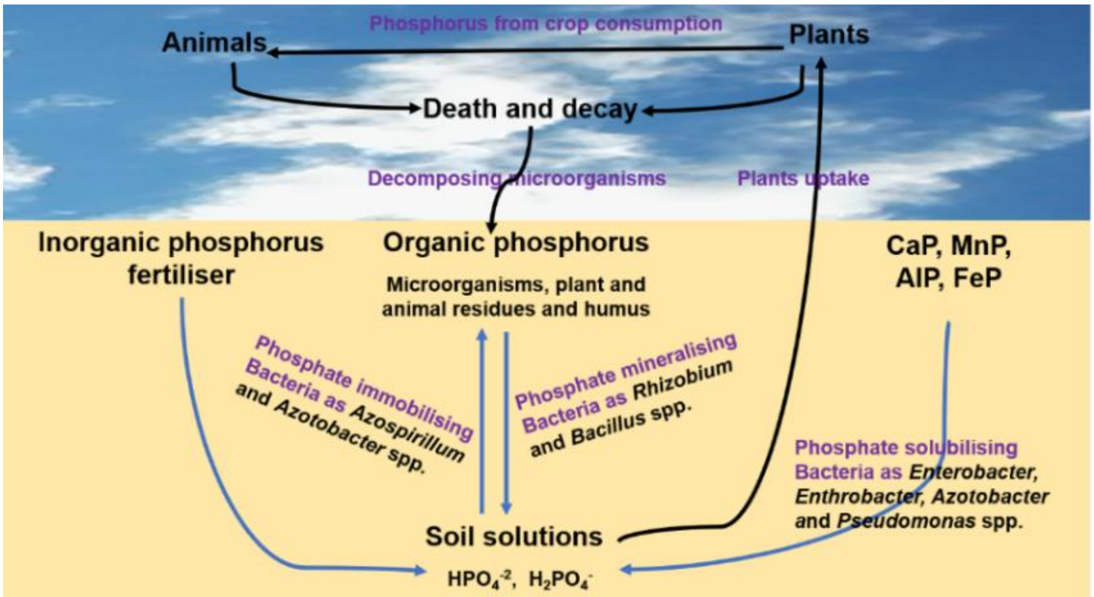


Figure 1. 3: Diagram showing the biogeochemical cycle of phosphorus and mechanisms involved in the promotion of plant growth by phosphate-solubilising and mineralising bacteria (modified from Khan et al. 2009; Ahemad and Kibret 2014; Sharma et al. 2013).

1.3.6 Potassium-solubilising bacteria

Apart from the two major nutrients required by plants such as nitrogen (N) and phosphorus (P), potassium (K) is an essential macronutrient for plant growth and development processes. Taken up by plants in the ionic form, K^+, potassium participates in many cell metabolism processes including enzyme activation, photosynthesis, sugar and water transport, stomatal activity, and protein and starch synthesis (Van Brunt and Sultenfuss 1998; Thomas and Thomas 2009; Patil et al. 2011; Pajapati and Modi 2012; Rawat et al. 2016; Etesami et al. 2017). As a major component of living cells, plant K deficiency causes poor root development, slow growth, poor disease resistance, late fruit maturity, reduced seed production and lower yields (Rawat et al. 2016).

Naturally, the total K content in the soil varies but is usually between 0.04 and 3% by weight. However, due to its insolubility, most K is not available for uptake by plants (Etesami et al. 2017). Additionally, the use of K from chemical fertilisers as NPK has resulted in the weakening of the soil, impacting negatively sustainability of the environment (Rawat et al. 2016; Etesami et al. 2017). Thus, potassium-solubilising bacteria (KSB) can solve the problem by solubilising some K-mineral rocks into the soil, making these soluble forms of K available to plants uptake as nutrients (Etesami et al. 2017). Bacteria such as *Bacillus mucilaginous, Bacillus edaphicus, Bacillus circulans, Acidithiobacillus ferrooxidans* and *Paenibacillus* and fungi such as *Aspergillus* can solubilise K from geologic sources and then, making it available to plants (Meena et al. 2016; Etesami et al. 2017). Many mineral rocks containing large amounts of K include muscovite, orthoclase, biotite, feldspar and mica, and are present in the soil in fixed forms that plants can not directly absorb (Meena et al. 2016; Rawat et al. 2016). Solubilising K bacteria (SKB) have an effective interaction between these mineral rocks and the plant root systems, facilitating the transfer of K^+ from rocks to plants (Calvaruso et al. 2006; Meena et al. 2016). The main known K solubilisation mechanisms include acidolysis, chelation, exchange reactions, complex lysis and organic acid production (Meena et al. 2016).

1.3.7 Production of plant growth regulators

Plant growth regulators (PGRs) are phytohormones controlling many plant physiological processes such as seed germination, fruit and shoot development, leaf formation and overall plant growth in terms of boosting crop productivity for sustainable agriculture (Saharan and Vehra 2011; Vejan et al. 2016). The proposed mechanism of PGRs actions could be associated with the production of plant hormones including indole-3-acetic acid or auxin (IAA), gibberellin (GA) and ethylene (Lugtenberg et al. 2002; Somers et al. 2004; Vejan et al. 2016). The efficacy of IAA produced by the strains of *Azospirillum*, *Agrobacterium*, *Bacillus*, *Enterobacter*, *Pseudomonas* and *Rhizobium* has been demonstrated by accelerating the growth and development of potato plants (*Solanum tuberosum*) (Ahmed and Hasnain 2010; Vejan et al. 2016). This plant hormone controls a wide range of plant developmental processes by stimulating primary root elongation, lateral root and absorbent hair formation, structure facilitating access of plant root system to soil nutrients (Spaepen et al. 2007; Overvoorde et al. 2010; Vejan et al. 2016).

The dominant physiological effect of GA on the plant is shoot elongation, but it can also control a wide range of processes such as seed germination, floral induction, flower and fruit development, and stem and leaf growth (Bottini et al 2004; Spaepen and Vanderleyden 2011). GA can improve plant growth and biomass while counteracting abiotic stress (Hedden and Kamiya 1997). Many strains of bacteria such as *Acetobacter diazotrophicus, Bacillus cereus, Bacillus macroides* and *Promicromonospora* sp. have demonstrated the potential for GA production (Bastian et al. 1998; Joo et al. 2004; Kang et al. 2012b).

Ethylene is a volatile plant hormone known to regulate many plant development processes such as fruit ripening, thus regulating plant stress responses. (Vejan et al. 2016; Houben 2019). The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase is a precursor of ethylene that plants synthesize in response to exposure to various environmental stress including cold, drought, flood, infections and heavy metals (Glick 2012; Vejan et al. 2016). Research has demonstrated that certain strains of *Rhizobium* and *Pseudomonas* synthesise ACC-deaminase that can improve the growth, physiology and quality of mung beans grown under salt-affected soil (Ahmad et al. 2012).

1.3.8 Production of plant biological control

Growing public interest in plant disease control has sparked renewed interest in beneficial bacteria that can improve soil quality and plant health (Gupta et al. 2015). For reasons of health and environmental protection, it is necessary to develop alternative biological pesticides that can reduce excessive use of chemical-based pesticides. Therefore, all possible non-chemical strategies to produce plant biological control substances from PGPR should be considered (Kremer and Kennedy 1996). PGPR can promote plant growth by producing metabolites that may have antibacterial and antifungal activities and used as plant defence systems (Vejan et al. 2016). These bacteria, when applied to seeds, seed fragments or roots, may colonise the surfaces of roots and channels, allowing physical access to the roots, thereby limiting the establishment of pathogens (Kulik 1995; Abdel-Raouf et al. 2012; (Kumar et al. 2010; Vejan et al. 2016). However, for many PGPR, the production of metabolites such as antibiotics, siderophores and hydrogen cyanide is known as a biological control mechanism (Weller and Thomashow 1993). The mechanism would involve the production of hydrolytic enzymes such as chitinase and beta-glucanase. These enzymes are synthesised by the strains of *Sinorhizobium fredii* and *Pseudomonas fluorescens* and can control and inhibit the growth *Fusarium udum*, a plant pathogenic fungus (Kumar et al. 2010; Vejan et al. 2016). *Pseudomonas* strains inhibit the spread of *Rhizoctonia solani* and *Phytophthora capsici*, two fungi known as the most destructive phytopathogenic agents (Arora et al. 2008; Vejan et al. 2016). Research has demonstrated the possibility of developing thin polymers of bactericidal and fungicidal products that could protect against soil-borne pathogens that attack seeds and plants (Kulik 1995; Abdel-Raouf et al. 2012).

1.4 Sustainable agriculture and ecosystem services

Soil organic carbon is crucial for sustainable agriculture because of its relevance to the composition and properties of the soil ecosystem, crop growth and productivity (Al-Omran et al. 2004). A small change in soil carbon content can negatively affect its structure and make the remediation processes more difficult (Lal 2015). Therefore, in the case of soil structure modification, the remediating solution could be done by using fertiliser amendments that can improve physicochemical properties, fertility and productivity. The remediation of the soil can be made in a synthetic and/or a natural way. The synthetic way includes water-soluble polymers and hydrogels, while natural way includes the use of clay minerals, algae and organic manure (Mohammadi et al. 2011; Mlambo 2014). Natural amendments are generally cost-effective solutions with lower negative environmental impacts, while large-scale use of synthetic soil is often not feasible due to high cost and environmental constraints (Al-Omran et al. 2004; Mlambo, 2014).

1.4.1 Plant essential nutrients

In drought environments, insufficient water availability is the main soil stress limiting crop growth (Basu et al. 2016). Thus, increasing the amount of salinity in the soil significantly reduces root and shoot elongation under relatively well-watered or non-drought conditions (Whalley et al. 2006; Whitmore and Whalley 2009; Powlson et al. 2011). Research showed that various parts of the root system can contribute to the specific signals present in the xylem depending on the relative degree of stress and hydration in different regions of the root system (Dodd et al. 2008; Powlson et al. 2011). However, in addition to plant water requirements, plants also need other essential nutrients such as non-mineral and mineral elements (Pandey 2015) as discussed in the following subsections.

1.4.1.1 Non-mineral nutrients

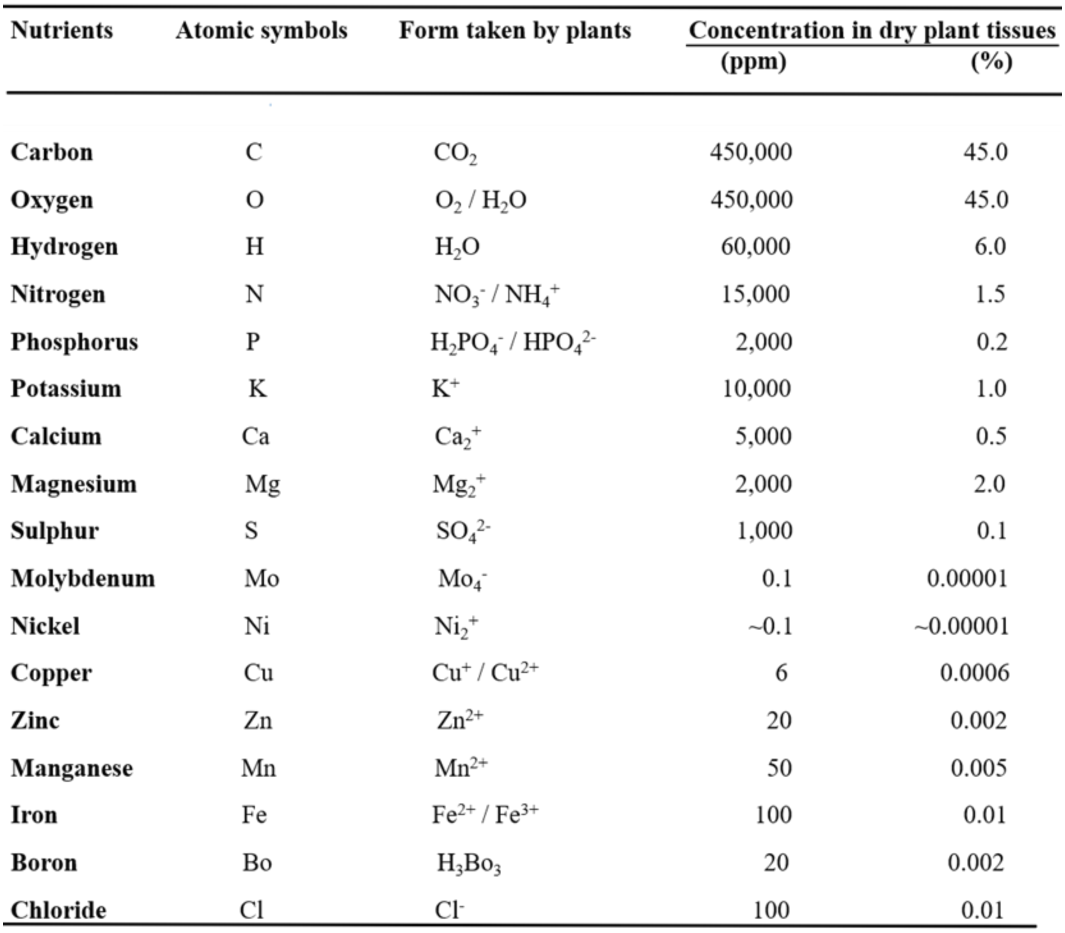
Non-mineral nutrients include carbon (C), hydrogen (H) and oxygen (O) (Table 1.2), which represent 95% of the dry weight of the plant. These non-mineral nutrients are absorbed by plants from the atmosphere, water and soil solutions (Stockdale et al. 1995; Vercesi 2000; Chisti 2006, Egamberdiyeva 2007; Mlambo 2014). Plants absorb atmospheric carbon in the form of carbon dioxide via the exchange of photosynthetic gas. Oxygen is taken also from the atmosphere as the respiratory gas exchange and hydrogen is absorbed by plants in the form of water by the root system (Goldy 2013).

1.4.1.2 Mineral nutrients

Mineral nutrients refer to macronutrients and micronutrients (Table 1.2). Therefore, for plant survival, macronutrients are the most important and represent 0.2 to 4.0% of the dry weight of the plant compared to micronutrients that represent less than 0.02% (Pandey 2015).

Nitrogen (N), phosphorus (P) and potassium (K) are primary macronutrients, while calcium (Ca), magnesium (Mg) and sulphur (S) are secondary macronutrients (Reece et al. 2011; Goldy 2013). Although the concentration of micronutrients needed for plant survival is small, these mineral nutrients are still necessary for normal plant growth and development. They include iron (Fe), copper (Cu), chloride (Cl), manganese (Mn), molybdenum (Mo), zinc (Zn), boron (B) and nickel (Ni) (Reece et al. 2011; Goldy 2013).

Table 1. 2: Plant essential nutrients; absorbed forms and concentration in dry plant tissues (modified from Pandey 2015).



Nutrients	Atomic symbols	Form taken by plants	Concentration in dry plant tissues	
			(ppm)	(%)
Carbon	C	CO_2	450,000	45.0
Oxygen	O	O_2 / H_2O	450,000	45.0
Hydrogen	H	H_2O	60,000	6.0
Nitrogen	N	NO_3^- / NH_4^+	15,000	1.5
Phosphorus	P	$H_2PO_4^-$ / HPO_4^{2-}	2,000	0.2
Potassium	K	K^+	10,000	1.0
Calcium	Ca	Ca_2^+	5,000	0.5
Magnesium	Mg	Mg_2^+	2,000	2.0
Sulphur	S	SO_4^{2-}	1,000	0.1
Molybdenum	Mo	Mo_4^-	0.1	0.00001
Nickel	Ni	Ni_2^+	~0.1	~0.00001
Copper	Cu	Cu^+ / Cu^{2+}	6	0.0006
Zinc	Zn	Zn^{2+}	20	0.002
Manganese	Mn	Mn^{2+}	50	0.005
Iron	Fe	Fe^{2+} / Fe^{3+}	100	0.01
Boron	Bo	H_3Bo_3	20	0.002
Chloride	Cl	Cl^-	100	0.01

1.5 Aims and objectives

This study aimed to investigate the plant growth promoting rhizobacterial bio-fertiliser potential of bacteria isolated from microalgae-bacterial flocs generated in high rate algal oxidation ponds of an integrated algal pond system for the treatment of domestic wastewater.

The specific objectives were then to:

1. Isolate, characterise and identify bacteria from high rate algal oxidation ponds of an integrated algal pond system for the treatment of domestic wastewater;
2. Determine the growth characteristics and culture conditions of bacterial strains isolated from the high rate algal oxidation ponds;
3. Investigate and select bacteria with plant growth promoting rhizobacteria potential to be used for the preparation and development of a bacterial bio-fertiliser.

Chapter 2: Isolation, purification, characterisation and identification of bacteria from high rate algal oxidation ponds

2.1 Introduction

High rate algal oxidation ponds (HRAOPs) biomass is composed of microalgae and bacteria that aggregate in a symbiotic relationship and called microalgae-bacterial flocs (MaB-flocs). This relationship can range from pathogen to mutualism and from facultative to obligatory (Lau et al. 2002). Symbiotic microorganisms such as bacteria are ubiquitous and can be found in animal intestines, soil, water and wastewater (Stott and Taylor 2016). One of the easiest ways to obtain these bacteria from MaB-floc samples is to perform isolation techniques using any solid culture medium. After the isolation, the colonies obtained must be purified. Purification is a necessary step in the basic bacteriological study leading to the morphological and biochemical characterisation that ultimately ends up in bacterial identification (Ruangpan and Tendencia 2004). The simple and effective method commonly used is to spread the bacterial sample onto a solid culture medium so that bacteria might grow as isolated colonies that can be observed (Ruangpan and Tendencia 2004; Johnson 2010).

The knowledge, characterisation and identification of the native bacteria composing the consortium MaB-flocs generated in the HRAOPs of the integrated algal pond system (IAPS) are necessary to understand the distribution and diversity of these microorganisms in this specific environment (Keating et al. 1995; Chahboune et al. 2011; Jimoh et al. 2019). Microalgae-bacterial flocs would have more value if its biomass could be used in addition to wastewater treatment and biogas production, as raw material to produce a bacterial bio-fertiliser (Woertz et al. 2009). *Paenibacillus polymyxa* a bacterium isolated from wastewater was used as a bio-fertiliser and had a positive effect on the growth of tea plants (*Camellia sinensis*) (Xu et al 2014).

In this study, efforts were made to better understand the composition of bacteria that populate the HRAOPs of IAPS for treating domestic wastewater. Samples of MaB-flocs were collected from HRAOP and transported to the laboratory for bacteriological analysis. After isolation and purification, the pure colonies obtained were characterised according to their morphological and biochemical properties and stored at 4°C in solid and liquid forms for further investigations such as faecal coliforms and bacterial purity tests. The molecular identity of the 12 selected bacterial strains was confirmed by BLAST analysis of the PCR product of DNA gene sequencing, at least at the genus level, using the NCBI online database.

2.2 Materials and methods

2.2.1 Integrated algal pond system configuration and operation

The integrated algal pond system (IAPS) used in this study is located at the Institute for Environmental Biotechnology, Rhodes University (EBRU) (Figure 2.1). This biological wastewater treatment system is an experimental station adjacent to the Belmont Valley Municipal Wastewater Treatment Plants in Grahamstown, (WWT; 33° 19’ 07’’ South, 26° 33’ 25’’ East). The system, established in 1995, consists of a primary facultative pond (PFP) incorporating an in-pond digester (ID) (Figure 2.1 A) and processing 75 - 100 m^3 of raw domestic wastewater per day. The hydraulic retention time (HRT) in the ID is 3 d while in the PFP it is 20 d. IAPS includes two 500 m^2 (60 x 8 m) high rate algal oxidation ponds (HRAOP A and HRAOP B) and two 12.5 m^2 algal settling ponds (ASP A and ASP B) (Figure 2.1 B). HRAOP A is connected in series with HRAOP B via ASP A (Figure 2.1.C). The HRT is 2 d in HRAOP A while it is 4 d in HRAOP B with a daily effluent rate of 75-100 m^3 d^{-1} and 37.5-50 m^3 d^{-1} respectively.

Mixing and turbulent flow are achieved by an eight-bladed paddlewheel powered by an electric motor (0.25 kWh) to maintain optimal conditions for maximum biomass productivity. The partially treated water of ASP A is pumped into HRAOP B after 4 d HRT and the effluent flows by gravity into ASP B which has a 0.5 d HRT. Here, the biomasses of microalgae and bacteria are settled. The settled microalgae-bacterial slurry is removed by pumping into drying beds, while treated water leaving ASP B is pumped back to the wastewater treatment works (WWTW).

Mixed liquor or MaB-floc samples for bacterial isolation were collected at the HRAOP B paddlewheel just before the inlet where the effluent was well mixed.

