QUALITY ASSESSMENT OF COMMERCIAL BIOFERTILISERS AND THE AWARENESS OF SMALLHOLDER FARMERS IN GAUTENG PROVINCE, SOUTH AFRICA

by

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Dedication

This work is dedicated to my beloved wife and children.

Declaration

- I **RAIMI ADEKUNLE,** hereby declare that the dissertation/thesis, which I hereby submit for the degree of **Masters in Environmental Science** at the University of South Africa, is my own work and has not previously been submitted by me or any other person for a degree at this or any other institution.
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- I declare that during my study I adhered to the Research Ethics Policy of the University of South Africa, received ethics approval for the duration of my study prior to the commencement of data gathering, and have not acted outside the approval conditions.
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Signed: Adekunle R. Raimi

Date: December, 2017

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Research output

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Summary

This study aimed to evaluate commercial biofertiliser quality and awareness amongst smallholder farmers in Gauteng Province, South Africa. Sixty-seven smallholder farmers were interviewed in Gauteng Province by using a survey method, while the physicochemical and microbiological properties of 13 biofertilisers were evaluated using laboratory experiments. The results showed that awareness and use of biofertiliser are very poor, with 96% of the smallholder farmers surveyed not having biofertiliser knowledge. Furthermore, the products lack basic quality parameters: 54% contained no biofertiliser strain while all the products contained microbial contaminants. The pH, moisture content and viable microbial densities were below the acceptable standards for some of the products. Two fungal and 58 bacterial operational taxonomic units were obtained from the 16S rRNA Sanger sequences while 5 791 OTUs were obtained from the Illumina Miseq system. Approximately 40%, 41% and 59% of the isolates were positive for nitrogen-fixation, siderophore production and phosphorous solubilisation. Overall, there is a need to improve awareness amongst farmers and promote good-quality biofertiliser products for increased crop productivity.

Keywords: Biofertiliser, contaminants, crop productivity, awareness, quality parameters.

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List of Abbreviations

AMF	Arbuscular mycorrhizal fungi
BLAST	Basic local alignment search tool
CFU	Colony forming unit
EC	Electrical conductivity
FAO	Food and Agricultural Organisation
IAA	Indole acetic acid
IFAD	International Fund for Agricultural Development
KEGG	Kyoto Encyclopedia of Gene and Genomes
MC	Moisture content
MPN	Most probable number
MR	Methyl Red
mS/m	Millisiemens per meter
NCBI	National centre for biotechnology and Information
Nfb	Nitrogen-free bromothymol blue
NGS	Next-generation sequencing
OTU.	Operational taxonomic unit
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
PMP	Predictive metagenomics profiling
PSB	Phosphate solubilising biofertilisers
PSM	Phosphate solubilising microorganisms
rRNA.	Ribosomal ribonucleic acid
SHF	Smallholder farmer

TVC	Total viable count
VP	Voges Proskauer
WHC	Water holding capacity

Chapter One

Introduction

1. Introduction

1.1 Background

South Africa's population growth rate is increasing and more than half of the population needs a substantial food supply, especially amongst the rural inhabitants who are living below the upper boundary of the poverty line (UBPL) (StatsSA, 2017). In order to adequately feed the population, it is necessary to increase food production more than twofold. However, South Africa has poor soil fertility and an erratic climate, coupled with other issues such as land acquisition, a struggling economy and a global concern for ecological balance (Goldblatt et al., 2010). Therefore, only an agricultural system that employs less capital and fewer land resources, with little or no fingerprint of ecological damage, will be the unlocking strategy. Such an agricultural system is entrenched in sustainable and smallholding agriculture (Lichtfouse et al., 2009; Vanlauwe et al., 2014).

Smallholder farmers (SHFs) mainly cultivate subsistence crops on small pieces of land and in some instances, commercial crops on a small-scale basis. They exist in various locations, ranging from rural areas to towns, and generally involve their family members in the labour force (Cousins, 2010). According to the International Fund for Agricultural Development [IFAD] (2013), more than 2 billion people in the world are supported by half a billion SHFs, with about 33 million operating in Africa. Smallholder farmers are very important for the agricultural and socio-economic development of any nation because of their contributions towards poverty alleviation and food security (Cacho et al., 2003; FAO, 2014). Currently, about 10% of agricultural land in the world are smallholder farms and interestingly they account for over 20% of total global food supply (Rudi, 2014).

However, the intensive farming practices of SHFs are not without their shortcomings. Where efficient nutrient management is not practised, soil nutrient depletion has been exacerbated. Therefore, there is a need for a replenishment strategy for sustainable crop productivity (Lahiff & Cousins, 2005). Presently, the major nutrient-management practice is the application of fertilisers, especially inorganic and in some cases organic fertilisers (Duarah et al., 2011). On the other hand, the use of chemical fertilisers is unsustainable amongst SHFs due to its high market price, caused by an unstable foreign exchange market and the high cost of production (Camara & Heineman, 2006). In addition, excessive applications of chemical and organic

fertilisers have been found to contribute to numerous ecological challenges such as leaching (Bationo et al., 2006), soil degradation, air and water pollution (Savci, 2012) and alterations in soil organic matter content (Bot & Benites, 2005). These challenges have a negative effect on farmers' productivity, as well as on plant, animal and human health (Savci, 2012).

Therefore, an ecologically friendly approach that will enhance sustainability in the farming system is required (Gruhn et al., 2000). Sustainable agricultural practices ensure efficient use of resources by integrating biological, chemical, physical and economic sciences to develop new practices that are safe and conserve the environment while supporting the development of plants and animals (Lichtfouse et al., 2009; Patel et al., 2014). The use of biological fertilisers such as biofertilisers has been suggested as a useful technology in nutrient management and sustainable agriculture (Kawalekar, 2013; Malusà & Ciesielska, 2014).

1.2 Rationale

The use of biofertiliser is vital for increased productivity amongst SHFs (Patangray, 2015; Patel et al., 2014). Surveys conducted in sub-Saharan Africa have shown that the application of biofertilisers on smallholder farms is very low compared to developed countries such as China, India, the United States of America and Canada (Masso et al., 2015). The low demand of biofertiliser products in Africa may be attributed to lack of awareness, product inaccessibility, poor quality products, a lack of technical experience as well as inadequate policies (Carvajal-Muñoz & Carmona-Garcia, 2012; Chianu et al., 2011). Consequently, it is necessary to improve awareness and quality in order for SHFs to fully benefit from the economic importance of biofertilisers (Ma et al., 2011; Masso et al., 2015).

Biofertilisers are substances that contain living microorganisms such as *Rhizobium, Azospirillum, Bacillus* and *Pseudomonas* (Rai, 2006). These microbes are able to improve plant growth and development through their participation in soil nutrient cycling and solubilisation, and through pest and diseases control (Kawalekar, 2013; Rai, 2006; Rose et al., 2014; Vessey, 2003). Biofertilisers may be solid or liquid. Solid biofertilisers are made with carrier materials such as peat, charcoal, humus and bagasse, while liquid biofertilisers are usually made with water, mineral oil or an oil-water solution. These materials support the growth and development of biofertiliser strains during storage. A good-quality carrier must be able to sustain microbes for a long period in order to guarantee the transfer of stipulated density of viable cells to the

field for effective functioning. Consequently, assessing the carrier materials' properties, and the types, density and the functional capabilities of beneficial microbes in biofertiliser products is important in defining the quality of the products and their potential efficiency on the field (Ansari et al., 2015).

Biofertiliser quality is also affected by the product's properties such as moisture content, pH, nutrient content and level of contamination. These parameters are usually defined by the product quality standard (Malusá & Vassilev, 2014; Yadav & Chandra, 2014). Hence, evaluating the quality of biofertilisers is an opportunity to ascertain the product conformity to quality standards and consequently determining the level of quality control management among manufacturers. In addition, it may be established that poor products are not sold to end users (Lupwayi et al., 2000; Mujawar, 2014; N'cho et al., 2013). It has been observed that inefficient quality control management usually results in the influx of low-quality products into biofertiliser markets (Ghosh et al., 2001; Simiyu et al., 2013) leading to poor field performance and consequently contributing to the low productivity and loss of economic value of SHFs (Lupwayi et al., 2000). Therefore, this study will assess SHFs' awareness and the quality of commercial biofertilisers available in South Africa.