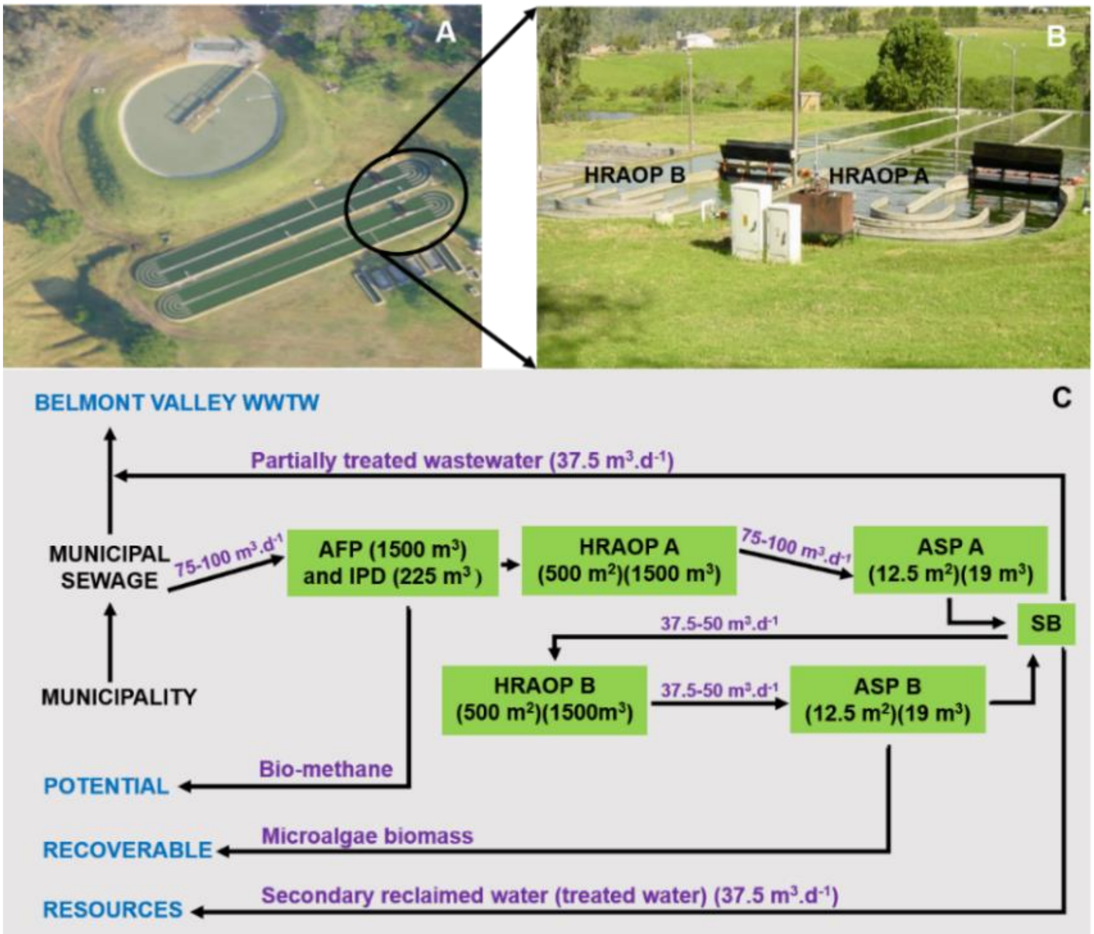


Figure 2. 1: Configuration and process flow of the EBRU integrated algal pond system (IAPS) used in this study. Aerial view of the EBRU IAPS (A); HRAOPs driven by the paddlewheel (B) and process flow (C). Advanced Facultative Pond (AFP); In-Pond Digester (IPD); High Rate Algal Oxidation Ponds A and B (HRAOPs A and B); Algae Settling Ponds A and B (ASPs A and B) and Splitter Box (SB).

2.2.2 Preparation of solid culture media

Three solid culture media, namely Nutrient Agar (NA), Luria-Bertani Agar (LBA) and American Type Culture Collection Agar (ATCC 111A) were prepared to perform isolation, purification and characterisations of bacteria from samples of MaB-flocs of HRAOPs. All solid culture media and material were autoclaved at 121°C and 1.5 kg cm^{-2} for 15 min using a Rexall autoclave (LS-2D, Taiwan).

2.2.2.1 Nutrient Agar

Nutrient Agar (NA) containing 5 g L^{-1} peptone (Fluka, Sigma-Aldrich, USA), 3 g L^{-1} yeast extract (Biolab, Merck, RSA), 8 g L^{-1} NaCl (Merck, KGaA, Germany) and 15 g L^{-1} agar

(Biolab, Merck, RSA) was prepared. The mixture was suspended in 1 L of distilled water, heated with frequent stirring to dissolve completely the medium and autoclaved.

Nutrient agar (NA) is known as a general microbial medium that could allow the growth of many bacteria (Himedia). This medium was prepared to evaluate the variety of bacterial population that inhabits HRAOPs.

2.2.2.2 Luria-Bertani Agar

Luria-Bertani Agar (LBA) containing 10 g L^{-1} tryptone (Biolab, Merck, RSA), 5 g L^{-1} yeast extract (Biolab, Merck, RSA), 10 g L^{-1} NaCl (Merck, KGaA, Germany) and 15 g L^{-1} agar (Biolab, Merck, RSA) was prepared. The mixture was also suspended in 1 L of distilled water, heated with frequent stirring to dissolve completely the medium and autoclaved.

Luria-Bertani agar (LBA) is also a general bacterial culture medium but better for *Bacillus* and *Pseudomonas* and some coliform bacterial species (Himedia). This could give a better understanding and knowledge of the *Bacillus*, *Pseudomonas* and some coliform bacterial strains populating the HRAOPs and that can be isolated.

2.2.2.3 American Type culture Collection Agar

American Type Culture Collection Agar (ATCC 111A) was prepared following two steps (EMCC): The first preparation step was performed by suspending in 800 mL of distilled water, 1 g of yeast extract (Biolab, Merck, RSA), 10 g of mannitol and 15 g of agar (Biolab, Merck, RSA). This first preparation constituted solution 1. The second step consisted of suspending in 200 mL of distilled water, 80 g of soil extract collected around Makana municipality WWTW land and 0.2 g of Na_2CO_3. This second preparation was called solution 2. The solution 2 was autoclaved at 121℃ and 1.5 kg cm^{-2} for 1 h before being mixed into solution 1. After autoclaving the solution 2, it was filtered through cotton wool to remove the soil suspension (EMCC). For the preparation of ATCC 111A as a bacterial culture medium, 200 mL of solution 2 was poured into 800 mL of solution 1. The mixture was then autoclaved at 121℃ and 1.5 kg cm^{-2} for 15 min.

American Type Culture Collection Agar (ATCC 111A) is a selective medium for the isolation of *Rhizobium leguminosarum phaseoli* (EMCC). The medium was used to evaluate whether nitrogen-fixing bacteria can be part of the microorganisms that populate the MaB-flocs biomass.

2.2.3 Collection of samples, isolation and purification of bacteria

The collection of microalgae-bacterial flocs (MaB-flocs) samples was carried out from HRAOP B using sterile 500 mL Erlenmeyer flasks. Sampling was conducted once a week from 9 a.m. to 1 p.m., July to October 2017 and from January to April 2018. These samples were taken to the site laboratory for bacteriological analysis (isolation and purification). Before isolation of bacteria, serial dilutions were prepared by measuring 1 mL of the sample in a 10 mL test tube and made up to 10 mL marked volume with distilled water to form a stock solution of 10^{-1} dilution. The gradient concentrations of 10^{-2} to 10^{-4} were prepared from the stock solution and the 10^{-1} dilution was chosen as better for the plate spreading techniques due to the expected number of colonies obtained as well as 30 °C was better for incubation. The spreading plate method consisted of pipetting 0.1 mL of 10^{-1} dilution onto the centre of the agar plate and using a glass L-rod, the sample was spread over the surface of the agar and incubated at 30 °C for 2 to 4 d (NA) and 2 to 5 d (LBA and ATCC 111A). Bacterial isolates were screened on NA, LBA and ATCC 111A media using the standard spreading plate method (Sujatha et al. 2012). For purification of bacterial isolates, a colony was selected using a sterile inoculation loop and series of non-overlapping parallel streaks were made on the agar to allow the growth of single colonies. Pure colonies should have the same characteristics and not overlap. Therefore, once bacterial colonies were confirmed pure, morphological and biochemical characterisations were examined (Ruangpan and Tendencia 2004).

2.2.4 Morphological characterisation of bacteria

2.2.4.1 Characterisation of bacterial colonies

Some bacteria grow faster than others while producing colonies of different appearances and shapes. The conventional methods based on shape, colour, diameter, surface, margin, elevation, opacity and consistency have been used to characterise these isolated bacteria colony varieties (Gupta et al. 2012; Acharya 2013; Zahid et al. 2015).

2.2.4.2 Scanning electron microscopy

To perform the scanning electron microscopy (SEM), NB was prepared. The pure bacterial colony of each NA plate was picked using a sterile inoculation loop and was inoculated into a flask containing 50 mL of NB. Flasks were incubated for 3d at 30 °C in a rotary shaker at 120 rpm. Scanning electron microscopy (SEM) sample preparation was performed by pipetting 1 mL of fresh grown NB bacterial culture into 2 mL graduated microtubes (SSIBio, 1310-00, USA). The samples were centrifuged using a Labnet centrifuge, 24 places rotor (Prism R, USA) with 9402xg for 5 min. The supernatant of each sample was removed, and the pellet left was washed four times using a phosphate buffer saline (PBS) to remove interstitial water containing residual growth medium. The bacterial pellet was then fixed in 0.5 mL of 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer and kept at 4 °C in the refrigerator for 24 h.

The fixation being completed, glutaraldehyde solution was removed by discarding the supernatant after centrifugation at 3920xg for 2 min using a Costar mini centrifuge, 6 slots rotor (10 MVSS-07343, USA). The samples were immersed twice in 0.1 M sodium phosphate buffer for 10 min, then the buffer supernatant was again removed by centrifugation at 3920xg for 2 min followed by dehydration of the pellets using ethanol. To perform this dehydration, the graduated series of ethanol of different concentrations (30%, 50%, 70%, 80%, 90% and 100%) were used. Pellets were resuspended in each ethanol dilution for a time interval of 3 min followed by centrifugation at 3920xg to decant the ethanol supernatant. Dehydration being completed, the samples were immersed in 0.5 mL of 99% hexamethyldisilazane (Sigma Aldrich, USA) for 10 min. Following immersion time, hexamethyldisilazane was removed by centrifugation and decanting, and the pellets left were air-dried at room temperature in a fume cupboard for 2 h.

The samples were finally mounted on stubs with double-sided conductive tape, gold-coated using Ashford Kent Coater (Q 150 RS, England), and viewed through a Vega analytical SEM Tescan (LMU -TPD 011, Germany) (Figure 2.2).

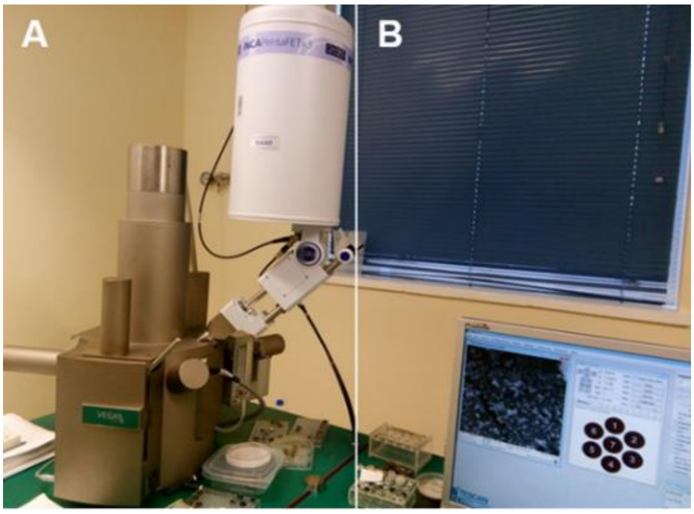


Figure 2. 2: Picture showing the Vega analytical scanning electron microscope (SEM) Testcan (LMU- TPD 011, Germany) (A) used to view on the computer’s screen (B) the 12 isolated bacterial strains.

2.2.5 Biochemical characterisation of bacteria

Following the morphological observations, the bacterial strains were identified according to their Gram staining reaction using an Olympus optical microscope (U-CMAD3, T2, Japan). They were also identified according to the catalase production using 3% hydrogen peroxide (H_2O_2) as a reagent (Bumunang et al. 2014). These two aspects were considered in the study of the biochemical characterisation of selected bacterial strains as detailed in the following subsections.

2.2.5.1 Gram staining reaction

The Gram staining technique discovered in 1884 by a Danish naturalist and physician Hans Christian Joachim Gram, consists of dividing the microorganisms into two groups including, Gram-positive and Gram-negative bacteria (Richard 2006). The difference lies in the structure of the bacterial cell walls. While the former have thicker peptidoglycan layer and lower lipid content and, therefore, are not discoloured by acetone-alcohol and remain as violet, the later are covered with a thin layer of peptidoglycan that cannot maintain crystal violet. These cell walls are then discoloured by alcohol, losing violet colour and when safranin is added, they appear as pink as the colour of the reagent (safranin) (Sandle 2004; Richard 2006). The following Gram staining standard procedure was used to characterise all the 12 isolates obtained (Sandle 2004; Sharma et al. 2011).

1. A portion of the bacterial colony (smear) of the NA plate was picked up, spread on a glass slide and air-dried before being heat-fixed;
2. Gently, crystal violet was poured onto the smear (cells) and allowed to react for 30 sec to 1min. This stained bacterial cells blue;
3. Gram iodine was also added and allowed to react for 30 sec to 1 min. The iodine solution enters the cells and forms an insoluble complex with the crystal violet dye;
4. The slide was slightly tilted and gently rinsed with distilled water;
5. The smear was discoloured with acetone-alcohol dropwise until the decolouring ran almost clear. Only Gram-positive cells remained stained;
6. Safranin was gently flooded onto the smear and allowed to react for 30 to 45 sec. The slides were washed again with distilled water and air-dried;
7. The bacterial cells were examined using an Olympus optical microscope (U-CMAD3 T2, Japan) under oil-immersion;

8. Gram-positive bacteria appeared purple whereas Gram-negative showed a pinkish-red colour.

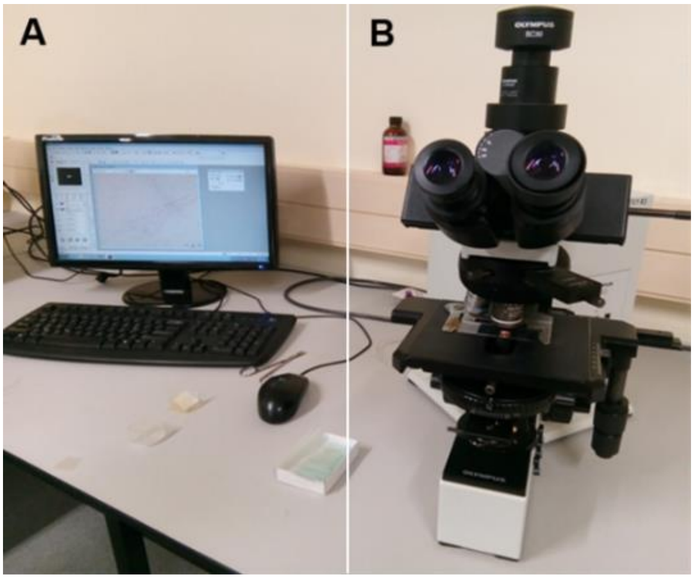


Figure 2. 3: Gram staining characteristics of the 12 isolated bacterial strains viewed on the computer's screen (A) connected to an Olympus optical microscope (U-CMAD3 T2, Japan) (B).

2.2.5.2 Catalase activity

Catalase activity was screened by adding a drop of 3% H_2O_2 to each freshly cultured bacterial colony (Bumunang et al. 2014). The production of O_2 in the form of gas bubbles indicated the enzyme activity of bacterial catalase that breaks down H_2O_2. This was evidence that the bacteria forming the colony were catalase-positive. Catalase positive bacteria may be strict aerobes or facultative anaerobes. However, the lack of O_2 bubbles could mean a lack of catalase production and indicate that the bacteria forming a colony were negative for catalase production or were anaerobes. The enzymatic reaction leading to the decomposition of H_2O_2 to O_2 and H_2O by strict aerobes and facultative anaerobes is detailed in equation 8 (Acharya 2013).

$$\mathbf{2\ H_2O_2 \xrightarrow{\textit{Catalase}} 2\ H_2O + O_2} \tag{8}$$

2.2.6 Checking for faecal coliform pathogens

Chromocult coliform agar (CCA) is an effective medium used to identify *Escherichia coli* and certain other coliforms without the need for extensive biochemical testing (Finney et al. 2003). This solid culture medium (34.5 g L^{-1}) (Merck KGaA, Germany) was prepared to check whether isolates of the faecal coliform pathogen could exist among the bacterial colonies from HRAOP. According to the manufacturer, the medium was heated to a boil and immediately poured into sterile Petri dishes without being autoclaved.

For the plate streaking method, a pure isolated colony was picked, and parallel streaks were performed on CCA. The colony colour was inspected as after 1 or 2 d incubation.

2.2.7 Checking the purity of bacterial strains isolated

The purity of bacterial isolates was checked every two months. The original bacterial culture colonies were sub-cultured on NA containing 5 g L^{-1} peptone (Fluka, Sigma-Aldrich, USA), 3 g L^{-1} yeast extract (Biolab, Merck, RSA), 8 g L^{-1} NaCl (Merck, KGaA, Germany) and 15 g L^{-1} agar (Biolab, Merck, RSA) and incubated at 30 °C for 2 to 4 d. Strains that were still pure should develop the same colony morphologies as the original. The new pure bacterial strains on agar plates were resealed and stored as stock cultures at 4 °C for further studies.

2.2.8 Storage conditions of bacterial strains isolated

Some of the pure isolates were selected, sub-cultured on a slanted NA, labelled and incubated at 30 °C for 2 to 4 d. These NA slants also served as stock cultures and were refrigerated at 4 °C, while other pure isolates were sub-cultured in NB containing 5 g L^{-1} peptone (Fluka, Sigma-Aldrich, USA), 3 g L^{-1} yeast extract (Biolab, Merck, RSA), 8 g L^{-1} NaCl (Merck, KGaA, Germany) and incubated for 3 d at 30 °C. These isolates in liquid culture were also stored in 30% (v/v) glycerol at -80 °C.

2.2.9 Molecular characterisation of bacterial strains isolated

2.2.9.1 Deoxyribonucleic acid extraction

The extraction of deoxyribonucleic acid (DNA) was performed by the boiling method (Queipo-Ortuño et al. 2008). Bacterial isolates were sub-cultured on NA plates and incubated at 30 °C for 2 d. A sterile inoculation loop was used to select 2 to 3 individual colonies (~2 mm ø) from each NA plate and suspend them in 2 mL graduated microtubes (SSIBio, 1310-00, USA) containing 200 µL sterile distilled water. The samples were vortexed and boiled at 100 °C for 10 min using a Labnet AccuBlock (D1100, USA) and then centrifuged at 15890xg for 10 min using a Labnet centrifuge, 24 places rotor (Prism R, USA). After centrifugation, the supernatant of each sample was carefully removed and a Nanodrop 2000 (Thermofisher Scientific, Waltham, USA) was used to quantify the DNA concentration. Finally, the samples were stored at -20 °C for PCR confirmation (Titilayo et al. 2015; Olawale 2017).

2.2.9.2 Deoxyribonucleic acid gene sequence analysis

After the DNA extraction, purity and quality were determined by pipetting from each sample 5 µL of bacterial DNA for electrophoresis in a 1% (w/v) agarose gel with TBE (×1) containing 0.05 mg mL^{-1} of ethidium bromide in gel and visualised under UV light with a FireReaderXs (Uvitech, France). DNA molecular weight was estimated by comparison with a DNA marker using Kapa Express Ladder (Kapa Biosystems). The amplification of DNA was performed using polymerase chain reaction (PCR) for sequencing.

Two oligonucleotide primers used for this 16S rRNA gene were universal prokaryotic (16S-515F 5'- GTGYCAGCMGCCGCGGTAA-3' forward primer (Parade) and (16S-926R 5'-CCGYCAATTYMTTTRAGTTT-3' reverse primer (Quince). These primers amplified a region of approximately 480 bp for each sample. The volume of the PCR was 20 µL, containing 10 µL of Go Taq enzymes, 0.25 µM of each primer and 50 to 100 ng of DNA. The quantification of the DNA was performed again using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, USA). The amplification was carried out using a Veriti 96 Well thermocycler (Applied Biosystems, Carlsbad, USA) and consisted of an initial denaturation step of 3 min at 94 °C followed by 35 cycles of 45 sec, 1 min and 1:30 min at 94 °C, 50 °C and 72 °C respectively. The final extension step of 10 min at 72 °C was also performed. After PCR, the samples were cleaned according to the EXOSAP protocol (Werle et al. 1994).

Cyclic sequencing was performed using the Big Dye sequencing kit (Applied Biosystems, USA). The reaction volume was 10 µL and contained 1µL of Big Dye sequencing reagent, 1.5 µL of Big Dye Sequencing buffer, 3.2 µM primer and 3 to 10 ng of DNA. The PCR thermal cycle for sequencing consisted of an initial denaturation step of 1 min at 96 °C followed by 25 cycles of 10 sec each at 96 °C and 50 °C with a final extension step of 4 min at 60 °C. The samples were cleaned using the specific ethanol-EDTA precipitation method for the Cycle Sequencing Product Cleaning Kit and then air-dried. Sequencing was performed on the Genetic Analyser (Applied Biosystems 3500, USA) of the Genetic Laboratory of the South African Institute for Aquatic Biodiversity (SAIAB). The edited sequences were used as queries in BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the nearest identifiable match found in the complete GenBank nucleotide database.

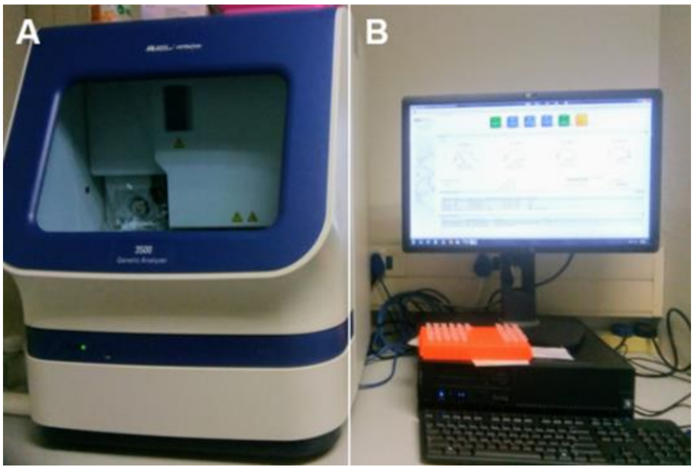


Figure 2. 4: Picture showing the Genetic Analyser (A) (Applied Biosystems 3500, USA) used for sequencing and viewing on computer's screen (B) the DNA genes of the 12 bacterial strains isolated.

2.3 Results

2.3.1 Isolation and purification of bacterial colonies

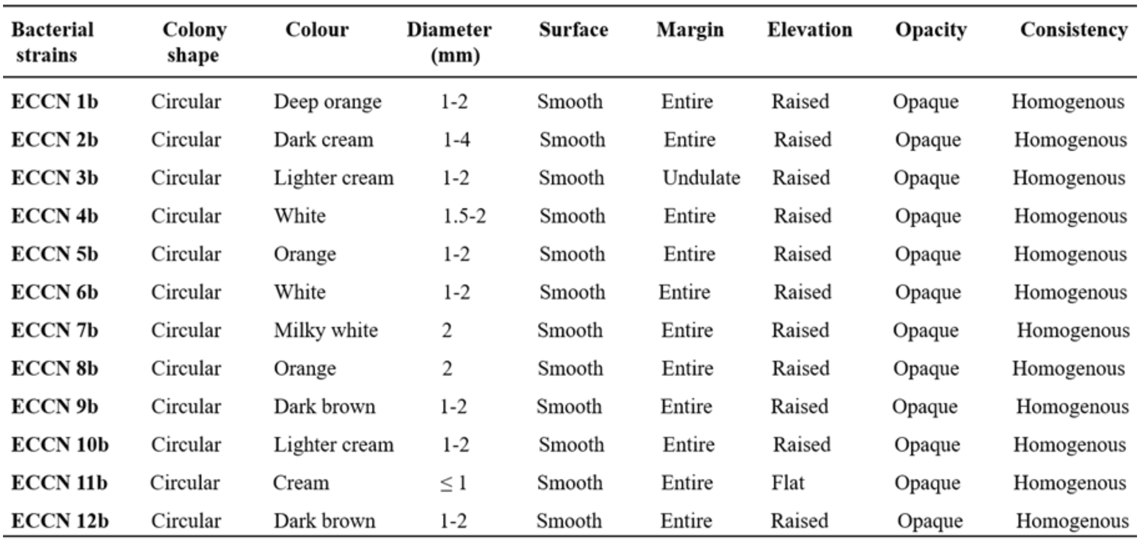
The samples of MaB-flocs from HRAOP B were collected and transported to the laboratory for bacterial isolation using three different solid culture media known as Nutrient agar (NA), Luria-Bertani agar (LBA) and American type culture collection agar (ATCC 111A). Following bacterial isolation and purification, nearly 16 bacterial isolates were obtained, and their morphological differences were examined in order to select colonies that would constitute the bacterial strains.

2.3.2 Morphological characterisation of bacterial isolates

2.3.2.1 Bacterial colony analysis

Since bacterial colonies could present similar morphology and shape while different strains, it was necessary to select one typical solid medium that would serve as a standard for morphological examination. The 16 bacterial isolates were able to grow on the 3 types of solid media, however, due to the better development of bacterial colonies on NA, this medium was chosen as a reference for running morphological characterisation. After streaking the 16 bacterial isolates on NA plates, 12 bacterial isolates were found to be morphologically different and were then selected as different strains. These 12 different bacterial strains were assigned as EBRU Culture Collection bacteria (ECCN b) and correctly labelled as ECCN 1b, ECCN 2b, ECCN 3b, ECCN 4b, ECCN 6b, ECCN 7b, ECCN 8b, ECCN 9b, ECCN 10b, ECCN 11b and ECCN 12b as detailed in Table 2.1.

Table 2. 1: Morphological characteristics of bacterial strains isolated from the second-high rate algal oxidation pond (HRAOP B).



Bacterial strains	Colony shape	Colour	Diameter (mm)	Surface	Margin	Elevation	Opacity	Consistency
ECCN 1b	Circular	Deep orange	1-2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 2b	Circular	Dark cream	1-4	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 3b	Circular	Lighter cream	1-2	Smooth	Undulate	Raised	Opaque	Homogenous
ECCN 4b	Circular	White	1.5-2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 5b	Circular	Orange	1-2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 6b	Circular	White	1-2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 7b	Circular	Milky white	2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 8b	Circular	Orange	2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 9b	Circular	Dark brown	1-2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 10b	Circular	Lighter cream	1-2	Smooth	Entire	Raised	Opaque	Homogenous
ECCN 11b	Circular	Cream	≤ 1	Smooth	Entire	Flat	Opaque	Homogenous
ECCN 12b	Circular	Dark brown	1-2	Smooth	Entire	Raised	Opaque	Homogenous

By analysing bacterial colonies using the standard conventional methods based on morphology viz. shape, colour, diameter, surface, margin, elevation, density, and consistency (Gupta et al. 2012; Acharya 2013; Zahid et al. 2015); it was found that these bacteria all appeared circular, smooth, opaque, and homogenous. The colours of the colonies were of creamy (ECCN 2b, ECCN 3b, ECCN 10b, and ECCN 11b); orange (ECCN 1b, ECCN 5b, and ECCN 8b); brown (ECCN 9b and ECCN 12b), and white (ECCN 4b, ECCN 6b, and ECCN 7b). Their diameters varied between 1 and 4 mm apart from the strain labelled ECCN 11b which presented a colony diameter that was less than 1 mm. Finally, the elevation of 11 colonies was raised except for the ECCN 11b which was flat.

2.3.2.2 Scanning electron microscopy (SEM) of bacterial strains

Scanning electron microscopy (SEM) was also used to analyse the shape of each isolate among the 12 strains of bacteria at magnifications of 5 µm and 10 µm. Figures 2.5 shows the scanning electron micrographs of the 12 nearly rod-shaped bacterial isolates. Some bacterial cells were small rod-shaped (ECCN 3b, ECCN 4b, ECCN 7b, ECCN 9b and ECCN 12b), rod-shaped (ECCN 5b, ECCN 6b and ECCN 8b) and long rods (ECCN 1b and ECCN 10b). However, ECCN 2b was composed of long-chain rod-shaped, whereas some bacteria appeared mainly in the form of single rod-shaped cells (ECCN 1b, ECCN 3b, ECCN 4b, ECCN 6b and ECCN

12b). In addition, ECCN 11b appeared as curved rod-shaped cells. Finally, all cells were easily visualised, except ECCN 7b.

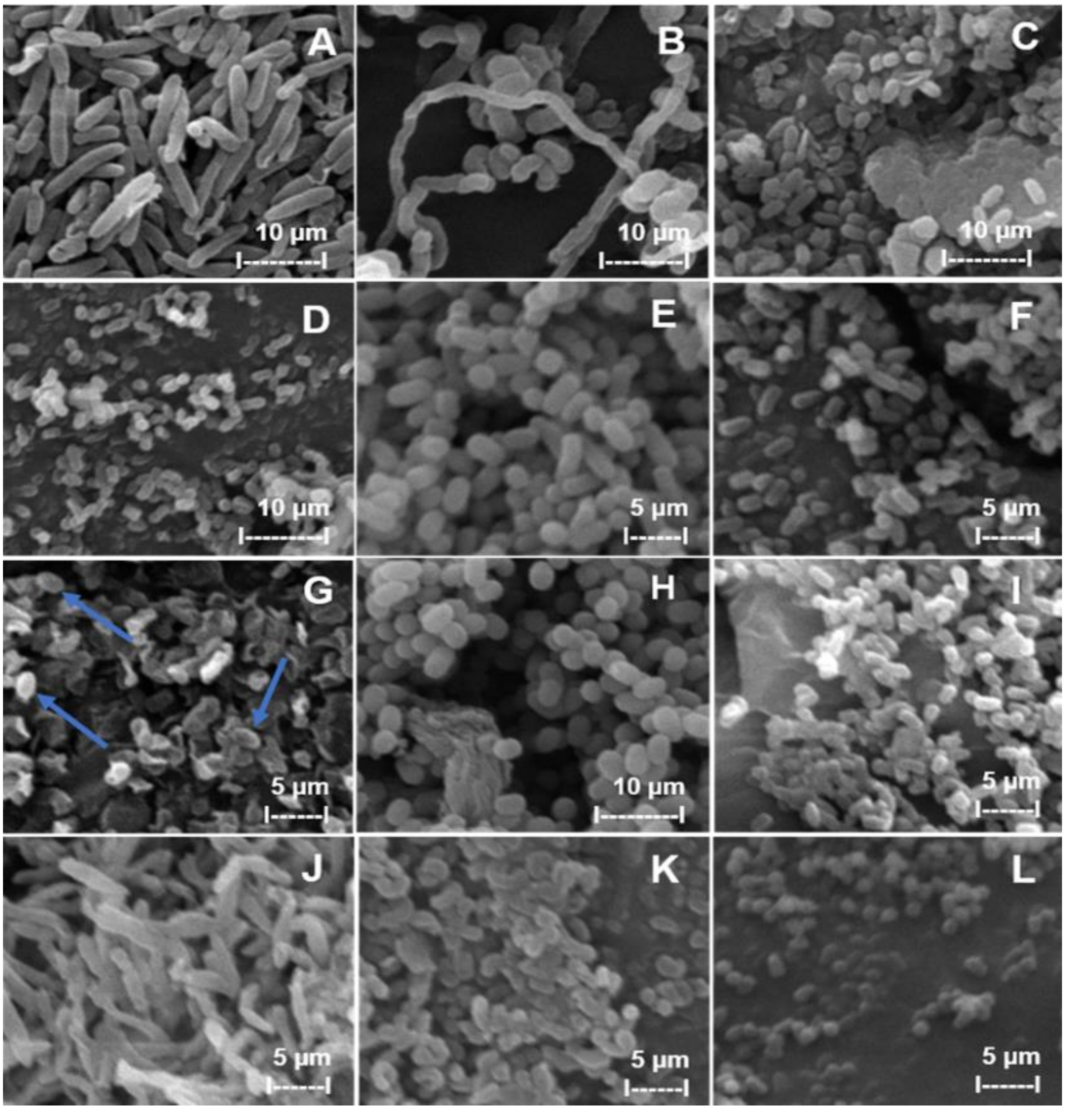


Figure 2. 5: Scanning electron micrographs of 12 isolated bacterial strains. ECCN 1b (A); ECCN 2b (B); ECCN 3b (C); ECCN 4b (D); ECCN 5b (E); ECCN 6b (F); ECCN 7b (G); ECCN 8b (H); ECCN 9b (I); ECCN 10b (J); ECCN 11b (K) and ECCN 12b (L).

2.3.3 Biochemical characterisation of bacterial isolates

2.3.3.1 Gram staining test

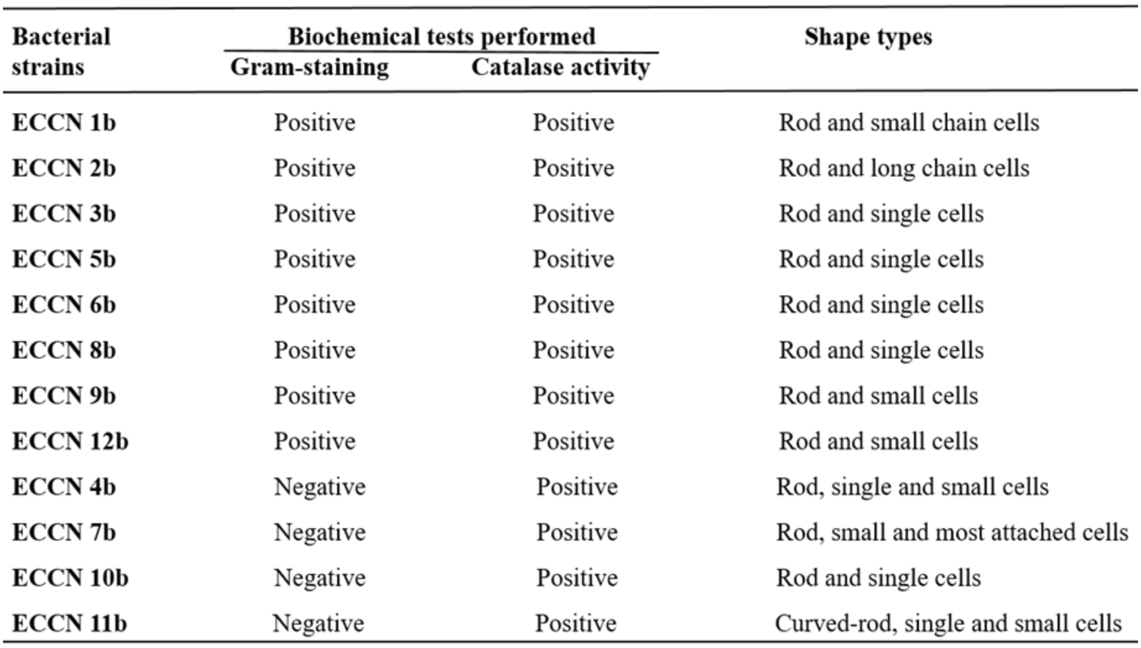
The biochemical characterisation in terms of Gram staining assay was performed by selecting a fresh bacterial colony from each NA plate. The colony was subjected to the Gram staining procedure and finally, the stained cells were visualised using an Olympus optical microscope (U-CMAD3, T2-Japan) as illustrated in Appendix A, Section A.1 and Figure A.1.1.

The details in Table 2.2 showed that 4 isolates namely, ECCN 4b, ECCN 7b, ECCN 10b, and ECCN 11b were Gram-negative bacteria, while 8 bacteria such as ECCN 1b, ECCN 2b, ECCN 3b, ECCN 5b, ECCN 6b, ECCN 8b, ECCN 9b, and ECCN 12b appeared Gram-positive.

2.3.3.2 Catalase test

The catalase activity assay was examined as the ability of isolates to produce catalase enzyme. Catalase-positive bacteria produce the enzyme that reacts with H_2O_2 and releases easily detectable oxygen (O_2) in the form of gas bubbles. Commercial H_2O_2 3% was used for this standard test of catalase-producing bacteria (Reiner 2010). After subjecting the 12 bacterial strains to the catalase test, the results showed that all isolates were catalase-positive (Table 2.2).

Table 2. 2: Biochemical characteristics (shape, Gram stain, and catalase production) of bacterial strains examined using the Olympus optical microscope (U-CMAD3 T2, Japan).



Bacterial strains	Biochemical tests performed		Shape types
	Gram-staining	Catalase activity	
ECCN 1b	Positive	Positive	Rod and small chain cells
ECCN 2b	Positive	Positive	Rod and long chain cells
ECCN 3b	Positive	Positive	Rod and single cells
ECCN 5b	Positive	Positive	Rod and single cells
ECCN 6b	Positive	Positive	Rod and single cells
ECCN 8b	Positive	Positive	Rod and single cells
ECCN 9b	Positive	Positive	Rod and small cells
ECCN 12b	Positive	Positive	Rod and small cells
ECCN 4b	Negative	Positive	Rod, single and small cells
ECCN 7b	Negative	Positive	Rod, small and most attached cells
ECCN 10b	Negative	Positive	Rod and single cells
ECCN 11b	Negative	Positive	Curved-rod, single and small cells

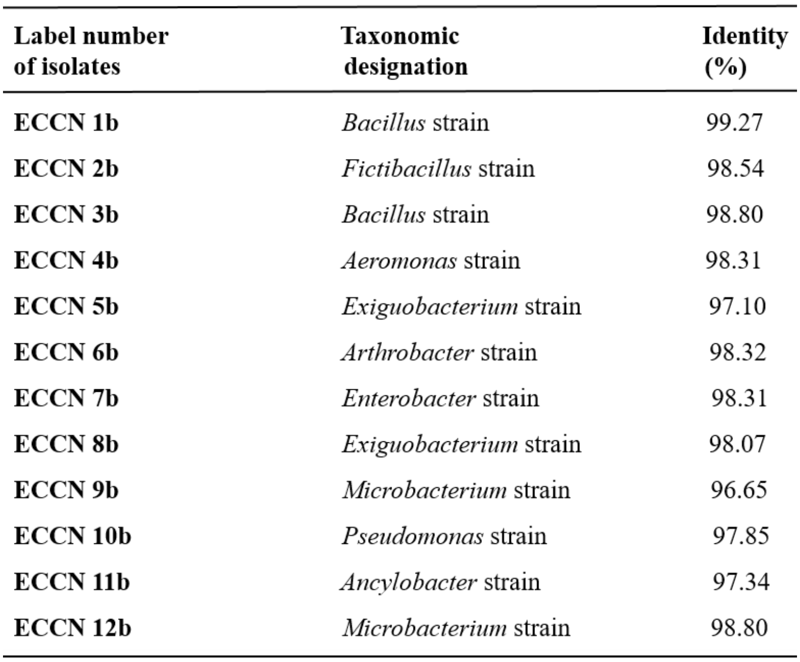
2.3.4 Determination of faecal coliforms

The pathogenic coliform test of 12 isolated bacteria was performed by plating each isolate on Chromocult Coliform Agar (CCA) (34.5 g L^{-1}). Based on the literature, the identification of faecal coliforms is determined according to the pigmentation of their colonies. *Escherichia coli* develops colonies from dark blue to violet, while other coliforms appear pink or red colonies. Non-targeted bacteria produce beige colonies, as well as yellow or, do not grow on CCA medium at all (Finney et al. 2003). The results revealed that of the 12 strains examined, only 3 known as ECCN 4b, ECCN 7b, and ECCN 10b were able to grow on CCA. The bacteria that grew on this selective solid medium developed pigment completely different from those expected. They were ranged from light pink (ECCNb4) to dark pink (ECCN 7b) and cream (ECCN 10b).

2.3.5 Molecular identification of bacterial strains

The molecular identity of the 12 bacterial strains using 16S rRNA was screened and established after DNA extraction and PCR (Appendix A, Section A.1, Figure A.1.2). The DNA sequencing result of each sample was recorded from the Genetic Analyser (Applied Biosystems 3500, USA) and BLAST analysis confirmed the taxonomic identity of each strain at the genus level using NCBI online database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as illustrated in Table 2.3.

Table 2. 3: Molecular identity of bacterial strains isolated from high rate algal oxidation ponds (HRAOPs).



Label number of isolates	**Taxonomic designation**	**Identity (%)**
ECCN 1b	*Bacillus* strain	99.27
ECCN 2b	*Fictibacillus* strain	98.54
ECCN 3b	*Bacillus* strain	98.80
ECCN 4b	*Aeromonas* strain	98.31
ECCN 5b	*Exiguobacterium* strain	97.10
ECCN 6b	*Arthrobacter* strain	98.32
ECCN 7b	*Enterobacter* strain	98.31
ECCN 8b	*Exiguobacterium* strain	98.07
ECCN 9b	*Microbacterium* strain	96.65
ECCN 10b	*Pseudomonas* strain	97.85
ECCN 11b	*Ancylobacter* strain	97.34
ECCN 12b	*Microbacterium* strain	98.80

Based to the BLAST analysis information recorded (Appendix A, Section A.1, Figure A.1.3 and Table 2.3), the % identity of each isolate was: 99.27% matched with *Bacillus* strain ECCN 1b, 98.54% corresponded with *Fictibacillus* strain ECCN 2b, 98.80% matched with *Bacillus* strain ECCN 3b, 98.31% corresponded with *Aeromonas* strain ECCN 4b, 97.10% matched with *Exiguobacterium* strain ECCN 5b, 98.32% matched with *Arthrobacter* strain ECCN 6b, 98.31% matched with *Enterobacter* strain ECCN 7b, 98.07% corresponded with *Exiguobacterium* strain ECCN 8b, 96.65% matched with *Microbacterium* strain ECCN 9b, 97.85% matched with *Pseudomonas* strain ECCN 10b, 97.34% corresponded with *Ancylobacter* strain ECCN 11b and 98.80% corresponded with *Microbacterium* strain ECCN 12b.

2.4 Discussion

This chapter describes laboratory experiments performed to isolate, purify, characterise and identify bacteria from the MaB-flocs produced in the HRAOPs of IAPS. The bacterial morphology analysed on NA showed that all colonies were circular, smooth, opaque, and homogenous. In fact, some colonies were cream (ECCN 2b, ECCN 3b, ECCN 10b, and ECCN 11b); orange (ECCN 1b, ECCN 5b, and ECCN 8b); brown (ECCN 9b and ECCN 12b), and white (ECCN 4b, ECCN 6b, and ECCN 7b). The diameters could vary between 1 and 4 mm, except for the strain ECCN 11b which presented a diameter less than 1 mm. The elevation of colonies was raised for at least 11 isolates except again for ECCN 11b which was flat. It appeared that all the microorganisms were bacteria as the classification based on colony morphology established that all bacteria can form a variety of shapes ranging from translucent or opaque, to different colours such as orange, white, or red (Khan et al. 2018).

Scanning electron microscopy (SEM) also showed that 12 isolated strains had different shapes. They were either rods, long rods, short rods or curved rods. Young (2006) explained this observation by stating that some bacterial cells have uniform morphologies from among a wide range of possibilities, but that some cells can modify their shape as conditions change. There is, therefore, insufficient evidence to suggest why most isolates were rod-shaped. However, the Watve (1997) study demonstrated that a rod-shaped cell has a streamlined body that would be advantageous for swimming and for nutrient delivery that takes place through the cell surface, and as the rate of bacterial growth may depend on the rate of nutrient ingestion.

Of the 12 strains tested for Gram staining, 8 isolates such as ECCN 1b, ECCN 3b, ECCN 3b, ECCN 5b, ECCN 6b, ECCN 8b, ECCN 9b and ECCN 12b were Gram-positive while the remaining 4 isolates were Gram-negative. All isolates tested positive for catalase activity. The low number of Gram-negative bacteria in the wastewater environment may be explained by their less adaptability under water-stress conditions (e.g. alkalinity) due to their thin cell walls. Research has shown that Gram-negative bacteria are generally the smallest and most sensitive to water-stress, while Gram-positive bacteria are much larger and their thicker cell walls are resistant to water-stress (Dick 2009; Hoorman 2016). Therefore, the 100% catalase-positive bacteria demonstrated that these strains may come from HRAOPs an oxygen-rich environment.

The absence of faecal pathogenic coliforms clearly showed that the disinfection potential of the domestic wastewater in HRAOPs was and is still successful. Rose et al (2002); Cowan et al (2016) and Butler et al (2017) indicated that, if properly maintained, HRAOPs could play a leading role in the biological treatment of wastewater by removing not only minerals, but pathogen microorganisms such as *Escherichia coli*, fungi, and viruses.

Finally, by using the BLASTN searches of the DNA gene sequencing results from the Genetic Analyser (Applied Biosystems 3500, USA) it was possible to identify 12 bacterial strains at least at the genus level as *Bacillus* (ECCN 1b and ECCN 3b), *Fictibacillus* (ECCN 2b), *Aeromonas* (ECCN 4b), *Exiguobacterium* (ECCN 5b), *Arthrobacter* (ECCN 6b), *Enterobacter* (ECCN 7b), Exiguobacterium (ECCN 8b), *Microbacterium* (ECCN 9b), *Pseudomonas* (ECCN 10b), *Ancylobacter* (ECCN 11b) and *Microbacterium* (ECCN 12b). Consistent with these identification results, this work has discovered that all these strains have been already isolated from different aquatic environments such as marine sediments, municipal sewage and saline wastewater (Ferreira da Silva et al. 2007; Vaz-Moreira et al. 2008; Kumar et al. 2014; Agunbiade et al. 2017; Piotrowska et al. 2017; Remonsellez et al. 2018). This indicated that these bacteria might be common to both aquatic environments such as marine, wastewater and biological wastewater. However, only a few bacterial isolates including *Bacillus* and *Pseudomonas* strains from wastewater have been screened for PGPR purposes (Strock 2008; Saharan and Nehra 2011; Xu et al. 2014; Khan et al. 2018).