1.3 Justification

Biofertilisers have been found to offer cheap and environmentally friendly alternatives to soil and crop nutrient replenishment (Patel et al., 2014; Vessey, 2003). In addition, biofertiliser-production technologies are relatively simple and cost-effective, which has encouraged an increase in commercial production (Mohammadi & Sohrabi, 2012). A key factor in evaluating the quality of biofertiliser is the source of the products (Yadav & Chandra, 2014). This is because various manufacturers often have different formulations for biofertiliser products (Masso et al., 2015). For instance, many of the imported products have been formulated in consideration of the quality standards, crops, soil and environmental factors of the manufacturing country. Thus, biofertiliser efficiency may not be optimal when used outside the environment in which it was manufactured (Huising, 2013).

The quality of biofertiliser is considered a major factor affecting SHFs' productivity, especially in the developing countries where awareness and acceptance of biofertiliser are low (Chianu et al., 2011). Many biofertilisers imported and produced in South Africa are rarely subjected to

standards or specifications; hence, some of the biofertilisers may be substandard and not efficient for sustainable agriculture (Lupwayi et al., 2000; Simiyu et al., 2013). However, in order to achieve significant biofertiliser usage among the SHFs, there is a need for a more focused and stringent national strategy (Masso et al., 2015). As SHFs continue to adopt and use biofertiliser, it will be beneficial to understand the various types and quality of biofertiliser products available for crop cultivation. This will improve knowledge that reduces costs of production, protects end users and promotes the perfect choice of biofertiliser products amongst SHFs. An increase in awareness and use of good-quality biofertilisers will improve and sustain crop productivity, which will subsequently promote South African agricultural development (Gentili & Jumpponen, 2006).

1.4 Hypotheses

- ✓ Commercial biofertiliser products available to smallholder farmers in Gauteng Province, South Africa are of poor quality.
- ✓ There is a low level of awareness and adoption of commercial biofertilisers among smallholder farmers in Gauteng Province, South Africa.

1.5 Aim and objectives

The study aimed at investigating the quality of commercial biofertiliser products available to SHFs in South Africa.

Specific objectives of the study include the following:

- ✓ To determine the physicochemical characteristics of various commercial biofertiliser products;
- ✓ To investigate the microbial diversity in commercial biofertiliser products using culture-dependent and culture-independent techniques;
- ✓ To evaluate the biochemical profile and functional attributes of microbial isolates from commercial biofertiliser products; and
- ✓ To investigate the awareness, adoption and types of biofertilisers used by SHFs in South Africa.

Chapter Two

Literature Review

2. Literature review

2.1 Introduction

The world population has been projected to grow to over 9.5 billion by 2050 (Godfray et al., 2010). Unfortunately, Africa especially sub-Saharan Africa, is predicted to be one of the major contributors to this increase (Sasson, 2012; Jayne & Ameyaw, 2016). The rapidly growing population of Africa has increased competition for every aspect of human life such as land, housing, water and food (Rukuni, 2002). Thus, there is a need to improve crop productivity to meet the high food demand. The industrial and green revolutions triggered a marked increase in food production in the past half-century; however, it has not alleviated the food-security challenges in Africa (Gregory et al., 2005). The African economy and the continent's natural resources must, therefore, be effectively utilised to increase productivity at a higher rate than the population growth rate (Daily & Ehrlich, 1992).

For centuries, the agricultural sectors of many African nations have been largely driven by SHFs, all the while faced with challenges of low-fertile soils, low or non-usage of external farm inputs, environmental degradation and lack of governmental support (Holt-Giménez & Shattuck, 2009). These challenges have caused a significant economic loss to farmers and have consequently hindered rural development (Diao et al., 2012). Collier and Dercon (2014) enumerated the strategies for rural development and one such strategy emphasised the support of SHFs. Resource-poor farmers should be supported financially and with various agricultural inputs such as fertilisers and high-yield seeds for improved productivity (Vink, 2012).

2.1.1 Smallholdings and Smallholder Farmers

Smallholding agriculture is a common practice in developing countries. A smallholding is a small plot of land used for cultivating crops where the number of plots owned by an individual farmer varies, subject to availability of farmlands (Salami et al., 2010). The smallholding size differs between countries and agro-ecological regions. In highly populated regions, less than 2 Ha of land is cultivated whereas more than 10 Ha may be cultivated in less-populated areas (Dixon et al., 2003). According to Fan et al. (2013), about half-a-billion farmers in the world cultivate less than 2 Ha due to declining arable lands. In addition, most of the lands are communally owned, which impacts negatively on their commercial value (Fan et al., 2013).

Recently, many countries have identified smallholding agriculture as one of the strategies for national economic development, stability and food security (Birner & Resnick, 2010; Dioula et al., 2013). Generally, SHFs have few or no resources and are often vulnerable to agricultural risk and climate change (Harvey et al., 2014). They are considered informal economy players, who are not registered and lack social protection (Curtis, 2013). It has been reported that smallholding agriculture supplies about 20% of the world's food supply. Therefore, investing in smallholding agriculture is an impetus to strengthen and develop the South African national economy (Rudi, 2014).

2.1.2 Smallholder farmers in South Africa

In South Africa, SHFs have contributed immensely to economic development through poverty alleviation and food supply (Livingstone et al., 2011). The contributions are so significant that the government is willing to invest more than R7 billion in smallholder agriculture (Rudi, 2014). Despite the contributions, most South African SHFs are economically highly deprived. They are less educated and reside mostly in the villages, which are characterised by less developed infrastructures (Jacobs & Baiphethi, 2015).

Figure 2.1: Female smallholder farmers cultivating the land

Source: http://www.actionaid.org/australia/6%20things-small-holder-women-farmers

According to Statistics South Africa [StatsSA], (2011), below 2% of households in South Africa are practicing in smallholder agriculture. With about 38% of all households in South Africa being headed by females, and the increasing need to improve the household economy, the population of women in smallholder agriculture has continued to increase. Smallholder farming has become an additional livelihood strategy for women to earn extra income and provide food for their families (Fig. 2.1) (Thamaga-Chitja & Morojele, 2014).

2.1.3 Smallholder economic importance

Increasing populations as well as economic and income growth lead to an escalation in commercial demand, thereby requiring complementary agricultural supply (Neven, 2014). Smallholder farmers are an important part of the emergence and modernisation of agricultural value chains (AVCs), which have contributed to the economic development of many nations. According to Barrett et al. (2012), one of the ways to achieve a seamless success in AVCs is the employment of contract farming arrangements (CFA) with smallholders. Many policymakers have utilised this to encourage rural economic growth.

Smallholder farmers are predominantly producers of subsistence crops such as maize, wheat, millet, groundnuts, rice, beans and potato. They also produce vegetables and fruits as well as cash crops such as coffee, cotton, tobacco, tea and cocoa, which contribute to the national export earnings of most nations (Livingstone et al., 2011; Salami et al., 2010). Therefore, the synergy between economic development and agriculture could improve the gross domestic product (GDP) if properly managed (Cervantes-Godoy & Dewbre, 2010). Consequently, it is imperative for SHFs to think beyond feeding their households alone and to produce in surplus in order to play a key role in African food security and economic development (Vink, 2012). In sub-Saharan Africa, smallholding agriculture employs over 65% of the African labour force while also contributing up to 90% of agricultural production (Asenso-Okyere & Jemaneh, 2012; Wiggins & Keats, 2013). In the 1980s, South African maize production increased by three-fold as a result of smallholders' contribution to the Grain Marketing Board intake. The huge increase was attributed to a number of factors such as improved agricultural research, extension management and government support, as well as the availability of improved technologies which included hybrid seeds, storage technology and fertilisers (Costa, 2014; Muzari et al., 2012). Developing smallholder agriculture with improved technologies can lead to a sustainable food system (Dioula et al., 2013).

2.1.4 Smallholder challenges

Obsolete and unsustainable farming practices amongst SHFs need to be improved to operate optimally. Excessive cultivation without nutrient management has caused loss of soil nutrients and quality. According to Bationo et al. (2006), the elevated nutrient loss experienced in parts of southern and northern Africa was attributed to unsustainable agricultural practices. There is no doubt that lack of technical know-how of cutting-edge scientific developments and nutrient-management systems has hindered increased productivity (Muzari et al., 2012). Most resource-poor farmers are illiterate or have low-level education, hence the adoption of new technologies that can bring about improved productivity is hindered. In addition, most African soils are not fertile and cannot naturally support optimal crop yield. Coupled with the scarcity of rainfall, farmers cannot increase per capita food production (Abraha et al., 2015; Okalebo et al., 2006).

Similarly, many agricultural policies do not favour smallholders. Infrastructure, financial support and extension services are rarely provided for farmers (Rosegrant et al., 2005; Wiggins & Proctor, 2001). In addition, declining cultivable land is another challenge. Arable lands are being used for social developmental purposes such as shopping malls, event centres and residential areas. Therefore, it is important that the available arable land is maximised through efficient nutrient management that can revitalise the soil for increased productivity (Gruhn et al., 2000). Until now, the major nutrient management system has been the use of inorganic fertilisers (Silva & Uchida, 2000). Common inorganic fertilisers include urea, ammonium sulphate, diammonium sulphate, potash, potassium phosphate and superphosphate. Mineral fertilisers have been able to support optimal crop productivity; however, it has had an adverse effect on the environment (Savci, 2012).

2.2 Challenges in the use of inorganic and organic fertilisers

Over-application of inorganic fertilisers has contributed to environmental degradation, leaching of nutrients, eutrophication and soil microbial floral alteration as well as soil pH distortion (Savci, 2012). Fertilisers are easily dissolved and washed away by rain or irrigation water. Nitrogen and phosphorous fertilisers are leached as nitrate (NO_3^-) and phosphate (PO_4^{3-}), respectively into water bodies where they support the excessive growth of algae (Savci, 2012). This causes a lack of oxygen in water bodies, leading to the formation of dead zones, which kills the aquatic organisms in the water body (Fig. 2.2). In addition, the acidity or alkalinity of

the soil has also been affected by the application of inorganic fertilisers, thereby causing a reduction in soil fertility (Hermary, 2007).

Figure 2.2: Algae bloom in a water body killing the aquatic organisms.

Source: Ahearn (2015)

Furthermore, a major challenge in the production process of mineral fertilisers is energy utilisation. It has been reported that the energy required to produce 1 kg of mineral fertiliser is about 80 JM, 40 JM and 12 JM for nitrogen, phosphorous and potassium fertilisers, respectively (Bhattacharyya, 2014). This is rather uneconomical, considering the challenges and costs of generating power in many African countries, especially South Africa. This has made inorganic fertilisers more expensive for SHFs to use (Chianu et al., 2011). Moreso, phosphorus, which is one of the limiting macro-elements, has been forecasted to run out in the next few decades (Cho, 2013). If this is not mitigated, it will cause a spontaneous hike in the price of phosphorous and its related products. To this end, there is a genuine need to discover a more economical and environmentally friendly method of nutrient management.

Another important nutrient-management practice that has been in use for decades is organic fertilising. Organic fertilisers are made from the remains of plants and animals such as plant leaves, cow dung, poultry manure and crop residues (Rosen & Bierman, 2005). However, challenges of availability, cost and management have decreased their use among SHFs. For instance, there are not enough poultry farms to supply the actual nutrient needs of the crops in any region of the world. Unfortunately, the little that is produced is wasted due to lack of technical expertise (Abbas, 2016). For example, South Africa produces about 3 million tons of animal manure annually, which can supply approximately 13% nitrogen, 28% phosphorous and 10% potassium of the needed soil nutrients. However, only 25% is utilised while the remaining is unexploited due to management constraints (Harris, 2002; Okorogbona & Adebisi, 2012).

The pungent smells associated with some organic fertilisers, especially animal manure, have made them difficult to work with (Gerber et al., 2007). Apart from this, compost, cow dung, and poultry waste are potential havens for pathogenic microbes (Heinonen-Tanski et al., 2006) and heavy metal contamination (cadmium, mercury, lead, cobalt and nickel) in agricultural soil (Moreno-Caselles et al., 2002). This encourages pests and diseases attack on plants and animals, leading to the creation of more challenges than benefits (Moreno-Caselles et al., 2002). Moreover, the cost of transporting manure from the source point to the farmland is high and coupled with the bad road networks to rural farms in many African countries, organic fertilisers have become uneconomical to use. The enumerated challenges have caused the need for more efficient nutrient management, which will not only benefit SHFs but will also be more sustainable in the long run.

2.3 Awareness and application of biofertiliser among South African smallholder farmers

Biofertiliser holds great potential to improve SHFs' crop productivity and their economic importance in South Africa (Bloem et al., 2009). To realise these benefits, there is a need to improve awareness, knowledge and usage amongst farmers. The level of knowledge about a technology is, therefore, a factor that can significantly influence adoption decisions. The initial awareness of a technology, which includes its potential benefits and economic characteristics, is an essential phase in the adoption of technology amongst farmers (Floyd et al., 1999). Many developed countries have realised the benefits of biofertiliser technology through increased awareness and scientific knowledge development (Chianu et al., 2011; Masso et al., 2015). Commercialisation and effective regulation of the biofertiliser industry in developed countries

have also enhanced the availability and adoption of biofertiliser products. On the other hand, lack of infrastructure, skill and a supportive regulatory framework have caused the low level of biofertiliser awareness and usage in sub-Saharan Africa (Simiyu et al., 2013), including South Africa.

2.3.1 Factors affecting awareness and use of biofertilisers among smallholder farmers

Low adoption rates of various agricultural practices amongst farmers are often a challenge for agronomists and extension managers. The major limitations to the adoption of new technology are time and the ability of farmers to integrate new ideas (Llewellyn, 2007). In addition, technologies may not be adopted due to lack of information and research-based evidence (Ochola et al., 2013). Moreover, the costs of seeking information and learning may be a challenge. Therefore, quality and readily available information with high reliability and relevance to the SHFs are important strategies for increasing awareness and use of biofertilisers (Santos Ordóñez, 2011). Other constraints on the use of improved nutrient-management systems are lack of institutional factors and non-adaptation of the technology to farmers' economic situations, as well as poor extension services (Sanginga & Woomer, 2009). Effective extension management is key to providing timely, adequate and relevant information to farmers (Tiwari et al., 2003). Individuals' awareness of problems, possible solutions and decision-making on adopting and using a particular technology is a function of the experience gained through different learning and experimental phases (Tiwari et al., 2003). Both economic and non-economic factors of the individual farmer may also be responsible for attitudes towards new technology (Liberio, 2012). Farmers' socioeconomic factors, knowledge, farm characteristics, institutional factors and biofertiliser accessibility were examined in this study as factors influencing awareness and use of biofertiliser technology.

Socioeconomic factors such as age, gender, education and types of crops grown are possible features that could influence awareness and use of biofertilisers amongst SHFs (Mutuma et al., 2014; Ochola et al., 2013). Farmers' level of education and experience also play significant roles in the awareness and adoption of biofertilisers. Generally, the more years of experience farmers have, the more knowledgeable they are over time. Perhaps the learning-process stages involving problem observation, evaluation, experimentation and the final-solution stage have improved farmers' proficiency, which affects adoption attitude (Llewellyn, 2007).