In conclusion, this study aimed at better understanding the composition of the bacterial population that constitutes part of the MaB-flocs generated in HRAOPs of IAPS located at EBRU. The taxonomic designation approach used in this study did not identify bacteria at a species level. However, the identification of 12 native bacteria at the genus level was an important step that can be furthered to study their growth characteristics and culture conditions.

Chapter 3: Growth characteristics and culture conditions of isolated bacterial strains

3.1 Introduction

Knowledge of the responsiveness of bacterial growth to a wide range of environmental factors, such as temperature, pH, carbon substrates and others, is important for bacteriological and biotechnological studies. According to the Food and Drug Administration (FDA) (2001), pH significantly affects the generation time of bacteria and their overall optimal growth rate is around 9. The carbon source significantly increases the biosynthesis of the enzymes responsible for direct oxidation and is active under aerobic conditions (Tejera et al. 2004). Glucose is mainly known as a carbon source commonly used for the growth of many microorganisms. However, it is also necessary to study the effect of other carbon substrates on the growth and activity of bacterial strains that can be developed for biotechnological purposes (Bren et al. 2016). Knowing the bacterial cell concentration used as inoculants is also important in the study of bacterial growth. It is then necessary to determine the known bacterial cell density as a colony-forming unit (CFU mL^{-1}) for use as a seed culture (Zahid et al. 2015). Bacterial cell counts can be performed by plating a known volume of the cell culture on the surface of the agar plates. If the cells are correctly distributed on the agar, it can be assumed that each cultivated cell will give rise to a single colony which can then be counted (Sanders 2012).

Every environmental condition is important for the study of microorganisms. Although bacteria have significant potential for adaptation to the environment, others are sensitive to changes in environmental conditions, such as temperature, acidity or alkalinity and may prefer a type of carbon source (O 'Callaghan et al. 2006). It is then necessary to establish the optimal pH and the preferred carbon substrate of each isolated bacterial strain (Sadi and Masoud 2012).

The objective of this chapter was to evaluate the growth characteristics and adaptability of the 12 bacterial strains subjected to changes in environmental conditions, such as pH and different carbon substrates. To achieve this goal under controlled laboratory conditions, the growth rate of bacteria was examined in NB with a pH set at 9 and measured at 1 h intervals for 14 h. However, the tolerance of bacterial strains to pH was investigated as a growth rate response using also NB, but at different pH values set from 5 to 11. Finally, three different carbon substrates (glucose, sucrose and mannitol) were selected and supplemented in NB used to determine the preferred carbon source of each isolate and their effect on bacterial growth rates.

3.2 Material and methods

3.2.1 Determination of bacterial density as colony-forming units

It is important to know the titre of the bacterial cells, as a colony-forming unit (CFU), used to prepare a bacterial inoculum. Nutrient broth (NB) was used to prepare this bacterial inoculum and consisted of suspending in 1 L of distilled water; 5 g of peptone (Fluka, Sigma-Aldrich, USA), 3 g of yeast extract (Biolab, Merck, RSA), and 8 g of NaCl (Merck, KGaA, Germany) and pH values were set at 7 and 9.

An individual bacterial colony was picked from an agar plate and inoculated into an Erlenmeyer flask containing 50 mL of NB. Twelve inoculum samples were prepared. The cultures were incubated for 3 d at 30 °C and 120 rpm. After the incubation and before the direct counting methods, serial bacterial dilutions were prepared from each sample. These serial dilutions were performed by pipetting 1 mL of freshly grown culture into a 10 mL test tube, made up to 10 mL marked volume using either normal distilled water, saline water (8%) or HEPES buffer solution (10 mM) to form a bacterial suspension solution of 10^{-1} dilution. For this purpose, the bacteria were suspended in different liquids or solutions according to the preferences of each strain. The gradient concentrations of 10^{-2} to 10^{-7} were prepared and typically diluted samples 10^{-7} were chosen as better for the plate spreading techniques on NA and in triplicate. Each plate received 0.1 mL of 10^{-7} dilution and, using a glass L-rod, the sample was spread on the agar surface and incubated at 30 °C for 1 to 3 d. Following incubation, colonies were counted with the naked eye and a bacterial colony count of 35 to 250 CFU was optimally considered (Jung and Lee 2016). Knowing the average number of colonies, the dilution factor and the volume (mL) plated, the number of bacteria (CFU mL^{-1}) was calculated using the formula of equation 8 (Bunting 2017; Sieuwerts et al. 2008). For liquid culture screening, seed culture inoculum concentration of ~2.0×10^{9} CFU mL^{-1} (Suleman et al. 2018) was prepared for use.

$$\mathbf{CFU} = \frac{\textbf{Number of colonies}}{\textbf{Volume plated x Dilution factor}} \qquad (8)$$

3.2.2 Determination of bacterial growth

There are different ways to measure bacterial growth in a liquid culture medium that includes turbidity using a colorimetric spectrophotometer assay or serial dilution plating using colony-forming units (CFU mL^{-1}). The turbidity measurements are based on the fact that as the number of cells in the liquid medium increases, the liquid becomes cloudy (Bunting 2017).

Nutrient broth (NB) was chosen as a growth medium and its pH fixed at 9. The growth estimation was carried out using a UNICO 1100 spectrophotometer. The standard procedure of bacterial growth was studied by pouring 5 mL of NB into a 10 mL test tube and autoclaved at 121℃ and 1.5 kg cm^{-2} for 15 min. The experiments were performed in triplicate and the control used was also triplicate 10 mL test tubes containing 5 mL of un-inoculated NB.

On the day of use, 0.005 mL of bacterial suspension (inoculant) or seed culture (~2.0×10^9 CFU mL^{-1}) was pipetted into each tube containing 5 mL of NB (JoVE 2019) and the initial absorbance values of all the triplicated treatments were immediately measured at 600 nm. The incubation followed at 30 ℃ with constant agitation of 120 rpm. The optical density was measured at 1 h intervals at 600 nm for 14 h.

3.2.3 Effect of pH on bacterial growth

Several bacteria have specific pH values at which growth is optimal. The ability of bacterial strains to grow in NB at different pH values was evaluated by adjusting the pH to 5, 6, 7, 8, 9, 10 and 11 (before autoclaving) using NaOH (2.6 M and 5 M) and HCl (35%) (Datta et al. 2010). A calibrated portable pH meter 330 (WTW 82362, Germany) was used for this purpose. The volume of 50 mL of NB was poured into 250 mL flask, labelled and autoclaved at 121℃ and 1.5 kg cm^{-2} for 15 min. Twelve flasks were prepared according to the number of strains and the experiment was performed in triplicate. A triplicate 250 mL flask containing 50 mL of NB each was autoclaved and left uninoculated. This served as the control. From a liquid seed culture, 0.05 mL of each bacterial inoculant was pipetted to inoculate the corresponding flask containing 50 mL of NB, the initial values were measured, and the media incubated for 3 d at 30 ℃ on a rotary shaker at 120 rpm. After the incubation, 1 mL of freshly grown culture was pipetted into 1 mL spectrophotometric cuvettes and the optical density was determined at 600 nm (OD_{600}) using a Shimadzu spectrophotometer (UV-1201, Japan).

3.2.4 Effect of carbon source on bacterial growth

Three carbon sources (glucose, sucrose and mannitol) were selected to investigate how these sugars could affect the growth of the 12 bacterial isolates. These sole carbon substrates were added for the preparation of three separated liquid culture media namely glucose peptone broth (GPB), sucrose peptone broth (SPB) and mannitol peptone broth (MPB). The pH was always set at 9 and the treatments were triplicated. The same volume of 0.05 mL of bacterial suspension or seed culture (~2.0×10^9 CFU mL^{-1}) was pipetted into each flask containing 50 mL of carbon substrate broth. The incubation was set at 30 ℃ on a rotary shaker at 120 rpm for 3

d. The cell growth density was determined based on turbidity by measuring the optical density at 600 nm (OD_{600}) using Shimadzu spectrophotometer (UV-1201, Japan).

3.2.4.1 Glucose peptone broth

The glucose peptone broth (GPB) was prepared to test the ability of bacterial strains to utilise glucose as a carbon source. GPB was prepared by suspending in 1 L of distilled water; 5 g of peptone (Fluka, Sigma-Aldrich, USA), 3 g of yeast extract (Biolab, Merck, RSA), 8 g of NaCl (Merck, KGaA, Germany) and 20 g of glucose (Alpha, RSA) (Mohite, 2013). The pH was fixed at 9 and the medium autoclaved. After autoclaving, inoculation followed into a 250 mL flask containing 50 mL of GPB, the incubation was set at 120 rpm on a rotary shaker at 30 ℃ for 3 d. Optical density was measured at 600 nm.

3.2.4.2 Sucrose peptone broth

The sucrose peptone broth (SPB) was prepared to evaluate the ability of the isolates to use sucrose as a carbon source. This medium was prepared by suspending in 1 L of distilled water; 20 g of sucrose (Merck KGaA, Germany) (Mohite, 2013), 5 g of peptone (Fluka, Sigma-Aldrich USA), 3 g of yeast extract (Biolab, Merck, RSA) and 8 g of NaCl (Merck, KGaA, Germany). Inoculation of bacteria into each 250 mL flask containing 50 mL of SPB was performed and the culture was incubated at 30 ℃ on a rotary shaker at 120 rpm for 3 d. Optical density was measured at 600 nm.

3.2.4.3 Mannitol peptone broth

The bacterial strains were cultured on mannitol peptone broth (MPB) to study the ability of the isolated strains to use mannitol as a carbon source. The modified MPB was prepared by dissolving in 1 L of distilled water; 20 g of mannitol (Mohite, 2013), 5 g of peptone (Fluka, Sigma-Aldrich -USA), 3 g of yeast extract (Biolab, Merck, RSA) and 8 g of NaCl (Merck, KGaA, Germany). After inoculating bacteria into each 250 mL flask containing 50 mL of MPB the culture was incubated at 30 ℃ on a rotary shaker at 120 rpm for 3 d. Optical density was measured at 600 nm.

3.2.5 Data and statistical analysis

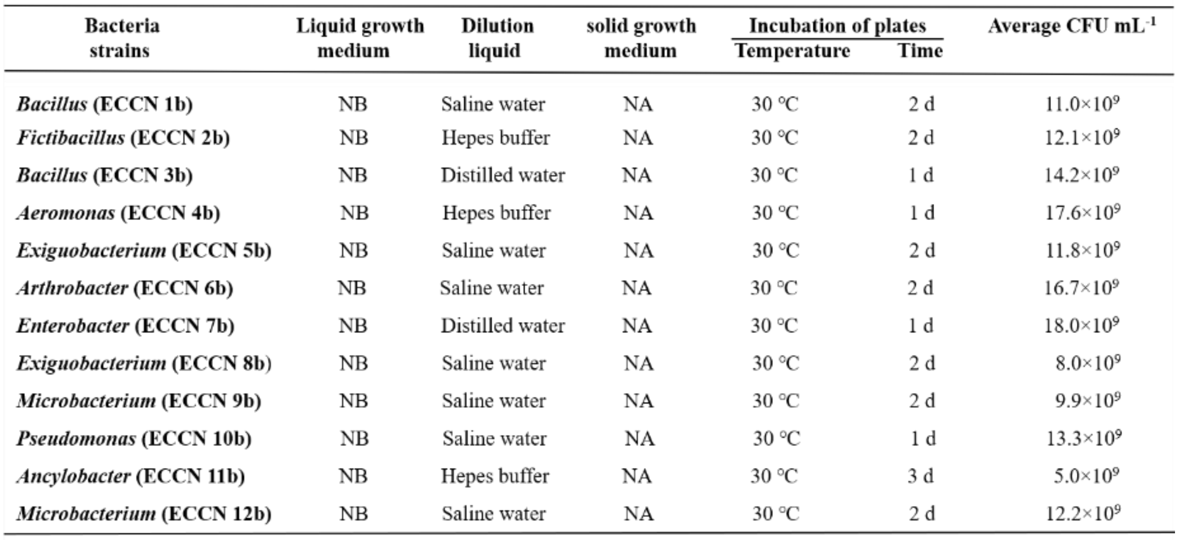
All data presented in this chapter were mean values and standard errors (SE) of at least three sample measurements that were collected as mentioned in Appendix B, section B.1, Tables B.1.1 to B.1.3. All statistical analyses were performed using Microsoft Excel 2016. Mean values were compared using analysis of variance (ANOVA) to test for significant differences between all datasets at 95% level of significance (Microsoft Excel, 2016).

3.3 Results

3.3.1 Bacterial count

For bacterial cell count, 0.1 mL of bacterial suspension (10^{-7} dilution) of each sample was plated on agar, spread using a glass L-rod and incubated for 3 d at 30 °C. After incubation, the isolated colonies were counted. Based on the known volume of culture plated, the cell concentration was calculated using the formula of equation 8 and the results noted (Table 3.1). The CFUs obtained ranged from 5.0×10^9 to 18.0×10^9 cells mL^{-1}. Knowing the average bacterial concentration of each freshly grown NB, bacterial inoculant at a constant concentration volume of ~2.0×10^9 CFU mL^{-1} was prepared from each sample using freshly sterilised NB as a suspension solution (inoculant). From this equivalent inoculum known as seed culture, the same volume of 0.05 mL was used to inoculate 50 mL of any liquid culture medium.

Table 3. 1: Culturing method and bacterial count estimation as CFU mL^{-1}.



Bacteria strains	Liquid growth medium	Dilution liquid	solid growth medium	Incubation of plates		Average CFU mL^{-1}
				Temperature	Time	
***Bacillus* (ECCN 1b)**	NB	Saline water	NA	30 °C	2 d	11.0×10^9
***Fictibacillus* (ECCN 2b)**	NB	Hepes buffer	NA	30 °C	2 d	12.1×10^9
***Bacillus* (ECCN 3b)**	NB	Distilled water	NA	30 °C	1 d	14.2×10^9
***Aeromonas* (ECCN 4b)**	NB	Hepes buffer	NA	30 °C	1 d	17.6×10^9
***Exiguobacterium* (ECCN 5b)**	NB	Saline water	NA	30 °C	2 d	11.8×10^9
***Arthrobacter* (ECCN 6b)**	NB	Saline water	NA	30 °C	2 d	16.7×10^9
***Enterobacter* (ECCN 7b)**	NB	Distilled water	NA	30 °C	1 d	18.0×10^9
***Exiguobacterium* (ECCN 8b)**	NB	Saline water	NA	30 °C	2 d	8.0×10^9
***Microbacterium* (ECCN 9b)**	NB	Saline water	NA	30 °C	2 d	9.9×10^9
***Pseudomonas* (ECCN 10b)**	NB	Saline water	NA	30 °C	1 d	13.3×10^9
***Ancylobacter* (ECCN 11b)**	NB	Hepes buffer	NA	30 °C	3 d	5.0×10^9
***Microbacterium* (ECCN 12b)**	NB	Saline water	NA	30 °C	2 d	12.2×10^9

3.3.2 Bacterial growth curves

Of the 12 isolated bacterial strains, 11 grew successfully in NB. The growth curves were evaluated for conventional growth profiles. The logarithmic growth curves obtained included the largest number of exponential phases, except for bacteria 5 and 8 (*Exiguobacterium* strains); which showed the rapid growth but that stopped early, reaching the stationary phase after 6 h (Figure 3.1).

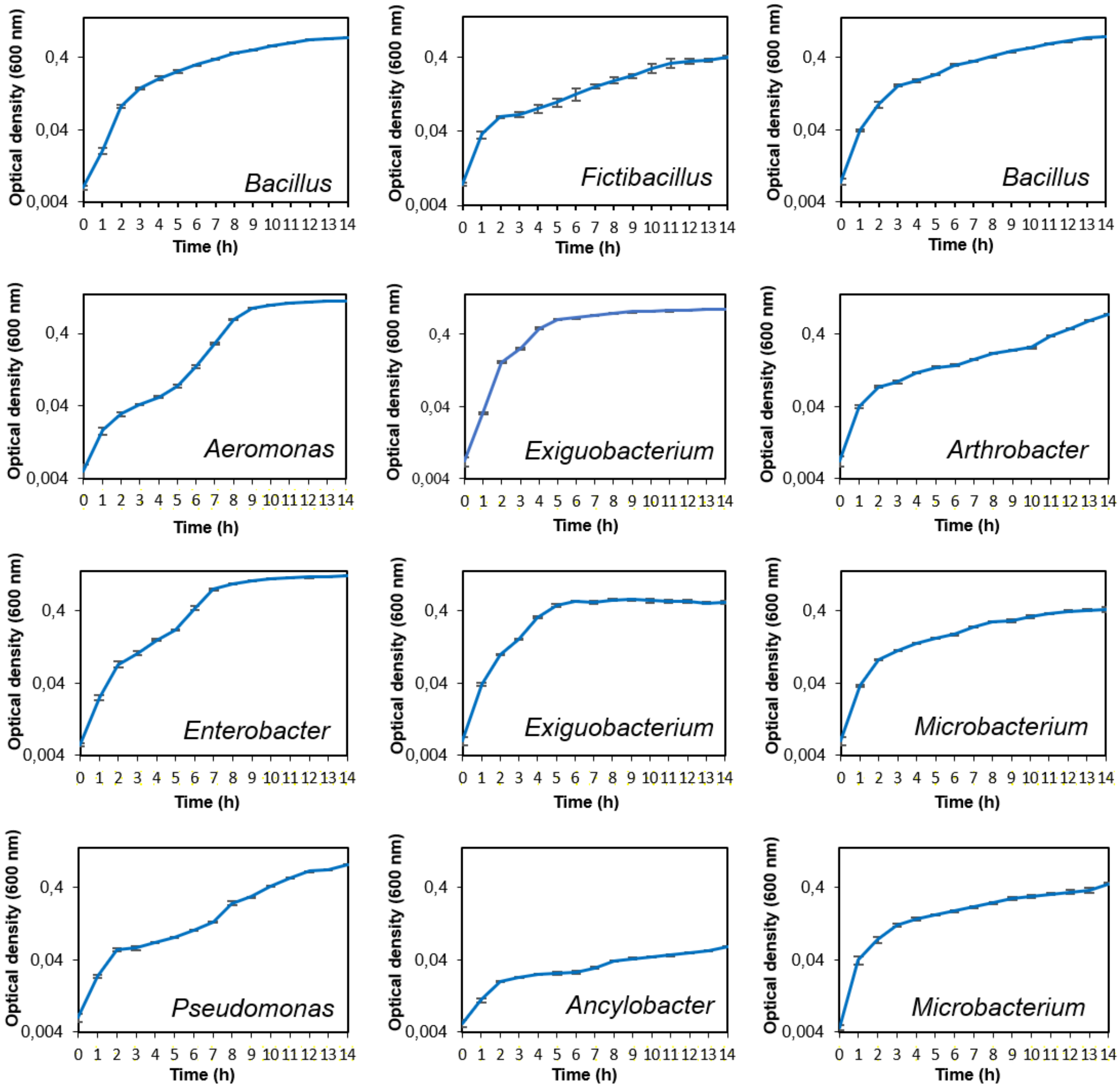


Figure 3. 1: Logarithmic growth curves of 12 genera of bacterial strains grown in NB. The pure colonies were inoculated and incubated at 30 °C, pH 9.2 and 120 rpm for 14 h. At 1 h intervals, growth was determined by measuring the OD_{600}. The data are represented by the mean and standard error of the 12 biological treatments.

Considering Figure 3.1 in terms of growth rates based on OD_{600} measured; 8 bacteria namely *Enterobacter* strain ECCN 7b (1.214), *Aeromonas* strain ECCN 4b (1.120), *Exiguobacterium* strain ECCN 5b (0.893), *Pseudomonas* strain ECCN 10b (0.841), *Bacillus* strain ECCN 3b (0.770); *Bacillus* strain ECCN 1b (0.760); *Arthrobacter* strain ECCN 6b (0.750) and *Exiguobacterium* strain ECCN 8b (0.530) were among the highest. *Microbacterium* strain ECCN 12b (0.452), *Microbacterium* strain ECCN 9b (0.420), *Fictibacillus* strain ECCN 2b (0.406) and *Ancylobacter* strain ECCN 11b (0.061) had the lowest growth rates. The differences in logarithmic growth curves were significant (P<0.05) (Appendix B; Section B.1 and Table B.1.1).

3.3.3 Bacterial pH tolerance curves

For the 12 bacterial strains grown in NB at pH values set between 5 and 11, the culture density of bacteria was quantified at 600 nm and the results plotted (Figure 3.2).