Types of crops grown also affect adoption and use of biofertiliser technology. Where cash crops are cultivated, high revenue may be realised which could possibly increase the financial capability of farmers to invest in new technology and bear the attendant risk (Santos Ordóñez, 2011). Farm characteristics such as farm size, agricultural practices, soil nutrient challenges and disease and pest occurrence also affect adoption (Tiwari et al., 2003). Furthermore, where the present farm practices have been efficient with optimal results, the adoption of new technology may not be an option, even with evidence of improvement.

2.4 Soil fertility

Soil is a living system containing millions of different creatures. These living organisms are essential for the recycling of soil nutrients, which occur through physical, chemical and biogeochemical reactions (Deepti & Mishra, 2014). Soil fertility is the ability of soil to supply vital nutrients and water in adequate amounts for plant growth in the absence of poisonous substances. Where the soil is toxic and essential nutrients are absent, plant growth is inhibited (Roy et al., 2006). This is why soil nutrient deterioration is considered a significant cause of reduced productivity of agriculture soil, especially in Africa (Sanchez, 2002). Soil deterioration occurs mainly through anthropogenic activities such as over-cultivation, unsustainable nutrient management or mechanised farming. To revitalise deteriorated soils, organic components, which supply the bulk of nutrients, must be augmented (Walworth, 2011). It is important that fertile soils have the appropriate physical and biological properties necessary for plant growth and development (Jones, 2012). Biological properties, which include the abundance and diversity of bacteria, blue-green algae and fungi, are good indicators of soil fertility. These microbes decompose organic wastes and their by-products improve the fertility of the soil (Biswas et al., 2000).

Fertilisers are substances that deliver plant nutrients in usable forms. The fertility of the soil is essential in the descriptions of fertiliser usage. This is because fertilisers are of different types and nutrient compositions. These define their various nutrient implications on plant growth and in the soil (Bationo et al., 2006; Jones, 2012). According to the Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Amendment Act 24, 1977, fertiliser is any material intended or used for improving or maintaining plant growth or soil productivity. Essentially, about 17 elements are necessary for proper plant development. A shortage or overdose in the

supply of any of these nutrients (Table 2.1) can result in severe damage to plant development. Primary macronutrients include nitrogen, phosphorous and potassium (Roy et al., 2006) while secondary macronutrients – calcium, magnesium and sulphur –, which are not so important for crop development, are usually sufficient in the soil. However, micronutrients such as iron, manganese, boron, nickel, zinc and copper, as important as they are, can become phytotoxic when present in excess (Mishra & Dash, 2014).

Table 2.1: Essential plant nutrient elements

Essential plant element	Elements	Symbol	Primary form
Non-mineral elements	Carbon	C	$CO_{2\ (g)}$
	Hydrogen	H	$H_2O_{\ (l)}$, H^+
	Oxygen	O	$H_2O_{\ (l)}$ $O_{2\ (g)}$
Mineral elements			
Primary macronutrients	Nitrogen	N	NH_4^+ NO_3^-
	Phosphorous	P	HPO_4^{2-}, $H_2PO_4^-$
	Potassium	K	K^+
Secondary macronutrients	Calcium	Ca	Ca^{2+}
	Magnesium	Mg	Mg^{2+}
	Sulphur	S	SO_4^{2-}
Micronutrients	Iron	Fe	Fe^{3+}, Fe^{2+}
	Manganese	Mn	Mn^{2+}
	Zinc	Zn	Zn^{2+}
	Boron	B	$B(OH)_3$
	Molybdenum	Mo	MoO_4^{2-}
	Chlorine	Cl	Cl^-
	Nickel	Ni	Ni^{2+}
	Copper	Cu	Cu^{2+}

Source: Parikh & James (2012).

Nitrogen is crucial for plant growth and optimum yield. When deficient, crops have limited growth and show signs of chlorosis, which is more pronounced in mature leaves (Bennett, 1993). The leaves show signs of yellow to tan colouration and eventually die. Some crops such as maize and tomatoes also exhibit purplish pigmentation on the stems and leaves when nitrogen is lacking (Baligar et al., 2001). Phosphorous is another important nutrient required for cell division and development. It is essential for photosynthesis, sugar and starch formation, energy

transfer, reproduction and movement of carbohydrate within the plant cells. Lack of potassium leads to stunted growth, delayed maturity and dark-green coloured young leaves, while the matured leaves become dark brown (Roy et al., 2006). On the other hand, potassium is necessary for protein, carbohydrate and fat production. It is also essential for chlorophyll and enzyme formation. Potassium helps to maintain the cell electrolyte balance and plant stomata functioning. When deficient, crops have irregular chlorotic leaves that appear burnt around the edges. Weak branches and stems appear in cereal grains while roots become rotten from microbial attack (Roy et al., 2006). Where these signs are visible, wind, rain or animals easily pull down the crops. When in short supply, other elements such as calcium, magnesium, sulphur, iron, zinc, copper and boron affect chlorophyll formation and cause dark green, twisted, necrotic and deformed leaves. Crops also produce seed disorder and stunted growth (He et al., 2005).

However, the increased emphasis on ecosystem quality has led to intensified sustainable agriculture (Gruhn et al., 2000), which is aimed at water and soil conservation, eliminating or reducing the use of chemical inputs and promoting crop and ecosystem biodiversity, as well as sustaining the economic stability of farms (Lichtfouse et al., 2009). Various agricultural techniques used in sustainable agriculture involve the cultivation of crops that can produce their nutrients such as legumes, which can fix atmospheric nitrogen, or the use of microbes that can fix soil nutrients or make nutrients available to crops. Such beneficial microorganisms are referred to as biofertilisers (Uribe et al., 2010). Biofertilisers have been widely used in sustainable agriculture for improved soil and crop productivity (Mahdi et al., 2010; Saba et al., 2013).

2.5 Biofertiliser

2.5.1 What are biofertilisers?

Biofertilisers are substances that contain live microorganisms, which when applied to plant surfaces, seeds, roots or soil stimulate plant growth by increasing the availability of plant nutrients and growth substances to the host crops (Figueiredo et al., 2010; Vessey, 2003). The term “biofertiliser” is used interchangeably with “inoculant” or “bioformulation” (Gupta et al., 2007; Hassen et al., 2016; Suyal et al., 2016). In South African fertiliser legislation, biofertiliser

falls under agricultural remedy substances. In the amendment Act 24 of 1977, an agricultural remedy includes any biological preparation or combination of any substance intended to be used as plant growth regulator, defoliant, desiccant or legume inoculant (Kotzé, 2006; SAFL, 1977). Biofertilisers may comprise of fungi, blue-green algae (BGA) and bacteria (separately or in combination), in liquids or carrier substances. The beneficial microbes may be rhizospheric–colonising the root surface or intercellular spaces of the plant roots– or endophytic –where they colonise the tissue or apoplastic space within the host plants (Malusà et al., 2016). The carrier materials sustain the microbial inoculants and allow the product to be stored for a longer period before field application (Boraste et al., 2009; Rashid et al., 2016).

Biofertilisers are not in any way the same as organic fertilisers such as animal manure, compost and plant manure or extracts (Mazid & Khan, 2014). However, if it causes an increase in crop yield (Banayo et al., 2012), increases crop accessibility to nutrients (Mujawar, 2014), replaces lost nutrients in the soil (Shridhar, 2012), or if the overall nutrient condition of crop and soil has been improved only by the beneficial microbes (Fig. 2.3), such a substance can be considered as a biofertiliser (Vessey, 2003).

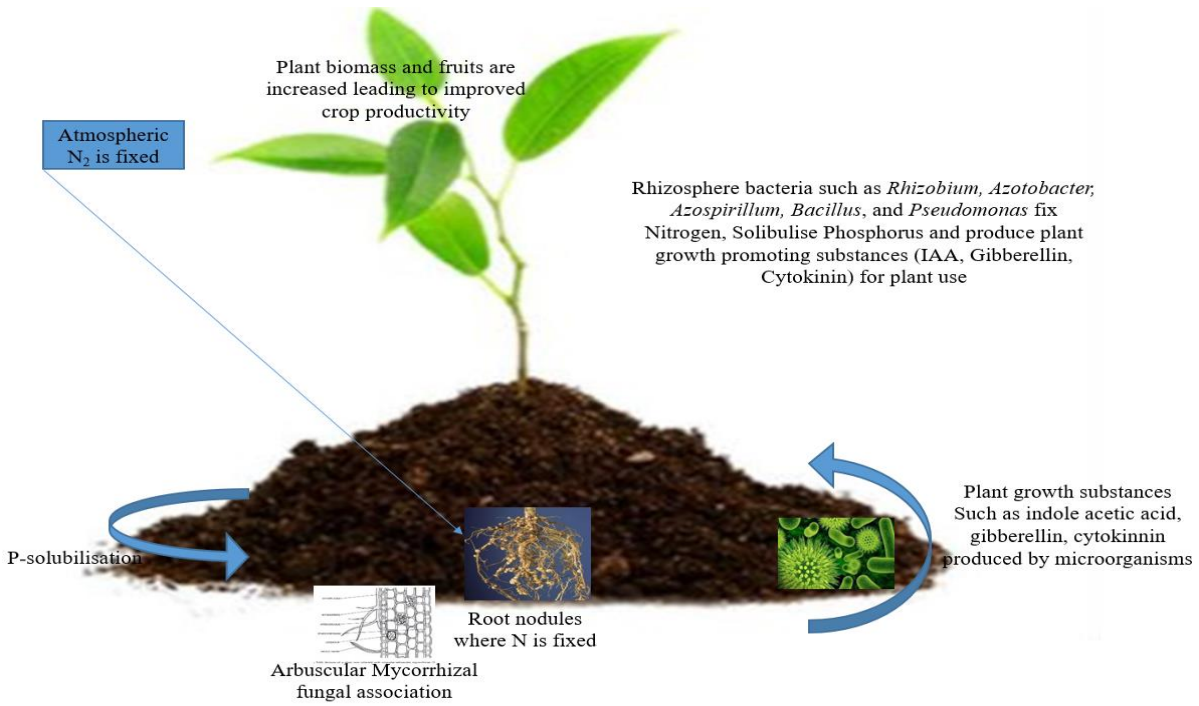

Figure 2.3: Plant and beneficial microbe interaction in the rhizosphere.
Source: Raimi et al. (2017)

2.5.2 Types of biofertiliser

The classification of biofertilisers depends on the microbial type and functional attributes established during their interactions with plants in the rhizosphere (Huang et al., 2014; Lucy et al., 2004). These functional attributes include nutrient fixing and solubilisation, biocontrol ability and production of plant growth-promoting substances (PGPS) (Gupta et al., 2012). According to Lesueur et al. (2016), biofertilisers should be classified based on the ability of the inoculum to perform two or more of the above functions. However, the basic classification based on strain types and functions are nitrogen-fixing, phosphate and micronutrient solubilising and plant growth-promoting biofertilisers (Fig. 2.4).

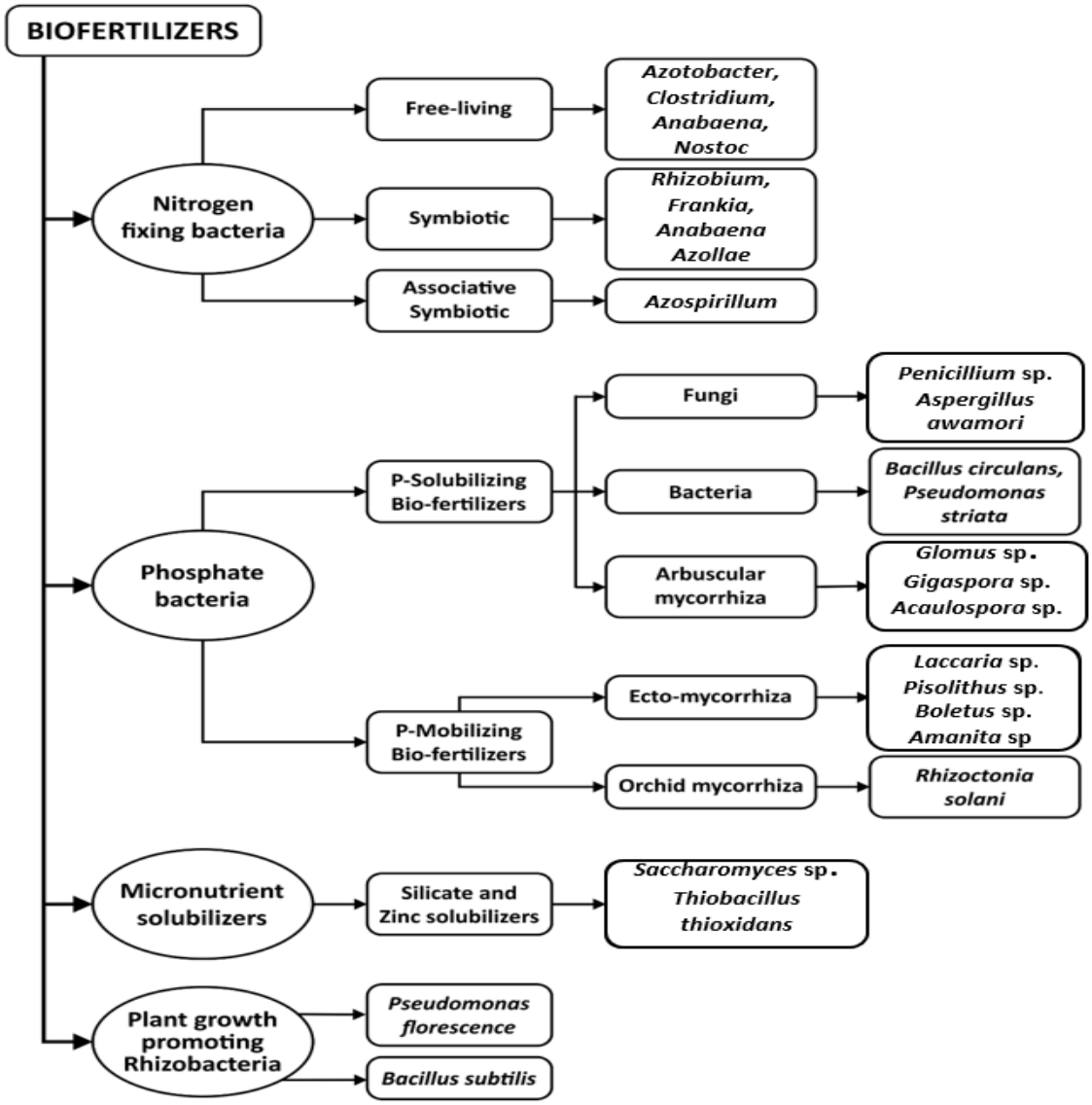

Figure 2.4: Classification of biofertilisers.

Adapted from Tamil Nadu Agricultural University, [TNAU] (2014).

2.5.2.1 Nitrogen-fixing biofertilisers

Nitrogen makes up approximately 78% of the atmospheric gases. It is a stable gas and inaccessible by organisms except when converted to compounds that can easily be assimilated (Guinness & Walpole, 2012). Nitrogen is recycled through various biological and chemical transformations involving different soil microbes as summarised in Fig. 2.6. In nitrogen deficient soils, diazotrophs fix nitrogen gas from abiotic to biotic environments using the enzyme nitrogenase. This oxygen-sensitive enzyme complex is composed of dinitrogenase reductase and dinitrogenase (Fig. 2.5), which reduce the dinitrogen into reactive forms such as ammonia and nitrate (Dighe et al., 2010).