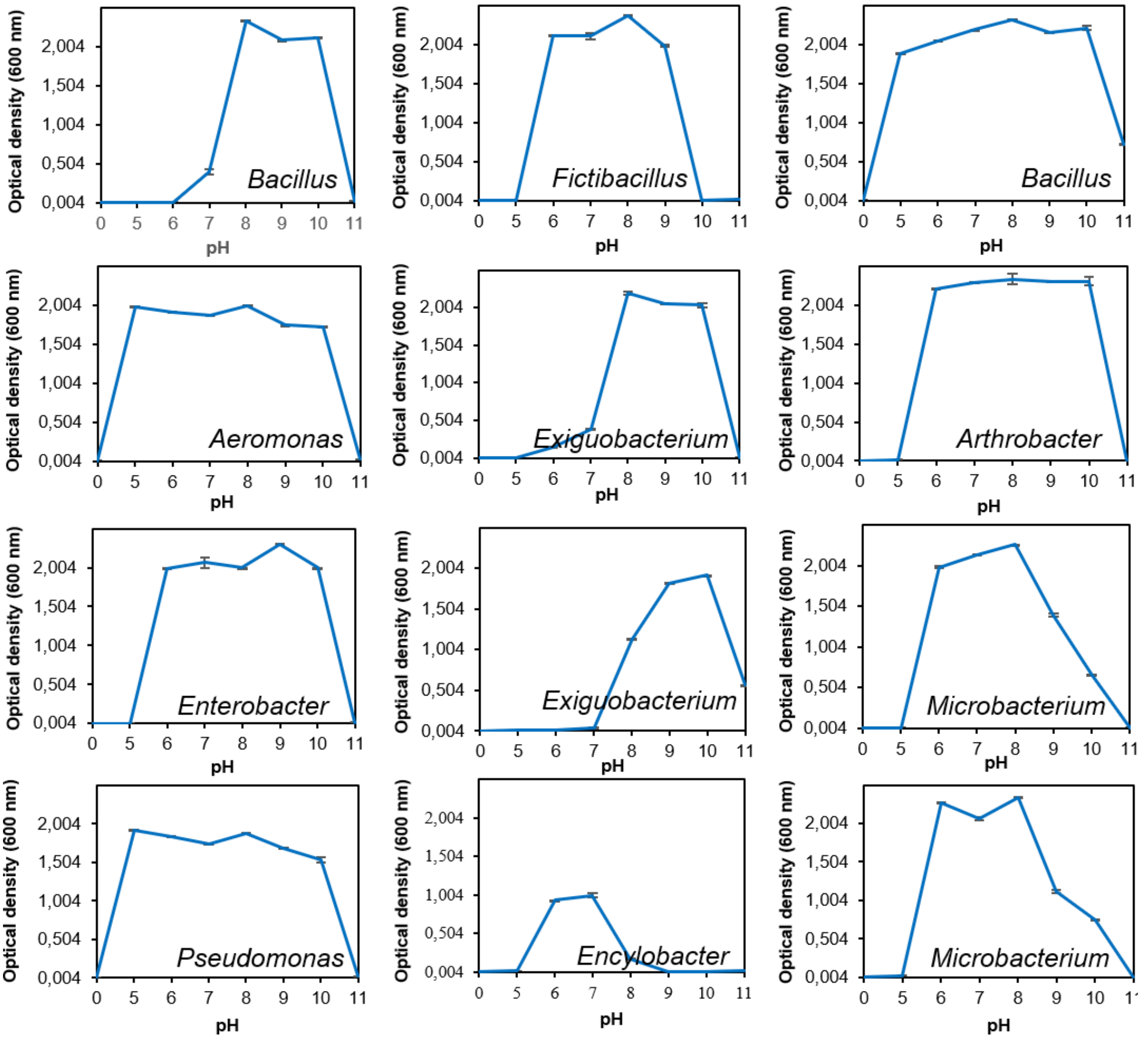


Figure 3. 2: Bacterial pH tolerance in NB at pH 5 to 11. The pure colonies were inoculated and incubated at 30 °C, pH 5 to 11 and 120 rpm for 3 d. After incubation, the bacterial culture density was determined by measuring OD_{600}. The data are represented by the mean and standard error of the 12 biological treatments.

Based on Figure 3.2, the lowest growth was recorded at pH 5 and pH 11. Growth was optimal between pH 6 and 10, but pH 8 and 9 displayed the best growth with the highest OD_{600} average values of 1.861 and 1.723 respectively. Data showed that of the 12 isolated strains, 9 had good growth at pH 8 and 9. By considering the bacterial pH tolerance, the strains were classified into

3 categories in terms of the average OD_{600} measured after 3 d of incubation. The first category of 4 isolates included *Bacillus* (ECCN 3b), *Arthrobacter* (ECCN 6b), *Aeromonas* (ECCN 4b) and *Pseudomonas* (ECCN 10b). Their A_{600} values were 1.939, 1.641, 1.615 and 1.523 respectively. The second category of 7 isolated strains included *Enterobacter* (ECCN 7b), *Fictibacillus* (ECCN 2b), and 2 strains of *Microbacterium* (ECCN 9b and ECCN 12b), *Bacillus* (ECCN 1b) and 2 strains of *Exiguobacterium* (ECCN 5b and ECCN 8b). They presented the OD_{600} values of 1.484, 1.233, 1.207, 1.073, 0.996, 0.973 and 0.640 respectively. The third and last category had a single isolated strain, namely *Ancylobacter* (ECCN 11b) which recorded a lower OD_{600} of 0.307.

Finally, the single ANOVA factor showed a significant difference ($P<0.05$) as a function of pH tolerance (Appendix B; Section B.1 and Table B.1.2), indicating that pH influences bacterial growth, but extremes in pH are unfavourable to their growth.

3.3.4 Use of carbon substrates by bacteria

The 12 isolated bacterial strains were cultured in 3 different NB. Each NB prepared was supplemented with one of the three-carbon substrates selected and known as glucose, sucrose and mannitol. The three liquid culture media prepared were designated glucose-peptone broth (GPB), sucrose-peptone broth (SPB) and mannitol-peptone broth (MPB).

Figure 3.3 showed that 6 bacterial strains demonstrated their ability to use glucose as a carbon source, 8 strains were able to use sucrose and 8 mannitol. The 6 bacteria that preferred glucose as a carbon substrate were: *Bacillus* (ECCN 3b), *Aeromonas* (ECCN 4b), *Arthrobacter* (ECCN 6b), *Enterobacter* (ECCN 7b), *Pseudomonas* (ECCN 10b) and *Ancylobacter* (ECCN 11b). The 8 isolates that used sucrose as a carbon source were also *Fictibacillus* (ECCN 2b), *Bacillus* (ECCN 3b), *Aeromonas* (ECCN 4b), *Arthrobacter* (ECCN 6b), *Enterobacter* (ECCN 7b), *Pseudomonas* (ECCN 10b) and 2 *Microbacterium* (ECCN 9b and ECCN 12b). The last group of 8 bacterial strains composed of 2 *Bacillus* (ECCN 1b and ECCN 3b), *Fictibacillus* (ECCN 2b), *Aeromonas* (ECCN 4b*), Arthrobacter* (ECCN 7b), *Enterobacter* (ECCN 7b), *Pseudomonas* (ECCN 10b) and *Ancylobacter* (ECCN 11b) were able to grow on mannitol broth using it as a carbon substrate. Sucrose and mannitol were the preferred carbon substrates relative to glucose, with the average OD_{600} peaks of 1.833 and 1.716 each. However, 5 isolates were able to use both sugars effectively. These are *Arthrobacter* strain ECCN 6b (2.594), *Aeromonas* strain ECCN 4b (2.507), *Pseudomonas* strain ECCN 10b (2.489), *Enterobacter* strain ECCN 7b (2.333) and *Bacillus* strain ECCN 3b (2.247).

The results showed significant differences ($P<0.05$) in terms of carbon substrate utilisation within and between bacterial treatments (Appendix B; Section B.1 and Table B.1.3) indicating how different bacteria showed different preferences for carbon sources.

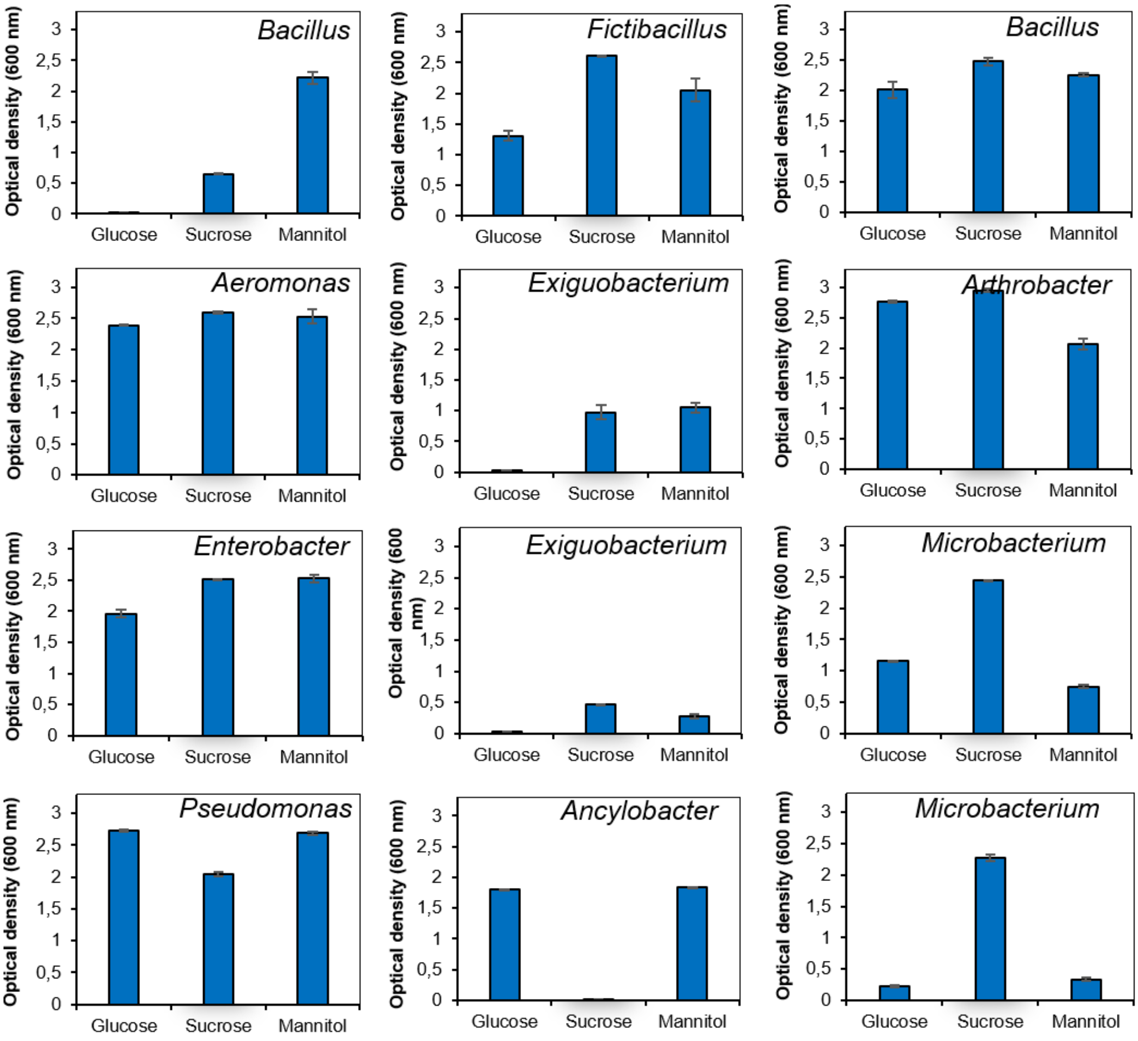


Figure 3. 3: Growth of bacterial strains in 3 different carbon substrate broths. The incubation was set at 30 °C and pH 9.1 for 3 d. After incubation, the bacterial culture density was determined by measuring OD_{600}. The data are represented by the mean and standard error of the 12 biological treatments.

3.4 Discussion

This chapter examined the growth characteristics and culture conditions of bacterial strains in terms of pH tolerance and carbon substrates by measuring their culture density as absorbance at 600 nm using spectrophotometric quantification assays.

The results demonstrated that at least bacterial strains such as *Enterobacter* (ECCN 7b), *Aeromonas* (ECCN 4b), 2 *Exiguobacterium* (ECCN 5b and ECCN 8b) and 2 *Bacillus* (ECCN

1b and ECCN 3b) had the highest growth rates of the all strains investigated. Also, the 12 isolates could use NB substrates as sources of carbon and nitrogen, but differences in growth rates could be due to pH tolerance. Studies have shown that various bacteria can grow in an acidophilic, neutrophilic or alkaline environment (Ñancucheo et al. 2016; Bren et al. 2016). Thus, when cultivated in an alkaline environment (pH 9) as in the case of this research, certain acidophilic or neutrophilic bacteria could not develop or grow well (Ñancucheo et al. 2016). This could be the case for *Ancylobacter* strain ECCN 11b. The recording of high growth rate at pH 8 and 9 showed that the bacterial strains had been isolated from HRAOP as part of the IAPS for treatment of domestic wastewater because, in this environment, the pH is almost closer to 9.

The results obtained in Figure 3.3, demonstrated that sucrose and mannitol were the preferred carbon substrates relative to glucose, with the averaged OD_{600} peaks of 1.833 and 1.716 each compared to glucose which had an OD_{600} averaged peak of 1.368. This confirmed again the findings of Bren et al (2016) that glucose is the most widely used carbon source for the growth of many microorganisms, but some may use other sugars as the better sources of carbon than glucose. However, 5 strains namely *Arthrobacter* (ECCN 6b), *Aeromonas* (ECCN 4b), *Pseudomonas* (ECCN 10b), *Enterobacter* (ECCN 7b and *Bacillus* (ECCN 3b) were able to grow in both carbon substrates broth showing their ability to use all these sugars as sources of carbon. Covert and Moran (2001) reported that due to the adaptation mechanisms, some bacteria might be able to metabolise monosaccharides or polysaccharides or both. They can express their hydrolytic ectoenzymes into the media by hydrolysing polymeric materials and particles which can be then transported into the cell as the available sources of carbon (Martinez et al. 1996). The confirmation of the presence of bacterial isolates from HRAOPs with such characteristics is a novelty of this research.

In conclusion, this chapter investigated the growth capacity and culture conditions of each of the 12 isolated bacteria. Of these isolates, 5 strains such as *Bacillus* (ECCN 3b), *Aeromonas* (ECCN 4b), *Arthrobacter* (ECCN 6b), *Enterobacter* (ECCN 7b), and *Pseudomonas* (ECCN 10b) showed an optimal pH tolerance and an ability to use both carbon substrates. These bacteria should, therefore, be studied in the field to investigate their potential and determine if they could play a beneficial role in the ecosystem remediation in terms of restoration of soil bacterial microbiota. This can have a positive economic impact on communities and especially in the sustainable agriculture sector.

Chapter 4: Screening of bacterial strains for plant growth promoting activity

4.1 Introduction

The beneficial bacteria for plants are known as plant growth promoting rhizobacteria (PGPR). These microorganisms play a critical role in plant growth and development by transforming, mobilising and solubilising matter into the soil, and then stimulating the uptake of these plant essential nutrients to promote plant growth and development that can boost crops production (Raimi et al. 2017). The application of a bacterial-based bio-fertiliser is now a viable alternative to chemical-based fertilisers, which, in addition to their high cost, increase the risk of environmental pollution. Therefore, strengthening and expanding the use of a bacterial bio-fertiliser may reduce the dependence on chemical fertilisers and, as a result, their negative environmental effects (Suyal et al. 2016). The effects of PGPR and their modes of action are increasing rapidly. Although these efforts are being made to exploit PGPR as a bio-fertiliser in South Africa, these microorganisms (bacteria) have already been commercially exploited in many developing countries, as plant growth promoters (Vessey 2003; Khan et al. 2018).

The isolation and purification of bacterial strains, the screening for desirable characters and the selection of efficient strains are important steps in the production of the bacterial inoculum (Bashan 1998; Trivedi et al. 2004). The use of bacterial isolates as a bio-fertiliser to improve crop yield and biological control is a proven technology for sustainable fertiliser production (Bhardwaj et al. 2014). However, the efficacy of the bacterial isolate and the good application technology are the two main aspects of successful inoculation (Bashan et al. 2014). Most of the bacterial inoculants used as a bio-fertiliser have been isolated from the soil, rhizosphere, root systems and nodules of plants (Bumunang and Babalola 2014; Etesami et al. 2017). This research studied the PGPR bio-fertiliser potential of isolates from HRAOPs of IAPS.

This chapter presents the research conducted to evaluate the PGPR capacities of the 12 bacterial strains isolated from HRAOP B of IAPS for the treatment of domestic sewage. It aimed to investigate their PGPR potential in terms of ammonium (NH_4^+) production, tri-calcium phosphate ($Ca_3(PO_4)_2$) and potassium aluminosilicate ($AlKO_6Si_2$) solubilisation, and manganese sulphate ($MnSO_4.H_2O$) oxidation. Also, it investigated the potential of PGR by assessing the ability of isolates to produce the plant hormone such as auxin, indole-3acetic acid (IAA). The investigations were carried out under laboratory conditions at pH 9 and by several well-known colorimetric bioassays using a standard ultraviolet Shimadzu spectrophotometer (UV-1201, Japan).

4.2 Materials and methods

4.2.1 Ammonium production

To determine the production of NH_4^+ by the bacterial strains, peptone water (PW) (Himedia, M028) was selected as a culture medium and Nessler's reagent (HgI_4K_2) (Himedia, R010) used as an indicator for NH_4^+. Peptone water was prepared by suspending in 1 L of distilled water, 10 g of peptone (Fluka, Sigma-Aldrich, USA) and 5 g of NaCl (Merck KGaA, Germany). The mixture was then autoclaved at 121℃ and at 1.5 kg cm^{-2} for 15 min. The indicator Nessler's reagent was prepared by suspending in 100 mL of distilled water, 10 g of ($HgCl_2$) (UnivAR, RSA), 7 g of KI (Holpro L, RSA) and 16 g of NaOH (Merck, India).

The bacterial inoculant volume of 0.05 mL ($\sim 2.0 \times 10^9$ CFU mL^{-1}) was pipetted and inoculated into 250 mL flask containing 50 mL PW and incubated in a rotary shaker at 30 °C and 120 rpm for 3 d. After incubation, 30 mL of the freshly grown culture of each treatment was centrifuged at 18900xg using an Avanti centrifuge-JA 20 rotor (J-E, USA) for 10 minutes. The supernatant obtained was used for NH_4^+ quantification by the colorimetric assay as follows:

5 mL of supernatant from each sample was pipetted into 10 mL test tubes and labelled. 0.5 ml of Nessler's reagent was added to each 10 mL test tube containing 5 mL of supernatant. After at least 5 min, the development of a brown colour as a positive indicator of NH_4^+ could be visible (Cappucino and Sherma 1992; Bumunang and Babalola 2014). For colorimetric quantification, 1 mL of each treatment sample was pipetted, poured into the respective cuvette and absorbance measured at 430 nm (Jeong et al. 2013). A stock solution of 12.5 mg L^{-1} was prepared for the calibration curve with NH_4Cl.

4.2.2 Phosphate solubilisation activity

4.2.2.1 Plate screening

Pikovskayas agar (PVK) was selected to study the ability of the isolated bacteria to solubilise phosphate and tri-calcium phosphate ($Ca_3(PO_4)_2$) as the sole source of insoluble inorganic phosphate (Sharma et al. 2011).

The modified PVK agar 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 of $Ca_3(PO_4)_2$ (Himedia, India), 0.5 g of $(NH_4)_2SO_4$ (Merck, Germany), 0.2 g of 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) (Himedia Technical Data; Sharma et al. 2011).

For bacterial inoculation, a colony portion of the agar plate was picked, inoculated at 4 different positions on the PVK agar and incubated at 30 ℃ for 9 d. After incubation, the solubilising activity of $Ca_3(PO_4)_2$ was determined by observing the development of clear areas around active bacterial colonies (Baliah et al. 2016).

4.2.2.2 Liquid culture screening

The preparation of the PVK broth included all the above ingredients without agar (Cerrato et al. 2010). An equal volume of bacterial inoculant (0.05 mL) from the seed culture was inoculated into 250 mL flask containing 50 mL of PVK broth and incubated at 30 °C on a rotary shaker at 120 rpm for 7 d. After incubation, 30 mL of freshly grown culture was pipetted and centrifuged at 18900xg using an Avanti centrifuge-JA 20 rotor (J-E, USA) for 10 min. For the colorimetric assay of solubilised phosphate in PVK broth, 5 mL of supernatant from each sample was pipetted into a 10 mL test tube. Indicators were then added according to the protocol of purchased phosphate test kits (Merck KGaA, 1.14848.0001, Germany). The resulting blue colour was measured as absorbance using a Shimadzu spectrophotometer (UV-1201, Japan) at 690 nm. A stock solution of 12.5 mg L^{-1} was prepared for the standard curve using K_2HPO_4.

4.2.3 Potassium solubilisation activity

4.2.3.1 Plate screening

Aleksandrow agar was selected to study the potential of the 12 isolated bacteria to solubilise potassium. Potassium aluminosilicate ($AlKO_6Si_2$) (Himedia, RSA) was selected as the sole source of insoluble inorganic potassium (Saha et al. 2016). The bacterial strains were plated on Aleksandrow agar containing per 1 L of distilled water, 0.5 g of $MgSO_4.7H_2O$ (Merck, RSA), 0.1 g of $CaCO_3$ (Pal Chemicals), 2 g of $AlKO_6Si_2$ (Himedia), 5 g of glucose (Alpha, RSA), 0.005 g of $FeCl_3.6H_2O$ (Saarchem, RSA), 2 g of $Ca_3(PO_4)_2$ (Himedia India) and 20 g of agar (Biolab, Merch, RSA). Plates were incubated at 30 ℃ for 9 d. After incubation, the solubilisation potential for potassium was identified by observing the development of clear zones around the active bacterial colonies.

4.2.3.2 Liquid culture screening

The Aleksandrow broth preparation consisted of the entire medium composition described above without agar (Cerrato et al. 2010). The volume of 0.05 mL of inoculant (seed culture) was inoculated into 250 mL flask containing 50 mL of Aleksandrow broth and then incubated at 30 °C on a rotary shaker at 120 rpm for 7 d. After incubation, 30 mL of freshly grown culture was pipetted and centrifuged at 18900xg for 10 min. The volume of 2 mL of supernatant from each sample was used to quantify potassium concentration in the broth. The resulting turbidity was measured spectrophotometrically at 690 nm following the protocol of K cell test kits (Merck KGaA, 1.14562.0001, Germany). A stock solution of 50 mg L^{-1} KCl was made for the standard curve.