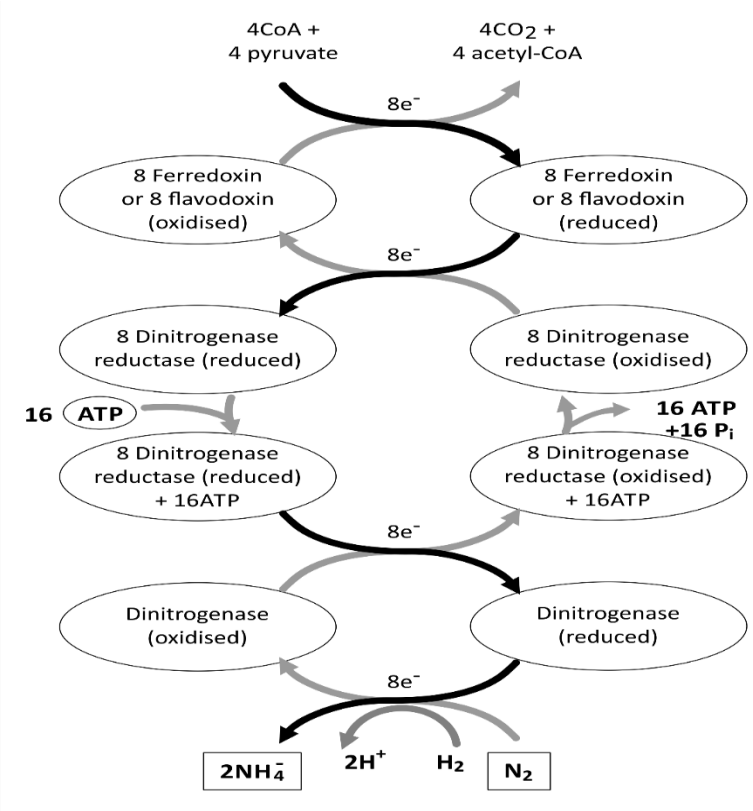

Figure 2.5: Dinitrogenase in nitrogen cycle (Dighe et al., 2010).

Biofertilisers are usually in symbiotic or non-symbiotic relationships with host plants. The symbiotic relationship is common in rhizobia such as *Rhizobium, Bradyrhizobium* and *Sinorhizobium,* which inhabit the root nodules of most leguminous crops such as bean, cowpea, soybean and groundnut (Martínez-Romero, 2009; Oldroyd et al., 2011). This association has huge ecological importance due to its substantial impact on global biological nitrogen fixation. The non-symbiotic free-living nitrogen-fixers include *Azotobacter, Beijerinckia* and

Clostridium, while associative nitrogen-fixers include; *Enterobacter* and *Azospirillum* species (Shridhar, 2012; Wagner, 2012). These microbes regulate the amount of soil-organic nitrogen through immobilisation and mineralisation during organic matter decomposition. During mineralisation, microbial cells are decomposed to release ammonia and nitrate, while immobilisation occurs when soil microbes take up ammonia and nitrate in a form that is unavailable to crops (Zehr & Kudela, 2011).

Another nitrogen-fixing biofertiliser of great economic importance is Cyanobacteria. It is comprised of *Anabaena* (or *Nostoc*) in association with *Azolla* (Benkeblia & Francis, 2014). Cyanobacteria are mainly used in rice cultivation, maintaining soil organic carbon and fixing nitrogen in the range of 40-100 kg/Ha when used for wet-rice cultivation (Paudel et al., 2012; Wagner, 2012). Azolla in rice cultivation can give about 15-18% yield increase, while also saving about 15-25 kg/Ha on nitrogen fertilisers (Jiao et al., 2015). Arbuscular mycorrhiza fungi (AMF) also play an important role in nitrogen fixation. Leigh et al. (2009) established that mycorrhizal association might supply up to 50% of plant nitrogen needs, making it very important in cultivating soils with low nitrogen, especially in sub-Saharan African countries such as South Africa.

2.5.2.2 Solubilising and mobilising biofertiliser

Phosphorous and potassium form stable compounds with elements such as iron, aluminium and calcium, which are not readily accessible by plants (Richardson et al., 2009). This has resulted in limiting nutrients, especially for phosphorous. Unfortunately, phosphorous has no large atmospheric deposit unlike nitrogen; therefore, solubilisation and mobilisation are important mechanisms used by phosphate solubilising microorganisms (PSM) in the phosphorus cycle (Mohammadi, 2012).

PSMs are mainly bacteria (*Pseudomonas, Klebsiella, Micrococcus, Flavobacterium* and *Bacillus*) and fungi (*Aspergillus, Fusarium and Penicillium*) (Sundara et al., 2002). According to Pindi and Satyanarayana (2012), the most efficient phosphorous solubilisers include; *Bacillus polymyxa, B. megaterium, Pseudomonas striata, P. rathonis, Aspergillus awamori, A. niger,* and *Penicillium digitatum*. They are able to mineralise organic phosphorous by secreting

phosphatases, which hydrolyse organic phosphorous and can save up to 30-50 kg/Ha of phosphorous pentoxide (P_2O_5) fertiliser (Richardson et al., 2009; Singh et al., 2014).

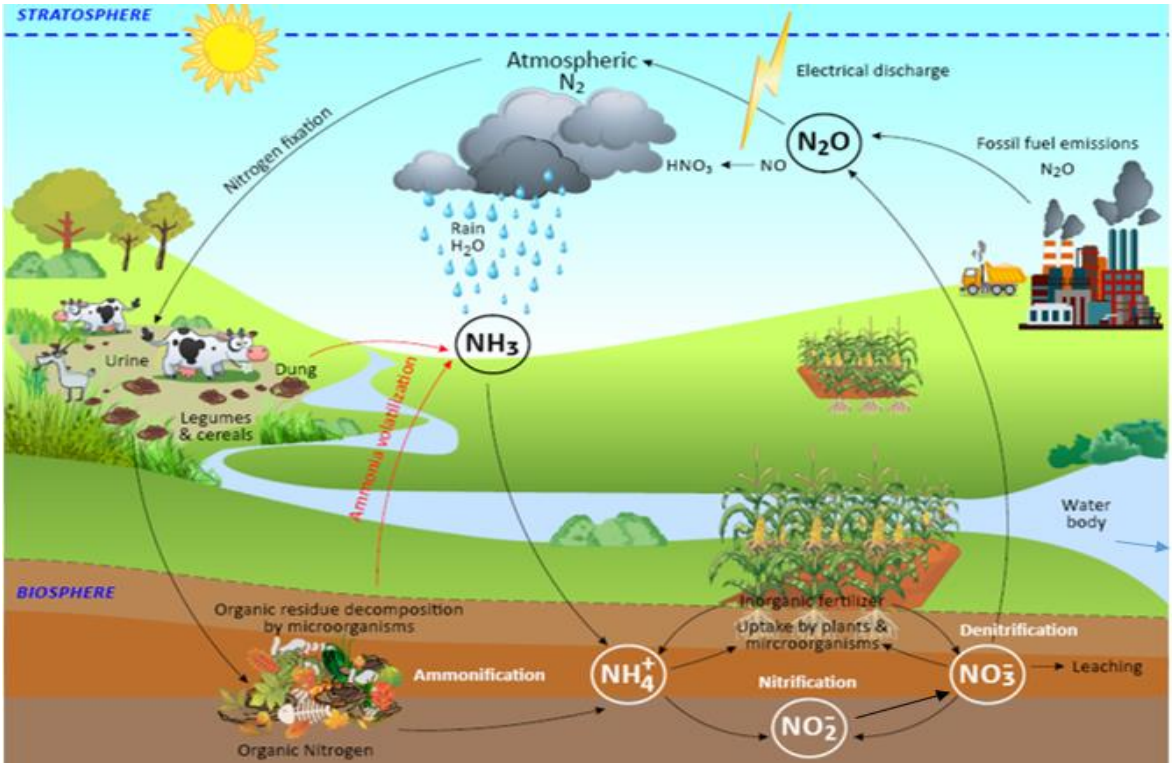

Figure 2.6: Nitrogen cycle

Adapted from the University of Waikato (http://www.waikato.ac.nz/__data/assets/image/0013/151033/NitrogenCycle.jpg).

Phosphate-solubilising microbes also produce organic acids such as gluconic, lactic, oxalic and citric acids to solubilise soil inorganic phosphorous (Fig. 2.7) (Malusà & Ciesielska, 2014). The genera *Bacillus, Actinomycetes* and *Pseudomonas* are non-symbiotic bacteria that are effective in solubilising inorganic phosphorous such as tricalcium and rock phosphate to monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions (Adeleke et al., 2017; Rai, 2006).

Phosphorous-mobilising microorganisms (PMMs) improve the ability of plants to acquire soluble phosphorous. Many plants, especially in phosphorus-deficient soil, develop increased root growth by the extension of the existing root systems. This may occur through mycorrhizal association or the phytostimulation effect, which involves hormonal stimulation (Richardson & Simpson, 2011). The plant-fungi symbiotic relationship is a major technique used by most

plants to alleviate phosphorous-limiting conditions. Here, fungal hyphae are able to mobilise and make phosphorous available to plants (Mujawar, 2014; Ramasamy et al., 2011). Arbuscular mycorrhizal fungi through specialised structures known as vesicles and arbuscules are able to increase a plant's exploitation of soil nutrients (Leigh et al., 2009; Smith et al., 2011). Another mechanism of phosphorous-mobilisation is through changes in the sorption balance of soil solution caused by microbial biomass turnover in the rhizosphere. This may lead to increased mobility and uptake of organic phosphorous or orthophosphate ions. Microbial metabolic processes may also directly mineralise and solubilise phosphorous (inorganic and organic) through the efflux of protons, organic ions and siderophore production (Fig. 2.7) (Richardson & Simpson, 2011).

Deficiency of phosphorous affects nodule development and total nitrogen fixation in legume crops, hence phosphorous biofertilisers are essential for legume development and its contribution to soil and plant-nitrogen content (Valentine et al., 2017). Legume cultivating countries such as South Africa, with high phosphorous-deficient soils, can use phosphorous biofertilisers to augment soil phosphorous to the required levels for crop use (Deckers, 1993).

Potassium-solubilising biofertiliser (KSB) solubilises compounds such as muscovite, illite, mica and biotite by producing organic ligands, hydroxyl anions, enzymes and biofilms in the rhizosphere (Bahadur et al., 2014; Shanware et al., 2014). Potassium-solubilising bacteria include *Pseudomonas, Burkholderia, Acidothiobacillus, Paenibacillus* and *Bacillus* sp.*,* and their ability to solubilise effectively is based on the type of soil, potassium complexes and microbial strains (Liu et al., 2010; Sangeeth et al., 2012).

2.5.2.3 Micronutrient biofertiliser

Micronutrients form complexes in the soil that are inaccessible by crops. For example, 75% of zinc applied as fertiliser forms insoluble complexes, while plants use only about 1-4% of total available zinc in the soil (Mahdi et al., 2010). However, biofertilisers such as *Rhizobium, Bradyrhizobium, Pseudomonas, Trichoderma, Bacillus and Saccharomyces* sp. can improve the availability and uptake of micronutrients in the soil (Ahsan et al., 2012; Esitken et al., 2010).

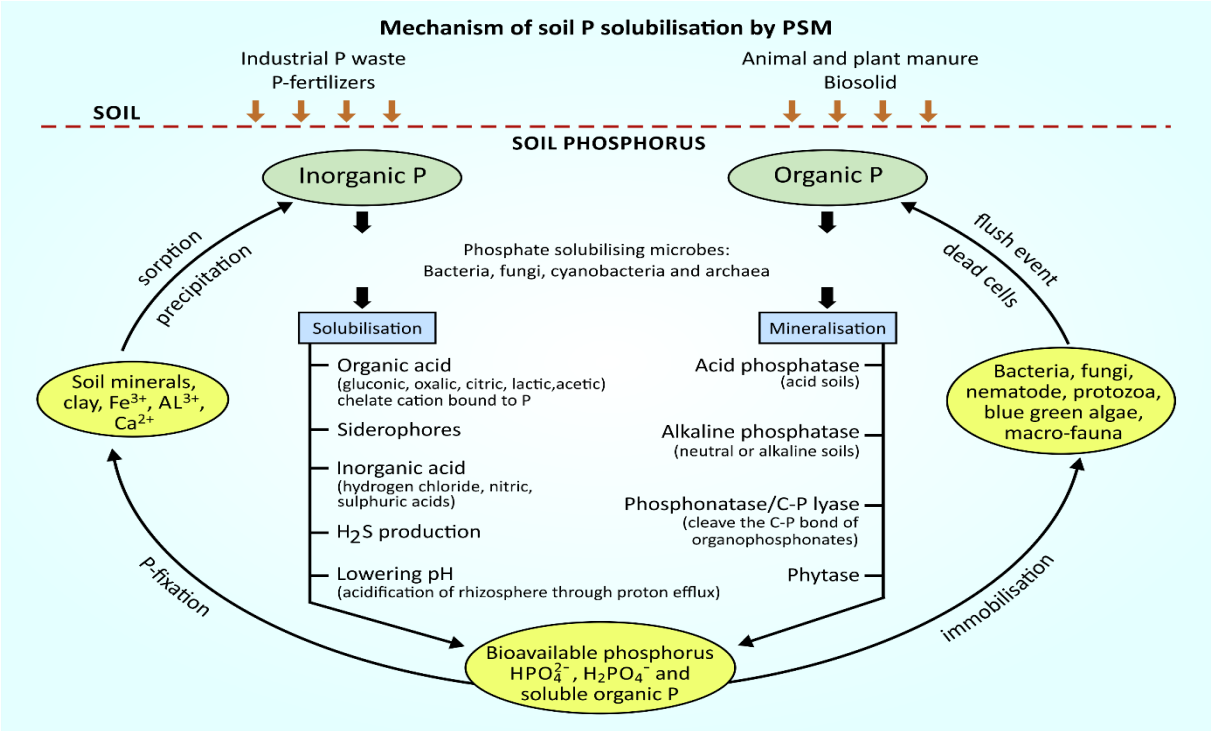

Figure 2.7: Phosphorous solubilisation mechanism in PSM (Richardson & Simpson, 2011).

In iron-immobilized soil, for example, bacteria produce siderophores that solubilise and chelate iron into complexes that can be easily absorbed by plants (Mathew et al., 2014). *Trichoderma harzianum*, a fungal species, solubilises minerals such as metallic zinc and manganese oxide by chelating and reducing mechanisms (Altomare et al., 1999). Vesicular-arbuscular mycorrhiza (VAM) are also able to solubilise zinc, iron, manganese and copper complexes in agricultural soil (Martino et al., 2003; Pal et al., 2015).

2.5.2.4 Plant growth-promoting rhizobacteria (PGPR)

Beneficial microorganisms that participate in nutrient cycling and produce growth-promoting substances in the rhizosphere are called plant growth-promoting rhizobacteria (PGPR) (Ahemad & Kibret, 2014; Bhattacharyya & Jha, 2012). This group of biofertilisers stimulates plant growth through one or more mechanisms. The growth-promoting substances may increase plant growth directly or indirectly (Hayat et al., 2010; Soltani et al., 2010). The indirect mechanisms involve the production of antimicrobial metabolites such as hydrogen cyanide, phenazines and tensin, which protect the plants against diseases. Plant growth-promoting rhizobacteria synergy with plant roots also elicit plant defence against bacterial, fungal and viral

pathogens, termed induced systemic resistance (Ahemad & Kibret, 2014; Vacheron et al., 2014). This group of heterogeneous beneficial rhizospheric microbes has multiple modes of action, which may include nutrient solubilisation, phytostimulation and biocontrol (Table 2.2). They include genera such as *Bacillus, Burkholderia, Serratia,* and *Pseudomonas.* (Bhattacharyya & Jha, 2012; Hassen et al., 2016).