4.2.4 Manganese oxidation activities

To determine the manganese oxidation activity of the 12 isolated bacteria, the manganese oxide broth (MOB) was prepared as a growth medium and benzidine reagent ($(C_6H_4NH_2)_2$) was included as an indicator of the Mn-oxide concentration in the broth (Krumbein and Altmann 1973; Cerrato et al. 2010; Akob et al. 2014). The source of manganese used in this study was manganese sulphate monohydrate ($MnSO_4.H_2O$). The composition of MOB consisted of the following per 1 L of 10 mM HEPES buffer solution (Sigma-Aldrich, Switzerland), 0.001 g of $FeSO_4.7H_2O$ (Holpro, RSA), 0.15 g of $MnSO_4.H_2O$ (Merck, RSA), 2 g of peptone (Fluka, Sigma-Aldrich, USA) and 0.5 of yeast extract (Biolab, Merck, RSA) (Cerrato et al. 2010). An inoculum volume of 0.05 mL of was seeded into 250 mL flask each containing 50 mL of Mn-oxide broth and incubated at 30 °C, at 120 rpm for 7 d. After incubation, the colorimetric quantification procedure of Mn-oxide was conducted as follows:

From each freshly grown culture, 1 mL was pipetted to be mixed with 5 mL of 0.04% benzidine (British Drug Houses, LTD, England) in 45 mM 99.5% acetic acid. The development of the blue colour indicated a positive test of Mn-oxide present in the broth. From each sample the mixture was immediately centrifuged at 18900xg for 2 min then 1 mL of supernatant was pipetted, placed in respective cuvettes and the blue colour was measured as absorbance at 620 nm (Cerrato et al. 2010). A stock solution of 150 mg L^{-1} was prepared for the standard curve using $KMnO_4$.

4.2.5 Indole-3-acetic acid production

To determine the production of IAA by the isolated bacterial strains, NB was supplemented with L-tryptophan ($C_{11}H_{12}N_2O_2$) as well as a combination of orthophosphoric acid and

Salkowski reagent was used as an indicator for testing the amount of IAA produced in the broth (Datta et al. 2015). The NB supplemented with L-tryptophan was prepared by suspending in 1 L of distilled, 5 g of peptone (Fluka, Sigma-Aldrich, USA), 3 g of yeast extract (Biolab, Merck, RSA), 8 g of NaCl (Merck, KGaA, Germany) and 1 g of L-tryptophan (Merck, KGaA, Germany).

The volume of 0.05 mL of bacterial inoculum was used to inoculate 50 mL of NB supplemented with L-tryptophan and incubated at 30 ℃ on a rotary shaker (120 rpm) for 4 d. After incubation, 30 mL of each freshly grown culture was centrifuged at 18900xg for 10 min and the cell-free supernatant was used for colorimetric IAA quantification following the procedure below (Datta et al. 2015):

From each sample, a volume of 2 mL cell-free supernatant was pipetted into test tubes and 2 drops of 10 mM of orthophosphoric acid and 4 mL of Salkowski reagent (50 mL, 35% perchloric acid, and 1.0 mL, 0.5 M $FeCl_3$ solution) were added. The mixture was incubated at room temperature for 20 min to get the maximum pink colour. After 20 min incubation, 1 mL of the mixture was pipetted into respective cuvettes and the absorbance measured at 530 nm. As IAA is not soluble in water but acetone, 10 mg IAA was pipetted in 10 mL acetone and stirred well until completely dissolved. This was the stock solution 1. From this stock solution 1, a stock solution 2 of 50 mg L^{-1} of IAA was prepared for the standard curve (Sarker and Al-Rashid 2013).

4.3 Data and statistical analysis

All data presented in this chapter were mean values and standard errors (SE) of at least three sample measurements that were collected as mentioned in Appendix B, section B.2, Tables B.2.1 to B.2.5. All statistical analyses were performed using Microsoft Excel 2016. Mean values were compared using analysis of variance (ANOVA) to test for significant differences between all datasets at 95% level of significance (Microsoft Excel, 2016).

4.4 Results

4.4.1 Ammonium production

The production of ammonium was quantified by measuring at 430 nm the absorbance of the brown colour developed after pouring Nessler’s reagent into the freshly grown culture supernatant (Cappucino and Sherma 1992; Bumunang and Babalola 2014). Of the 12 isolated bacteria tested to produce ammonium, 9 were positive. The amount of ammonium produced by each positive isolate after an incubation period of 3 d is summarized as follows: *Bacillus* strain ECCN 1b (7.403 mg L^{-1}), *Bacillus* strain ECCN 3b (7.221 mg L^{-1}), *Fictibacillus* strain ECCN 2b (7.392 mg L^{-1}), *Aeromonas* strain ECCN 4b (6.793 mg L^{-1}), *Exiguobacterium* strain ECCN 5b (6.616 mg L^{-1}), *Exiguobacterium* strain ECCN 8b (6.886 mg L^{-1}), *Arthrobacter* strain ECCN 6b (7.693 mg L^{-1}), *Enterobacter* strain ECCN 7b (4.779 mg L^{-1}) and *Pseudomonas* strain ECCN 10b (7.146 mg L^{-1}). Analysis of variance (ANOVA) showed that the amount of NH_4^+ produced between the bacterial treatments ($P<0.05$) was significantly different (Appendix B, Section B.2 and Table B.2.1), indicating that not all bacteria perform ammonification.

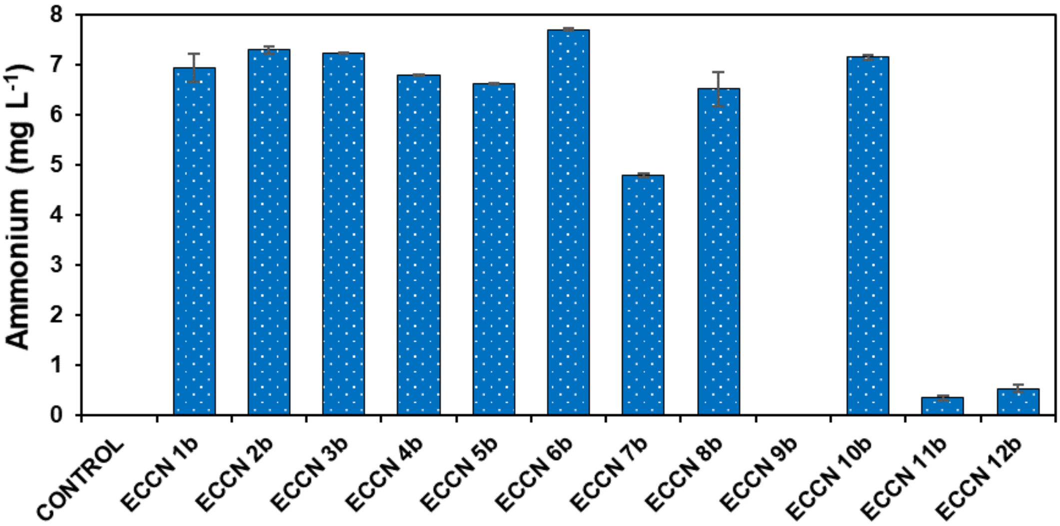


Figure 4. 1: The production of ammonium in PW. Bacterial strains were inoculated and incubated at 30 °C for 3 d. The data are represented by the mean and standard error of the 12 biological treatments.

4.4.2 Phosphate solubilisation

4.4.2.1 Solid culture

As indicated previously, the clear zones observed around bacterial colonies on PVK agar after an incubation period of 9 d were positive tests for $Ca_3(PO_4)_2$ solubilisation (Baliah et al. 2016). Of the 12 isolates screened for phosphate solubilisation, 5 strains such as *Bacillus* strain ECCN 3b, *Arthrobacter* strain ECCN 6b, *Enterobacter* strain ECCN 7b, *Pseudomonas* strain ECCN 10b and *Ancylobacter* strain ECCN 11b were able to grow on PVK agar, but only 1 strain (*Pseudomonas*, ECCN 10b) developed clear zones around bacterial colonies determining the phosphate solubilising potential.

4.4.2.2 Liquid culture

Colorimetric methods were used to estimate the amount of soluble phosphate available in PVK broth after 7 d incubation at 30 °C. Potential phosphate solubilising bacteria were tested, and the results showed a large variation in the solubilisation ability in a liquid culture medium assay (Baliah et al. 2016). Of the 12 strains tested, 7 were confirmed effective solubilisers of $Ca_3(PO_4)$. The amount of phosphate quantified at 690 nm in PVK broth was evaluated as follows: *Pseudomonas* strain ECCN 10b (2.206 mg L^{-1}), *Exiguobacterium* strain ECCN 8b (1.831 mg L^{-1}), *Exigobacterium* strains ECCN 5b (1.619 mg L^{-1}), *Aeromonas* strain ECCN 4b (1.402 mg L^{-1}), *Bacillus* strain ECCN 1b (1.386 mg L^{-1}), *Enterobacter* strain ECCN 7b (1.253 mg L^{-1}) and *Bacillus* strain ECCN 3b (1.149 mg L^{-1}).

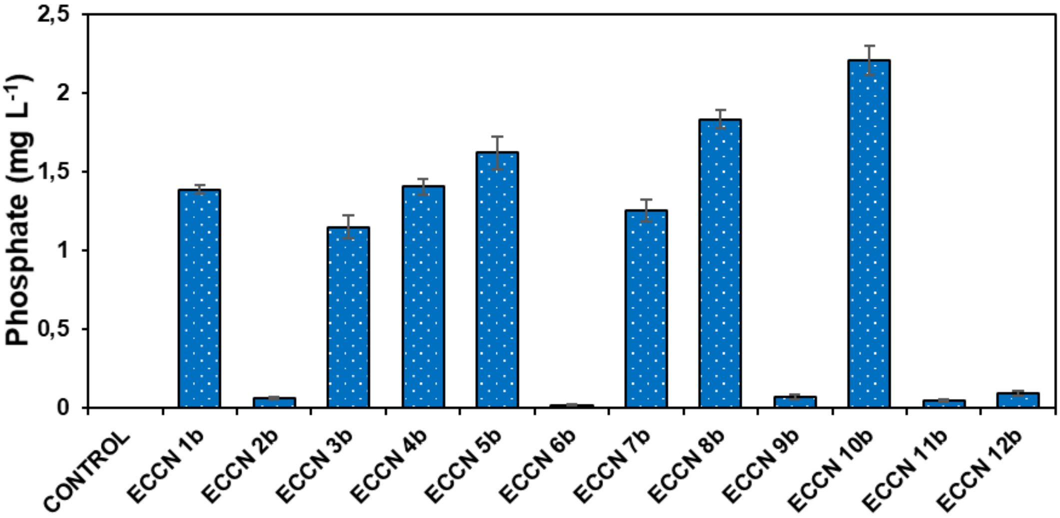


Figure 4. 2: The production of phosphate in PVK broth. Bacterial strains were inoculated and incubated at 30 °C for 7 d. The data are represented by the mean and standard error of the 12 biological treatments.

Based on the mean data collected, the experiment showed a significant difference between the amounts of phosphate solubilised by the bacterial isolates ($P<0.05$). Thus, different bacteria have different capacities for solubilisation of $Ca_3 (PO_4)_2$.

4.4.3 Potassium solubilisation

4.4.3.1 Solid culture

The methods used to test the solubilisation of $AlKO_6Si_2$ by isolated bacterial strains were the same as those used to test the solubilisation of $Ca_3 (PO_4)_2$. Solid Aleksandrow medium (SAM) was used to screen for the solubilising potential of bacteria. Bacteria were plated on SAM and after 9 d incubation, the development of clear zones around the colonies indicated the solubilisation potential of $AlKO_6Si_2$ (Saha et al. 2016). Of the 12 strains tested for potassium solubilisation potential, 3 isolates such as *Enterobacter* strain ECCN 7b, *Bacillus* strain ECCN 3b and *Pseudomonas* strain ECCN 10 b were able to grow on SAM, but only 1 strain (*Enterobacter*, ECCN 7b) showed the solubilisation ability of $AlKO_6Si_2$ by developing clear zones around colonies.

4.4.3.2 Liquid culture

A spectrophotometric method for quantifying the amount of soluble potassium in the liquid Alexandrow medium (LAM) after incubation of 7 d at 30 °C was performed at 690 nm. Figure 4.3 revealed that of the 12 bacterial strains tested, the same bacterium (*Enterobacter* strain ECCNb7) capable of solubilising $AlKO_6Si_2$ in the plate screening assay was also positive in the liquid culture screening.

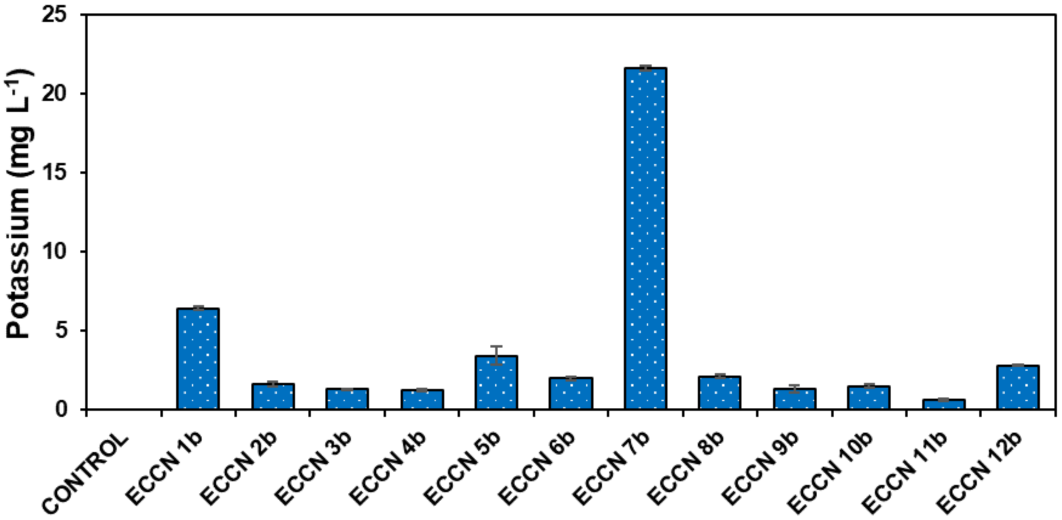


Figure 4. 3: The production of potassium in LAM. Bacterial strains were inoculated and incubated at 30 °C for 7 d. The data are represented by the mean and standard error of the 12 biological treatments.

The average amount of potassium available in the broth was evaluated at 21.581 mg L^{-1}. Also, *Bacillus* strain ECCN 1b produced a small amount of K evaluated at 6.409 mg L^{-1}. Indeed, analysis of variance (ANOVA) showed a significant difference ($P<0.05$) in terms of the amount of potassium solubilised by isolated bacteria.

4.4.4 Manganese oxidation

The ability of bacteria to oxidise manganese (Mn) rocks was tested by using $(C_6H_4NH_2)_2$ as an indicator that frequently reacts with the oxides of Mn (Krumbein and Altmann 1973; Akob et al. 2014). Among the 12 bacterial strains tested for the Mn-oxidation, 6 strains including *Bacillus* (ECCN 1b), *Fictibacillus* (ECCN 2b), *Bacillus* (ECCN 3b), *Exiguobacterium* (ECCN 5b), *Arthrobacter* (ECCN 6b) and *Exiguobacterium* (ECCN 8b) were positive in terms of oxidation of Mn from $MnSO_4.H_2O$ (Figure 4.4). However, 3 strains namely *Fictibacillus* (ECCN 2b), *Bacillus* (ECCN 1b) and *Bacillus* (ECCN 3b) were most effective by oxidising a maximum amount Mn evaluated at 27.109 mg L^{-1}, 26.107 mg L^{-1} and 24.732 mg L^{-1} (Table 4.1.). Finally, the significant difference ($P<0.05$) in terms of the amounts of Mn oxidised by bacteria showed that different bacteria have different capacities for oxidising $MnSO_4.H_2O$.

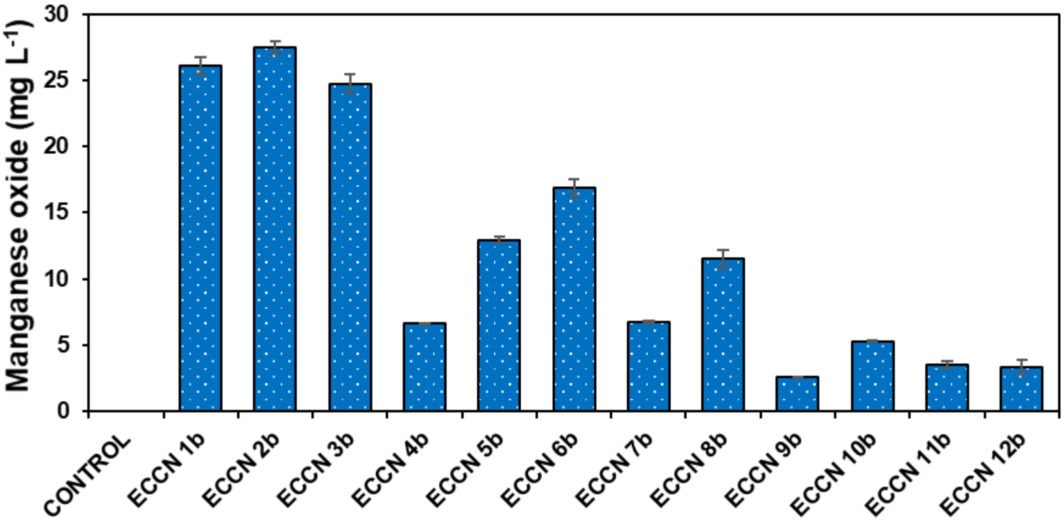


Figure 4. 4: The amount of manganese oxide in MOB. Bacterial strains were inoculated and incubated at 30 °C for 7 d. The data are represented by the mean and standard error of the 12 biological treatments.

4.4.5 Indole-3-acetic acid

The colorimetric quantification of IAA produced in NB supplemented with $C_{11}H_{12}N_2O_2$ was determined at 530 nm (Mohite 2013; Datta et al. 2015). As shown in Figure 4.5, of the 12 bacterial strains tested for IAA production, 3 isolates identified as *Aeromonas* strain ECCN 4b, *Arthrobacter* strain ECCN 6b and *Enterobacter* strain ECCN 7b were detected positive for the production of IAA.

The average amount of IAA (mg L^{-1}) produced by each bacterium that tested positive was 41.564 for *Enterobacter* strain ECCN 7b, 40.750 for *Aeromonas* strain ECCN 4b and 37.519

for *Arthrobacter* strain ECCN 6b (Table 4.1). The ANOVA single factor established the significant difference ($P<0.05$) concerning the amount of IAA produced in NB supplemented with L-tryptophan. This showed the different potential of bacteria isolated from HRAOPs in terms of IAA production.

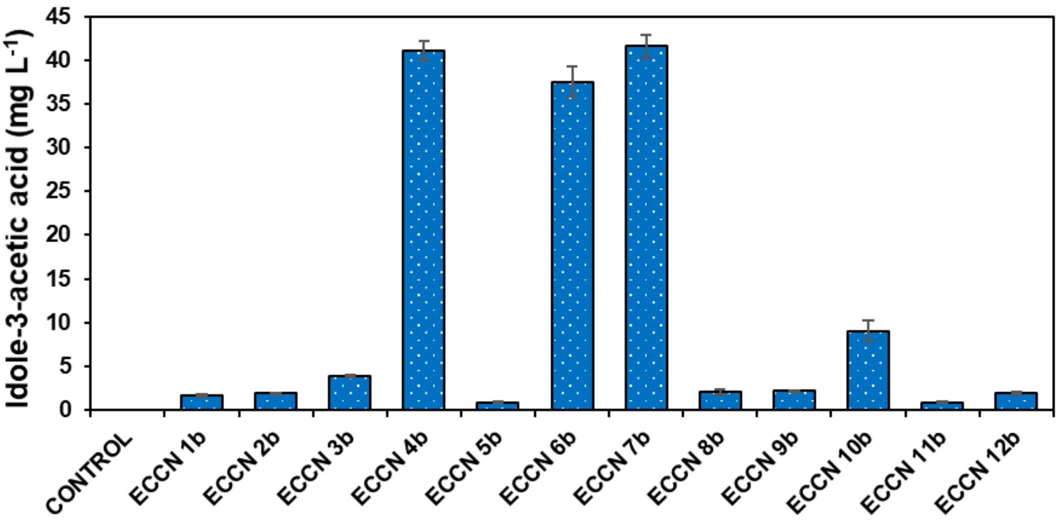


Figure 4. 5: The amount of indole-3-acetic acid produced in NB supplemented with L-tryptophan. Bacterial strains were inoculated and incubated at 30 °C for 3 d. The data are represented by the mean and standard error of the 12 biological treatments.

Table 4. 1: Summary of concentrations (mg L^{-1}) of plant essential nutrients and plant growth regulator produced by isolated bacterial strains.

Bacterial strains	Ammonium	Phosphate	Potassium	Manganese	Indole-3 acetic acid
Measurement	**mg L^{-1}**	**mg L^{-1}**	**mg L^{-1}**	**mg L^{-1}**	**mg L^{-1}**
Standard	**12.5**	**12.5**	**50**	**150**	**50**
***Bacillus* (ECCN 1b)**	7.403	1.386	6.409	26.107	1.696
***Fictibacillus* (ECCN 2b)**	7.393	0.062	1.659	27.109	1.948
***Bacillus* (ECCN 3b)**	7.221	1.149	1.287	24.732	3.907
***Aeromonas* (ECCN 4b)**	6.792	1.402	1.256	6.643	40.750
***Exiguobacterium* (ECCN 5b)**	6.616	1.619	3.426	12.937	0.917
***Arthrobacter* (ECCN 6b)**	7.693	0.017	1.984	16.899	37.519
***Enterobacter* (ECCN 7b)**	4.799	1.253	21.581	6.713	41.564
***Exiguobacterium* (ECCN 8b)**	6.841	1.831	2.093	11.561	2.085
***Microbacterium* (ECCN 9b)**	0.010	0.071	1.302	2.564	2.188
***Pseudomonas* (ECCN 10b)**	7.146	2.206	1.504	5.314	9.073
***Ancylobacter* (ECCN 11b)**	0.351	0.050	0.605	3.543	0.917
***Microbacterium* (ECCN 12b)**	0.509	0.092	2.806	3.333	2.154

4.5 Discussion

This chapter evaluated the characteristics of plant growth promoting rhizobacteria for the 12 bacterial strains isolated from HRAOPs of the IAPS treating domestic sewage. The results in Figure 4.1 showed the capacity of *Bacillus* strain ECCN 1b, *Bacillus* strain ECCN 3b, *Fictibacillus* strain ECCN 2b, *Aeromonas* strain ECCN 4b, *Exiguobacterium* strain ECCN 5b, *Exiguobacterium* ECCN 8b, *Arthrobacter* strain ECCN 6b, *Enterobacter* strain ECCN 7b and *Pseudomonas* strain ECCN 10b to produce NH_4^+ by decomposing organic nitrogen (proteins and amino acids) from peptone. These results confirm previous studies demonstrating that some bacterial strains (*Bacillus* and other ammonifying bacteria) can transform organic nitrogen into NH_4^+ making it available as nutrients to plants and other microorganisms (Strock 2008; Reece et al. 2011; Craggs 2014).

Based on the solubilisation of $Ca_3(PO_4)_2$ on PVK agar, one isolate, namely *Pseudomonas* strain ECCN 10b demonstrated active solubilisation by developing clear zones around bacterial colonies, while 4 other isolates (*Bacillus* strain ECCN 3b, *Arthrobacter* strain ECCN 6b, *Enterobacter* ECCN 7b and *Ancylobacter* ECCN 11b) grew on PVK agar but without developing any clear zones. However, the results of liquid screening were quite different. The number of bacteria that solubilised $Ca_3(PO_4)_2$ in a PKV broth increased to 8 strains (Figure 4.2). These results are consistent with the statement of DeAbreu et al (2017) that among the 60 isolated bacteria unable to solubilise $Ca_3(PO_4)_2$ on PVK agar, 19 showed the solubilisation potential in PVK broth. Marra et al (2012) explained these observations by demonstrating that the solubilisation of the substances may depend on the state of the culture media and the diffusion of organic acids involved in these mechanisms. On the contrary, the solubilisation of $AlKO_6Si_2$ on the plates and liquid screening assays gave the same results in potassium solubilisation screening. *Enterobacter* strain ECCN 7b that developed clear zones around colonies on SAM was the only isolated bacteria to show solubilisation potential of $AlKO_6Si_2$ in LAM assay.

Of the 12 bacteria isolates tested for Mn-oxidation, 3 strains all bacilli and known as *Bacillus* (ECCN 1b), *Fictibacillus* (ECCN 2b) and *Bacillus* (ECCN 3b) were prominent Mn-oxidising microorganisms in MOB screening.

Less prominent other isolates of *Arthrobacter* strain ECCN 6b and 2 *Exiguobacterium* strains ECCN 5b and ECCN 8b produced small amounts of Mn-oxides (Figure 4.1). The results of Tebo et al (2005) and Cerato et al (2010) found that Mn-oxidising bacteria are the most ubiquitous microorganisms composed of the strains of *Bacillus* and *Pseudomonas.* They might be involved in oxidising Mn rocks, therefore, releasing the forms of manganese (Mn^{+2}) that plants can use as nutrients (bio-fertilisation). Mn-oxidising microorganisms may also play a role in the filtration and detoxification of water and wastewater (Burger et al. 2008; Cerato et al. 2010). This could be a possible reason for finding these bacteria in HRAOPs environments.

Finally, of the 12 isolates used for the Salkowski test, 3 strains were able to perform IAA production in liquid culture screening. These 3 isolates identified as *Aeromonas* strain ECCN 4b, *Arthrobacter* strain ECCN 6b and *Enterobacter* strain ECCN 7b) were found to produce most amounts of IAA from L-tryptophan supplemented in NB. There is not sufficient evidence highlighting the production of IAA by bacterial isolates from HRAOP but Bharucha et al (2013) reported that bacteria from various environments can utilise L-tryptophan as a precursor to IAA production in the culture media. Xu et al (2014) isolated a strain of bacterium known as *Paenibacillus polymyxa* from sweet potato wastewater and which was studied as an IAA producing microorganism. Thus, it would not be surprising to find strains of bacteria capable of producing IAA in HRAOPs of IAPS for the treatment of domestic wastewater. This phytohormone may be involved in symbiotic interactions between microalgae and bacteria in this environment (Fuentes et al. 2016).

In conclusion, this chapter examined the abilities of 12 bacterial isolates from HRAOPs of the IAPS treating domestic sewage in terms of PGPR bio-fertiliser and PGR activities. The studies revealed that *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* ECCN 7b, *Exiguobacterium* ECCN 8b and *Pseudomonas* strain ECCN 10b were able to act as a potential bio-fertiliser by producing, solubilising and oxidising some amounts of plant nutrients such as ammonium, phosphate, potassium and manganese, and by producing auxin or indole-3-acetic acid as plant growth regulator. These results showed that PGPR bio-fertiliser could be developed from the HRAOPs of IAPS for treating domestic wastewater.

Chapter 5: General discussion and conclusion

South Africa is one of the 20 most arid regions in the world and is affected by water scarcity (Erasmus 2017). Much of the available water resources do not meet the needs of the community and especially farmers. To mitigate this crisis, efforts at recovering the available resources such as wastewater could be laudable. It was also mentioned that every person in South Africa uses approximately 235 L of water daily and this is much higher than the world average of 173 L per person per day (Erasmus 2017). This implies that a town of about 100 000 inhabitants generates approximately 23.5 ML of domestic wastewater daily (Johnson 2010). There are large volumes of domestic wastewater that can be treated to produce quality effluent suitable for re-use (Craggs et al. 2014; Mambo et al. 2014a; Butler et al. 2017; Jimoh et al. 2019). The implementation of full commercial-scale IAPS for wastewater treatment has been argued from the food-water-energy nexus point of view (Laxton 2010; Mambo et al. 2013) and seems to be an ideal technology for deployment in the peri-urban space (Cowan et al. 2019). Typical wastewater treatment technologies are designed to produce a treated effluent (water) of a quality that allows it to be discharged into environmental water bodies (Rose et al. 2007).

For many wastewater treatment plants in South Africa sludge handling, which includes treatment (to reduce the volume and stabilise the organic materials), disinfection, pelletising, and disposal either by transport to the landfill or stockpiling presents an added cost and environmental risk. Indeed, the implementation of IAPS not only mitigates this part of wastewater treatment, but it is also completely removed from the process flow (Mambo et al. 2014b; Cowan et al. 2016).

5.1 Wastewater as a resource for benefits

Communities can derive important benefits from wastewater, although knowledge about the treatment performance of the system is still limited (Higgins et al. 2018). From the primary processing unit, the treatment performance is still not significant in the advanced facultative pond (AFP). However, anaerobic digestion which reduces the organic load by fermentation produces biogas composed mainly of methane as a source of bioenergy (Mambo et al. 2014a; Craggs et al. 2014; Vasco-Correa et al. 2018).

The MaB-flocs generated in HRAOPs of IAPS as sludge can be used as resources for the production of nutrient-rich fertilisers, feed materials, soil improvers, amino acids, vitamins, polysaccharides, extracellular polymeric substances (EPS), plant growth promoting rhizobacteria (PGPR) and as a starting material for the production of plant growth regulators

(PGRs) (Aaronson et al. 1980; Borowitzka 1988; Rose et al. 2002; Wells 2005; Craggs et al. 2014; Butler et al. 2017; Xu et al. 2014; Kadmiri et al.2018). Finally, high-quality effluent (treated water) can be reused for irrigation (Rose et al. 2007; Higgins et al. 2018).

5.2 MaB-flocs as a bio-fertiliser

Sustainable soil bio-fertilisation to increase the quantity and to produce good quality of food is vital for everyone. Therefore, there is sufficient evidence to show that the demand for fertiliser is greater than its availability (Vejan et al. 2016). Due to global food shortages, the UN had set the 2016 goal for growth and consumption of pulses (A/RES/68/231) to ensure food security through an appropriate fertilisation approach that could minimise the risk to the environment caused by the excessive use of chemical fertilisers (Hansen 1996). HRAOPs are aerobic bioprocesses used in the treatment of wastewater and generate biomass in the form of a mixed liquor composed of microalgae-bacteria flocs (MaB-flocs). These microalgae and bacteria composing the MaB-flocs can be used to develop microalgal or bacterial bio-fertiliser, nutrient-rich organic fertiliser and to produce plant growth regulators (PGR) (Rose et al. 2002; Wells 2005; Mlambo, 2014; Xu et al. 2014; Fuentes et al. 2016; Chatterjee 2017).

Most of the bacterial bio-fertilisers developed and used in sustainable agriculture were isolated from soil, plant root systems and rhizosphere (Bumunang and Babalola 2014; Islam et al. 2016; Etesami et al. 2017), but a bacterial bio-fertiliser originated from HRAOPs has not been developed in South Africa. A study conducted previously at the Institute for Environmental Biotechnology, Rhodes University (EBRU) explored the fertilising potential of biosolids (algal biomass) from algae integrated wastewater treatment systems for cultivation of sugar bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*) plants (Mlambo 2014). Since the mixed liquor biomass (MaB-flocs) produced in conventional wastewater treatment has been demonstrated as a more than adequate feedstock for bioenergy and fertilisers production (Solovchenko et al. 2016; Cuellar-Bermudez et al. 2016). It seemed relevant to study the potential of HRAOPs to support a biomass of bacteria that can serve as a bio-fertiliser. Consequently, this study has investigated a bacterial bio-fertiliser potential of 12 isolates from HRAOPs using well-known bacteriological and biochemical laboratory tests. It sought to better understand the biotechnological mechanisms that could be involved in the process of producing a sustainable and environmental bacterial bio-fertiliser. The study was aimed to investigate the morphological and biochemical characteristics, the growth and culture conditions, and the PGPR bio-fertiliser and PGR like activities of 12 bacterial strains isolated from MaB-flocs generated in the HRAOPs of IAPS treating domestic wastewater. The 12 bacterial isolates were selected after purification and characterisation and were then identified using molecular studies as *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* ECCN 7b, *Exiguobacterium* strain ECCN 8b, *Microbacterium* strain ECCN 9b, *Pseudomonas* strain ECCN 10b, *Ancylobacter* strain ECCN 11b and *Microbacterium* ECCN 12b (Table 2.3). The growth characteristics and culture conditions were established using bacterial count (CFU), growth rates, pH tolerance and carbon substrates utilisation. To determine the PGPR bio-fertiliser and PGR like activity, the production, solubilisation and oxidation of essential plant nutrients such as ammonium, phosphate, potassium, manganese and the auxin, indole-3-acetic acid were measured.

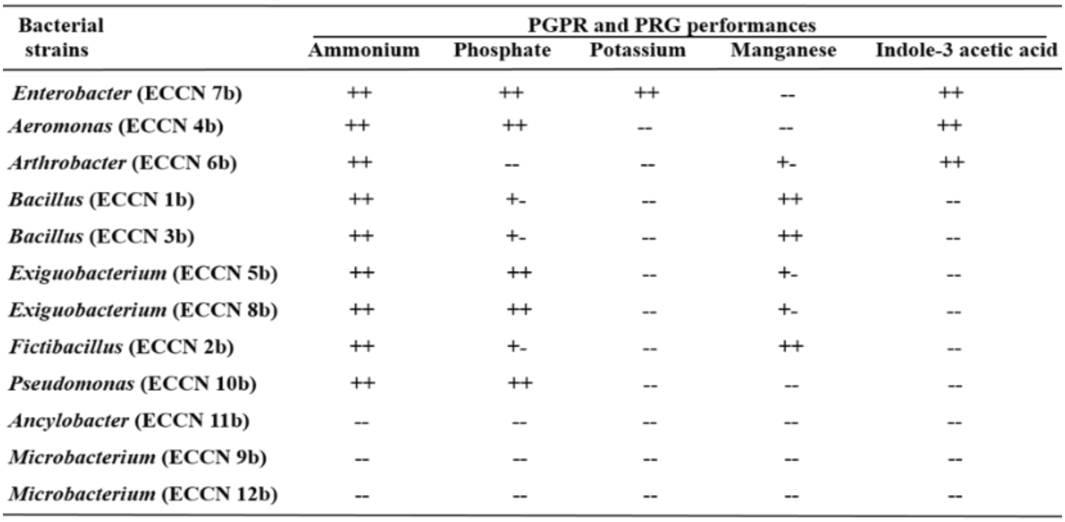
The morphological and biochemical characteristics in terms of bacterial colonies pigmentation showed differences. The colours were ranged from deep orange, orange to dark brown, while other colonies were white or cream-coloured. Gram stain showed that 8 isolates were Gram-positive. The Gram-positive bacteria seemed to be more adapted to survive in HRAOPs. The same results were found by Dick (2009) and Hoorman (2016) that, due to the larger size and thicker cell walls of Gram-positive bacteria, they may be more resistant to desiccation and other kinds of water stress such as alkalinity. All strains tested positive for catalase, suggesting that the isolates originated from an oxygen-rich environment (HRAOPs). Interestingly, during strain isolation and identification no faecal coliform bacteria or strains (*Escherichia coli*) were detected. This observation supports prior studies which showed that HRAOPs of IAPS are efficient in the disinfection of water (Rose et al. 2002; Cinara 2004; Mambo et al. 2014a; Jimoh and Cowan 2017; Butler et al. 2017).

The growth curves showed how different strains could respond differently to environmental variables. The optimal pH for growth was found to be between 8 and 9, but the growth rates were also good between pH 6 and 10. This confirms the previous reports of FDA (2001) showing that pH values close to 9 positively influence the generation time of bacteria. Baliat et al (2016) and Bren et al (2016) stated many parameters such as pH, carbon source, temperature, and the metabolic imbalance of each strain can affect bacterial growth. The growth of the isolates was influenced by the type of carbon substrates available. However, 5 isolates were successful in using both types of sugars as carbon sources. Studies demonstrated that some microorganisms can use different sugars and other nutrients provided in the culture media to produce ATP, which is necessary to maintain metabolism and reproduction (Covert and Moran 2001; Abbaszadeh-Dahaji et al. 2012; Ali et al. 2016). Sucrose and mannitol were found to be the best source of carbon than glucose in supporting bacterial growth. Bren et al (2016) stated that glucose is the most widely used carbon source for the growth of microorganisms, but some bacteria may have preferences that can make them utilise other sugars as carbon substrates rather than glucose.

The ability of 9 isolates to breakdown organic nitrogen (proteins and amino acids) of the peptone into NH_4^+ has shown the potential of these strains for ammonification. As previously observed by Strock (2008) and Reece et al (2011), some bacteria such as *Bacillus* strains can perform ammonification and then, if the amount of NH_4^+ produced was more than their own metabolic needs, the surplus is excreted into the environment and thus can so be used by plants and other microorganisms as nutrients. In this case, bacteria performing ammonification can be considered as PGRP. The solubilisation of $Ca_3(PO_4)_2$ was more effective in Pikovskayas broth than on agar plates. DeAbreu et al (2017) and Marra et al (2012) found the same results showing that the solubilisation of mineral containing phosphate or phosphorus can depend on the nature of culture media and the enzymes involved in the solubilisation processes.

Manganese plays a variety of roles in chlorophyll and carbohydrate synthesis (Mousavi et al. 2011). Of the 12 isolates tested for Mn-oxidation potential, 6 strains have been found to carry out Mn-oxidation. These isolates belonged to genera *Bacillus*, *Fictibacillus*, *Exiguobacterium* and *Arthrobacter*, but more commonly were Bacillus strains. Mn-oxidising bacteria are ubiquitous and are the most common species of *Bacillus* and *Pseudomonas* (Tebo et al. 2005; Cerato et al. 2010). In the treatment of effluents, they may reduce the toxicity caused by many soluble elements. It was also observed that Mn-oxide substances are powerful chelators of other trace metals and were effective in radium removal (Moore and Reid 1973), in retaining heavy metals like Co, Ni, Zn and thus, playing crucial role in the biogeochemical cycles of these minerals (Tebo et al. 2005; Vodyanitskii 2009; Sujith and Bharathi 2011). Mn-oxidising bacteria can also play the role of PGPR by promoting humus formation in the soil (Vodyanitskii 2009). Strains of *Aeromonas*, *Arthrobacter* and *Enterobacter* were found to produce IAA using L-tryptophan. Studies have isolated IAA producing bacteria such as *Paenibacillus polymyxa* from sweet potato wastewater and demonstrated that algal growth was stimulated by growth factors produced by bacteria including, IAA (Xu et al. 2014; Fuentes et al. 2016). All these results showed that certain bacterial strains isolated from HRAOPs of the IAPS could be used for the development of a bacterial bio-fertiliser as shown in the following Table 5.1.

Table 5. 1: Comparison of bacterial strains potential based on the production of plant essential nutrients and plant growth regulators and in terms of priority.



Bacterial strains	PGPR and PRG performances				
	Ammonium	**Phosphate**	**Potassium**	**Manganese**	**Indole-3 acetic acid**
Enterobacter (ECCN 7b)	++	++	++	--	++
Aeromonas (ECCN 4b)	++	++	--	--	++
Arthrobacter (ECCN 6b)	++	--	--	+-	++
Bacillus (ECCN 1b)	++	+-	--	++	--
Bacillus (ECCN 3b)	++	+-	--	++	--
Exiguobacterium (ECCN 5b)	++	++	--	+-	--
Exiguobacterium (ECCN 8b)	++	++	--	+-	--
Fictibacillus (ECCN 2b)	++	+-	--	++	--
Pseudomonas (ECCN 10b)	++	++	--	--	--
Ancylobacter (ECCN 11b)	--	--	--	--	--
Microbacterium (ECCN 9b)	--	--	--	--	--
Microbacterium (ECCN 12b)	--	--	--	--	--

Note: ++**,** good production; +-, low production and --, very low or no production.

5.3 Conclusion

The use of the MaB-flocs biomass generated in HRAOPs of the IAPS as a raw material for the production of a competitive and a sustainable bacterial bio-fertiliser is fundamental for the development of agricultural practices respectful of the environmentally. The study was able not only to isolate, characterise and identify the HRAOP bacteria but also to provide certain knowledge (protocol) on the conditions of growth and culture of the bacterial isolates. This is the first study seeking to assess bacterial strains isolated from MaB-flocs biomass as a sustainable source of a bio-fertiliser. As a recovery resource, bacteria with the performance of PGPR could be used to solve problems related to water scarcity, low biomass recovery and food security. Using a cost-effective bio-fertiliser from HRAOPs of the IAPS could significantly reduce dependence on chemical and fossil-derived fertilisers while protecting soil ecosystems. The development a bio-fertiliser from HRAOPs could change the negative attitude of communities towards wastewater treatment, which would make the system more attractive. Further studies should be undertaken to examine the PGPR bio-fertiliser and growth regulator activities of the effective isolated bacterial strains. These bacteria can be used to prepare seed inoculants and investigate the growth and development of cultivated crops from germination to harvest in pot or field trials. This should be done to confirm the positive results found during laboratory investigations and then the bio-fertiliser for use on a commercial scale can be prepared or made.

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Appendices

Appendix A.

Section A.1. Gram staining and molecular characterisation of bacterial strains

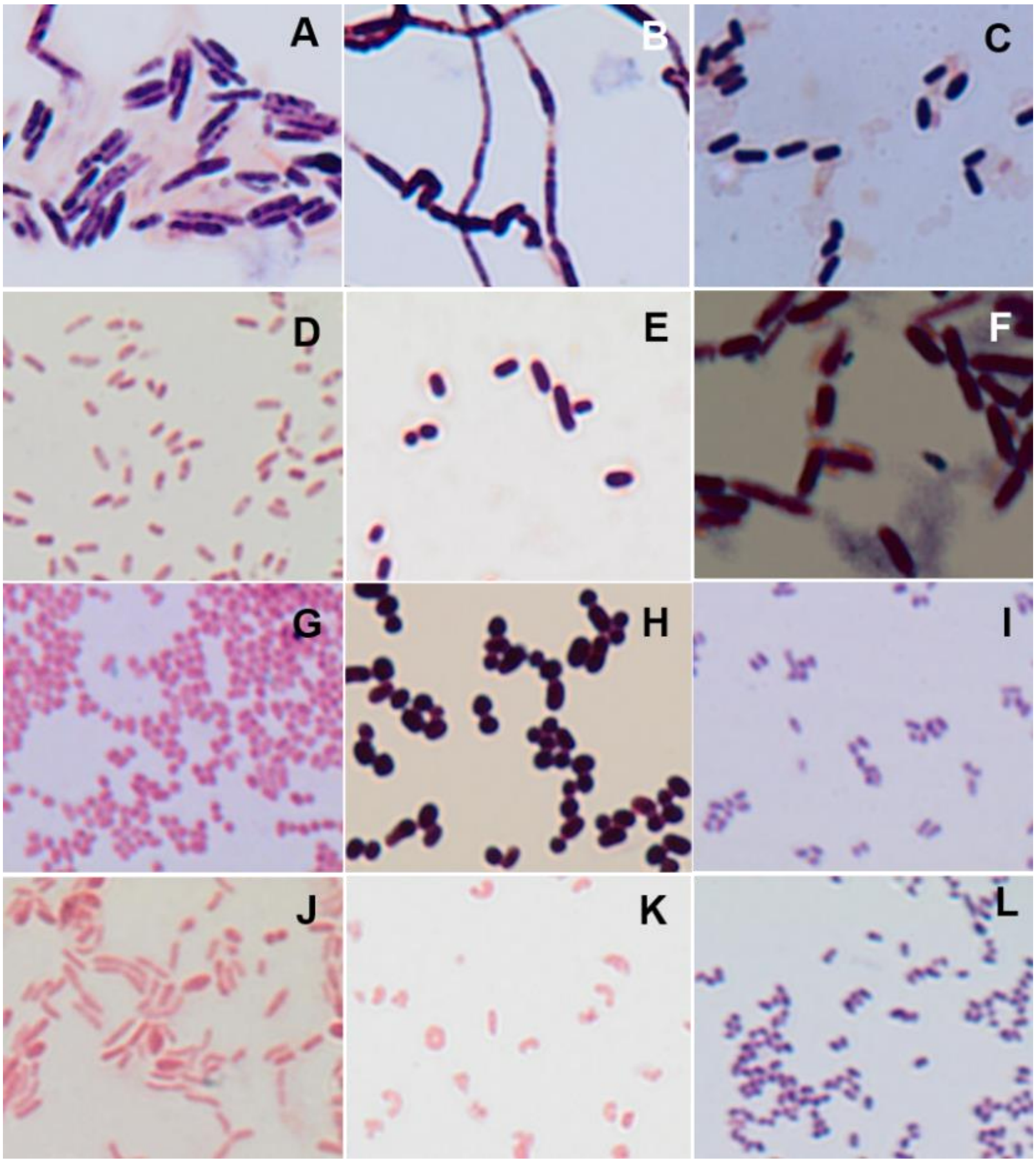


Figure A.1.1: Gram staining images of 12 bacterial strains isolated and viewed using an optical microscope (Olympus U-CMAD3 T2, -Japan) at magnification 1000x. ECCN b1 (A); ECCN b2 (B); ECCN b3 (C); ECCN b4 (D); ECCN b5 (E); ECCN b6 (F); ECCN b7 (G); ECCN b8 (H); ECCN b9 (I); ECCN b10 (J); ECCN b11 (K) and ECCN b12 (L).

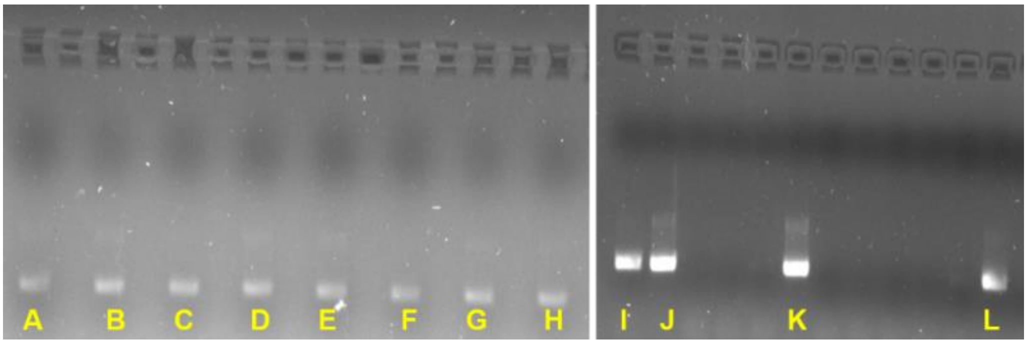


Figure A.1.2: PCR products for DNA sequencing to identify isolated bacterial strains. ECCN b1 (A); ECCN b2 (B); ECCN b3 (C); ECCN b4 (D); ECCN b5 (E); ECCN b6 (F); ECCN b7 (G); ECCN b8 (H); ECCN b9 (I); ECCN b10 (J); ECCN b11 (K) and ECCN b12 (L).

ECCN 1b identified as *Bacillus* strain

TTCGTCTGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC
TGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACT
GACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTTA
AATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGACA

ECCN 2b identified as *Fictibacillus* strain

GTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGTAATACG
TAGGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTT
CCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACT
GGGGAACTTGAGTGCAGGAGAGAAAAGTGGAATTCCACGTGTAGCGGTGAAATG
CGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGCCTGTAACTG
ACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTCCACCCTCAGTGCTGACGT
TAACACATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAT
AAATTGGCGGCTGTCTCTTATACACATCTCCGAGCCCACGAGA

ECCN 3b identified as *Bacillus* strain

GTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCCGCCGCGGTAATACG
TAGGTTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG
GGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTTAAA
TAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCAC

ECCN 4b identified as *Aeromonas* strain

TGTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGT
AATASGGAAGGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA
GGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATT
TAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGG
TGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA
AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCT
TCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAKGKTAAA
ACTTAAATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGACA

ECCN 5b identified as *Exiguobacterium* strain

TGTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGT
AATRSKTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
CGGCCTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGCCATTGG
AAACTGGGAGGCTTGAGTATAGGAGAGAAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGCCTAT
AACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTG
CTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCCGCAARRSKGRA
ACTTAAATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGACA

ECCN 6b identified as *Arthrobacter* strain

GCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGTAATASGTTAG
GGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGT
CGCGTCTGCTGTGAAAGCCCGGGGCTCAACCCCGGGTCTGCAGTGGGTACGGGC
AGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGC
AGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACG
CTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATG
CCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGC
TAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAARGCTAAAACTTAAAT
AAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGAC

ECCN 7b identified as *Enterobacter* strain

TGTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGT
AATACGGAGGGTGCAAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAG
GCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCG
AAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTG
AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAA
GACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTC
CGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGGCAARGTKAAAA
CTTAAATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGACA

ECCN 8b identified as *Exiquobacterium* strain

TGTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGT
AATACTGATAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCA
GGCGGCCTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGCCATT
GGAAACTGGGAGGCTTGAGTATAGGAGAGAAGAGTGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGCCT
ATAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAG
TGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGGSYGA
AACTYAAATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGA

ECCN 9b identified as *Microbacterium* strain

TAGTTGTTTTGTTGGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGTAATMSGTTAGGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTYTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTGAMAGCAGCTAACGCATTAAGTTCCCCGCCCTGGGGARKACGGCCGCAAGGCTAAAACTTAAATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGACA

ECCN 10b identified as *Pseudomonas* strain

TGTTTCGTCTGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGTAATCACAAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTAGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCCGGCAAAAGGKTAAAACTTAAATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGACA

ECCN 11b identified as *Ancylobacter* strain

TTTCGTCTGCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGTAATACGGAAGGGGGGCTAGCGTTGTTCGGAATCACTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAAGCCTGGAGCTCAACTCCAGAACTGCCCTTGATACTGGGTATCTCGAGTCCGGAAGAGGTAAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGGCGGCTTACTGGTCCGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGAGGCTAGCCGTTGGTGAGCATGCTCATCAGTGGCGCAGCTAACGCATTAAGCCTCCCGCCTGGGGAGTACGGTCCGGCAAGAKKAAAACTTAAATAAATTGACGGCTGTCTCTTATACACATCTCCGAGCCCACGAGACA

ECCN 12b identified as *Microbacterium* strain

GCAGCGTCAGATGTGTATAAGAGACAGGTGTCAGCAGCCGCGGTAATACGGTAG
GGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTC
GCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCA
GACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCA
GATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGC
TGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCC
CGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTRACGCAGCT
AACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTKAAATA
AATTGACGGCTGTCTCTTATACACATCTCCGAGCCCAC

Figure A.1.3: Genetic sequencing of isolated bacterial strains obtained using the Genetic Analyser (Applied Biosystems 3500, US).

<u>Section A.2.</u> Standard curves

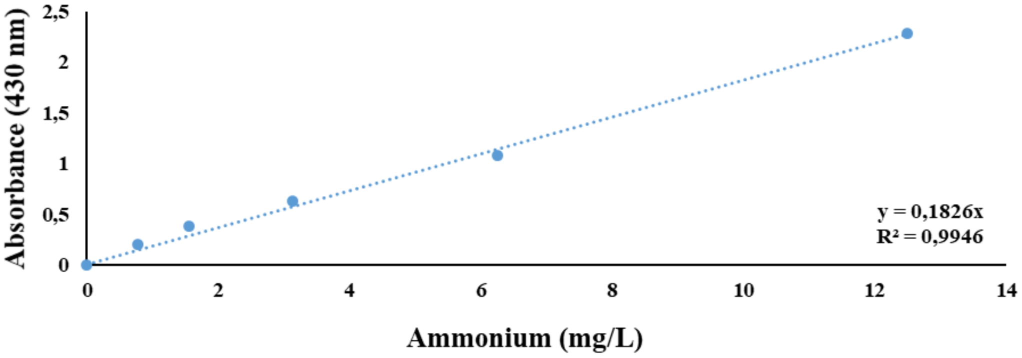


Figure A.2.1: The increasing concentrations of ammonium at a wavelength of 430 nm on ammonium chloride (NH_4Cl) assay. The curve was used to determine the unknown ammonium concentration in peptone water.

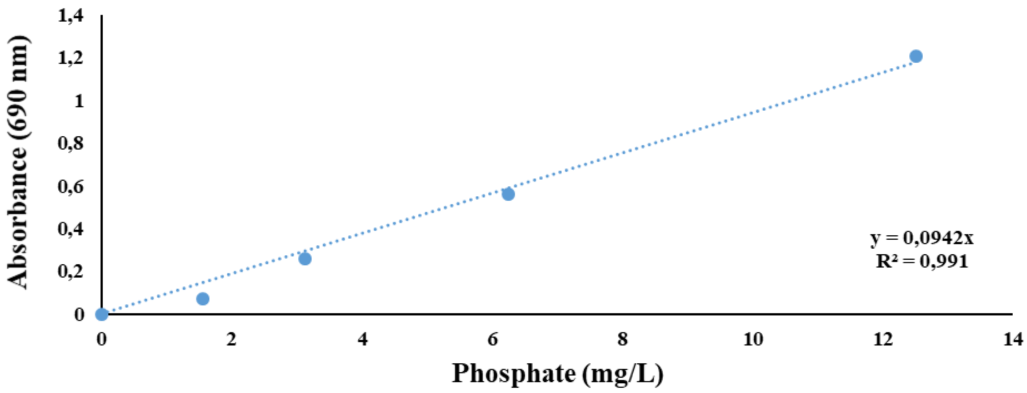


Figure A.2.2: The increasing concentrations of phosphate at a wavelength of 690 nm on potassium phosphate (K_2HPO_4) assay. The curve was used to determine unknown phosphate concentration in Pikovskayas broth.

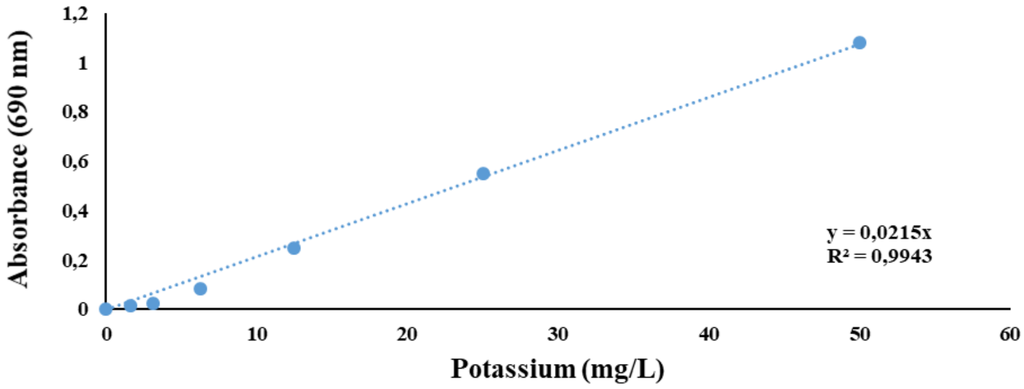


Figure A.2.3: The increasing concentrations of potassium at a wavelength of 690 nm on potassium chloride (KCl) assay. The curve was used to determine unknown potassium concentration in Aleksandrow broth.

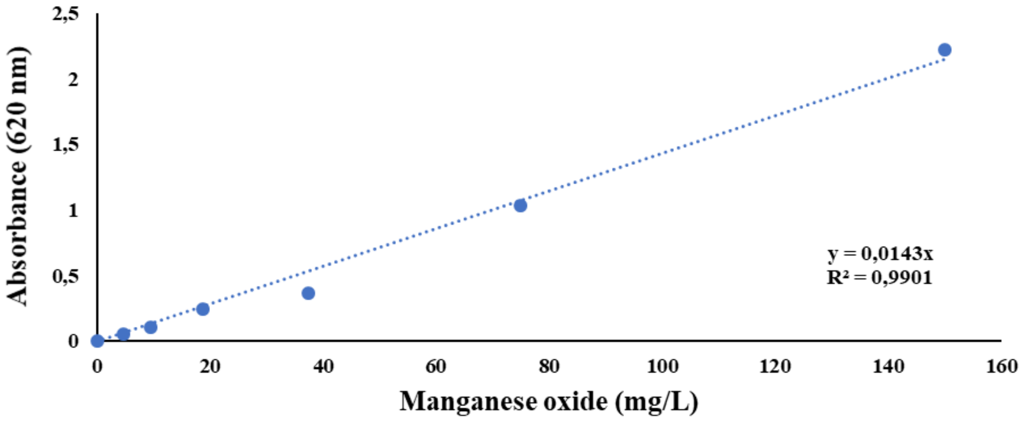


Figure A.2.4: The increasing concentrations of manganese oxide at a wavelength of 620 nm on potassium permanganate ($KMnO_4$) assay. The curve was used to determine unknown Mn concentration in manganese oxide broth.

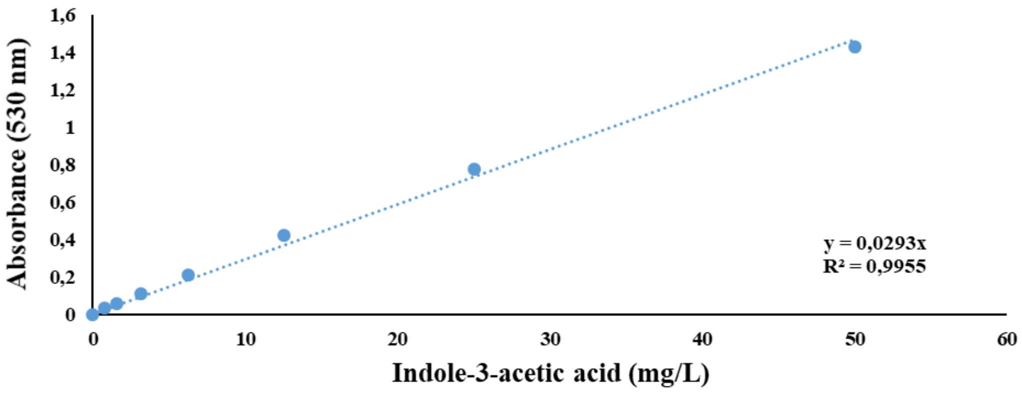
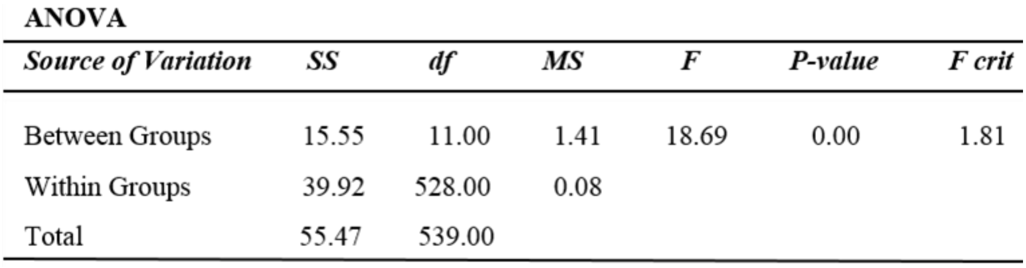


Figure A.2.5: The increasing concentrations of indole-3 acetic acid-IAA at a wavelength of 530 nm on IAA assay. The curve was used to determine the unknown IAA concentration in nutrient broth supplemented with L-tryptophan.

Appendix B.

Section B.1: Analysis of variance (ANOVA) showing the significant difference between all the isolated bacterial treatments in terms of bacterial growth and culture conditions.

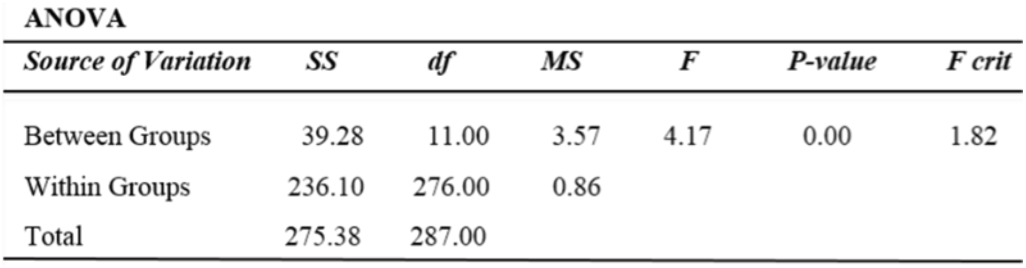
Table B.1.1: Growth curve



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15.55	11.00	1.41	18.69	0.00	1.81
Within Groups	39.92	528.00	0.08			
Total	55.47	539.00				

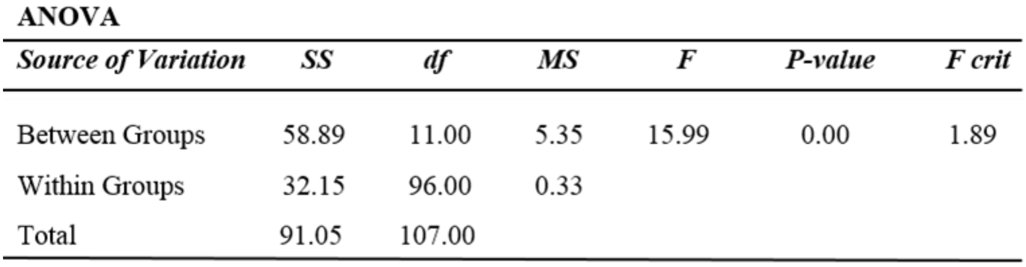
Table B.1.2: pH tolerances



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	39.28	11.00	3.57	4.17	0.00	1.82
Within Groups	236.10	276.00	0.86			
Total	275.38	287.00				

Table B.1.3: Carbon substrates

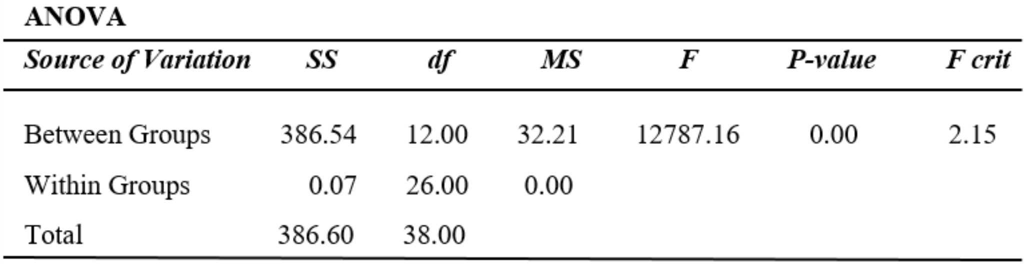


ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	58.89	11.00	5.35	15.99	0.00	1.89
Within Groups	32.15	96.00	0.33			
Total	91.05	107.00				

Section B.2: Analysis of variance (ANOVA) showing the significant difference between all the isolated bacterial treatments in terms of plant growth promotion and plant growth regulator activities.

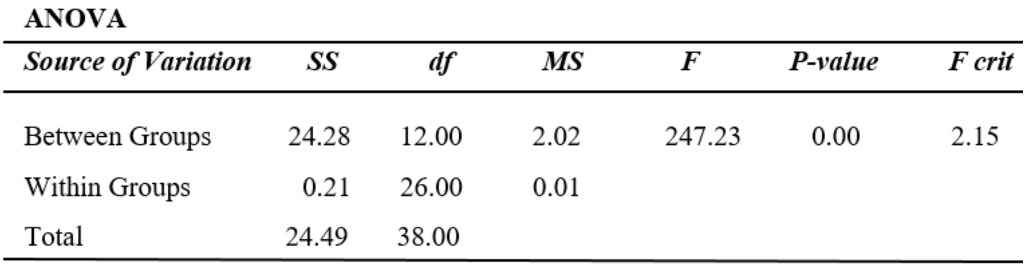
Table B.2.1: Ammonium



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	386.54	12.00	32.21	12787.16	0.00	2.15
Within Groups	0.07	26.00	0.00			
Total	386.60	38.00				

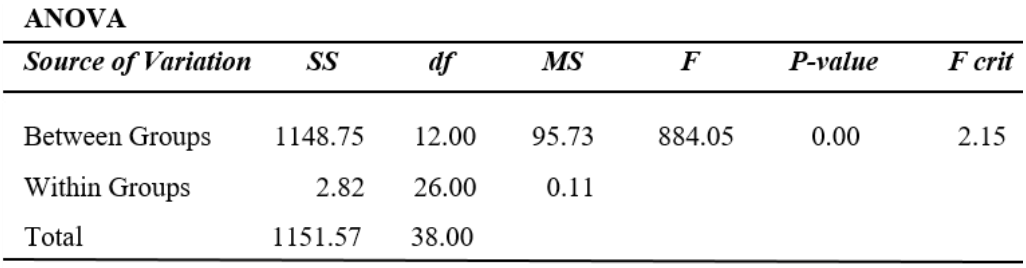
Table B.2.2: Phosphate



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	24.28	12.00	2.02	247.23	0.00	2.15
Within Groups	0.21	26.00	0.01			
Total	24.49	38.00				

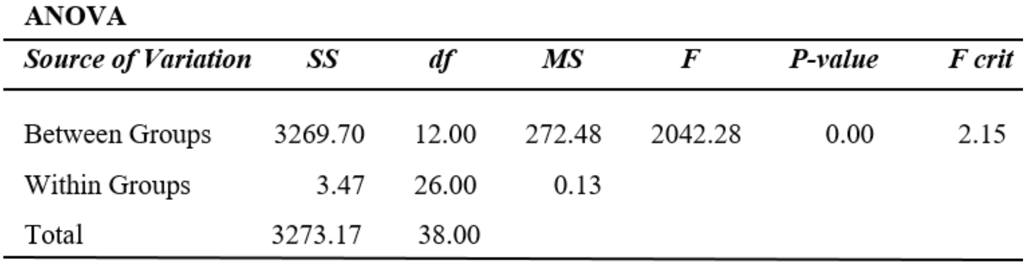
Table B.2.3: Potassium



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1148.75	12.00	95.73	884.05	0.00	2.15
Within Groups	2.82	26.00	0.11			
Total	1151.57	38.00				

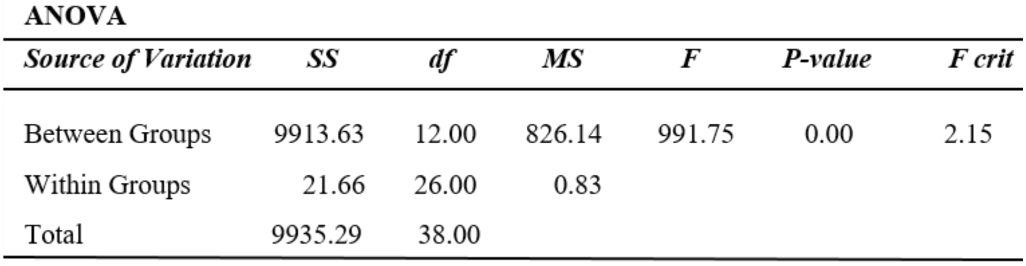
Table B.2.4: Manganese oxide



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3269.70	12.00	272.48	2042.28	0.00	2.15
Within Groups	3.47	26.00	0.13			
Total	3273.17	38.00				

Table B.2.5: Indole-3 acetic acid-IAA



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9913.63	12.00	826.14	991.75	0.00	2.15
Within Groups	21.66	26.00	0.83			
Total	9935.29	38.00				