Table 2.2: Plant growth-promoting rhizobacteria and their functions

Organisms	Function	Reference
Rhizobium cicero, R. phaseoli	Siderophore ,	Berraho et al. (1997)
	Fix- N, IAA	Zahir et al. (2010)
Sinorhizobium meliloti	Fix- N	Villegas et al. (2006)
Bradyrhizobium Japonicum, B. elkanii, B. canariense, B. betae, B. liaoningense,	Fix-N, P-solubilisation siderophore and IAA production	Antoun et al. (1998)
Azospirillum brasilense, A. lipoferum	Fix-N, P-solubilisation	Rodrigues et al. (2008)
A. amazonense, A. amazonense	IAA and siderophore production	Thakuria et al. (2004)
Azotobacter chroococcum	Fix-N, P-solubilisation, gibberellin, IAA, kinetin & siderophore production	Verma et al. (2001)
Azoarcus communis, A. indigens	N-fixer	Reinhold-Hurek et al. (1993)
Bacillus mucilaginous, B. megaterium	K & P-solubilisation, gibberellin, auxin, and	Parmar and Sindhu (2013)
B. licheniformis, B. edaphicus, B.	cytokinin production	Mohammadi and Sohrabi (2012)
subtilis, B. cereus, B. pumilus, B. circulans		Karadeniz et al. (2006)
Burkholderia unamae, B. tropica	1-aminocyclopropane-1-carboxylate (ACC) deaminase, N-fixer, IAA, P-solubilisation, and siderophore	Onofre-Lemus et al. (2009)
Enterobacter asburiae	IAA, P-solubilisation, siderophore ammonia,	Ahemad and Khan (2010)
Klebsiella sp.	IAA, P-solubilisation, siderophore ammonia	Ahemad and Khan (2011)
Pseudomonas putida, P. jessenii, P.	P-solubilisation, siderophore and IAA,	Parani and Saha (2012)
aeruginosa, P. chlororaphis.		Shaharoona et al. (2008)
Alcaligenes faecalis	P-solubilisation, IAA and siderophore production	Sayyed et al. (2010)
Acinobacter sp.	IAA, P-solubilisation and siderophore	Rokhbakhsh-Zamin et al. (2011)
Serratia marcescens	IAA, siderophore, HCN and P-solubilisation	Badawi et al. (2011)
Flavobacterium sp.	IAA,P-solubilisation	
Gigaspora ramisporophora, Glomus clarum, G. mosseae, G. etunicatum	Zn, Cu, Fe & P-solubilisation, Improve salinity tolerance	Garg and Chandel (2011)
Penicillium bilaiae, P. italicum	P- solubilisation, gibberellin, IAA, improve water and salinity tolerance	Waqas et al. (2012) Ahmad et al. (2008)
Aspergillus niger, A. terreus, A. flavus	P-solubilisation	Sharma et al. (2013)
Anabaena azolla	P-solubilisation, N-fixing, and detoxify heavy metal and gibberellin, cytokinin and IAA	Singh et al. (2016)

Adapted from Ahemad and Kibret (2014)

2.6 History of biofertiliser

The concept of biofertiliser might have originated from some of the ancient farming practices such as crop rotation and intercropping, which aimed at increasing yield and soil fertility (Peoples et al., 2009; Preissel et al., 2015). One of the major crops used in crop rotation is legume; this is because rhizobia develop symbiotic associations with leguminous plants, forming symbiosomes, intracellular compartments within root nodules where nitrogen is fixed (Oldroyd et al., 2011). The improved knowledge of the interactions in the soil between microbial communities and various plants has been the basis of biofertiliser development (Jensen et al., 2012). In addition, the fact that these beneficial microbes can be isolated, cultured and identified in the lab (*in vitro*), is also an important factor that has aided the development of biofertiliser (Carvajal-Muñoz & Carmona-Garcia, 2012). Biofertilisers are comprised of live beneficial microbes in a sustainable system for crop and soil inoculation, which support plant growth and soil-quality upgrades by way of the biological activity of the microbes in the soil.

Commercial biofertiliser production and use in the developed countries date back to 1895 with the first biofertiliser product patent registered in 1896 in the United Kingdom (Patent no. GB 189511460) (Chansa-Ngavej & Assavavipapan, 2007). In Africa, it is unclear when biofertiliser was first introduced, but according to literature, the history of its commercial production could date back to 1952 when commercial biofertiliser was first reported in South Africa (Strijdom, 1998). In addition, a documented report on large-scale afforestation of pine in Rhodesia (now Zimbabwe) also reveals that soil inoculants have been in use in Africa since 1928. Inoculated soil, most probably with mycorrhizal fungi, was a major strategy for pine-nursery growth and successful pine cultivation (Romberger & Mikola, 1964). Despite decades of inoculant existence, the production and use of biofertiliser in Africa are still very low. However, some countries such as Kenya, Malawi, Tanzania, South Africa, Zimbabwe and Zambia have small-scale inoculant production (Hardarson & Broughton, 2003).

2.7 Biofertiliser production in South Africa

In South Africa, the rapid expansion of the commercial biofertiliser market in 1952 necessitated the establishment of an independent quality-control body in the early 1970s. In conjunction with the Department of Agriculture, this body monitors the quality of the products produced (Strijdom, 1998). The Department of Agriculture stipulated some quality parameters for

biofertiliser registration. These include a six-month shelf life, maintenance of at least 5×10^8 rhizobial cells before the expiration date and the use of sterile peat as carrier material (Strijdom, 1998). There are various commercial biofertiliser producers in South Africa such as Soygro (Ltd) Ltd, which produce major products such as Mazospirflo and Rhizostim (*Azospirillum*), Peanutflo and Soyflo (*Bradyrhizobium*) and Nemablock (De Bruijn, 2015). Other producers include Mycoroot™ (Ltd) Ltd; Biological Control Products SA Ltd and Amka Product Ltd (Table 2.3).

Table 2.3: Biofertiliser products, microbial compositions and their manufacturers in South Africa

Biofertilisers	Active components	Manufacturers
Firstbase	*Bacillus* sp.	Microbial solution Ltd, South Africa
Biostart	*Bacillus* sp.	Microbial solution Ltd, South Africa
Landbac	*Bacillus* sp.	Microbial solution Ltd, South Africa
Waterbac	*Bacillus* sp.	Microbial solution Ltd, South Africa
Likuiq Semia	*Bradyrhizobium elkanii*	Microbial solution Ltd, South Africa
Nitrasec Alfalfa (Lucerne)	*Sinorhizobium meliloti*	Microbial solution Ltd, South Africa
Rhizatech	*Glomus mosseae, G. etunicatum, G. intraradices*	Dudu tech, Naivasha, Kenya
Symbion vam plus	*Glomus* sp. *Gigaspora* sp. *Bacillus megaterium* var. *phospaticum*	T. stanes and Company Ltd. India
Ectovit	Ectomycorrhizal	Symbiom Ltd., Czech Republic
Rhodovit	Not specified	Symbiom Ltd., Czech Republic
Mycoroot superGro	Arbuscular mycorrhizal fungi	Mycoroot Ltd, South Africa
Mycoroot Super Booster	Arbuscular mycorrhizal fungi	Mycoroot Ltd, South Africa
Mycoroot green	Arbuscular mycorrhizal fungi	Mycoroot Ltd, South Africa
Organico	*Bacillus* sp., *Enterobacter* sp., *Pseudomonas, Stenotromonas, Rhizobium, Bacillus subtilis, Bacillus thuringiensis, Azotobacter chroococcum, Pseudomonas florescens,*	Amka Products Ltd, South Africa
Soil Vital Q	*Lactobacillus* sp.	Biological Control Products SA Ltd
Nitrasec	*Rhizobium tropici*	Lage y Cía. S.A, Uruguay

Biofertilisers	Active components	Manufacturers
LifeForce	*Bacillus* sp.	Microbial solution Ltd, South Africa
Azo- N	*Azospirillum brasilense, Azospirillum lipoferum*	BioControl Products SA Ltd
Azo-N Plus	*Azospirillum brasilense, Azospirillum lipoferum, Azotobacter chroococcum*	BioControl Products SA Ltd
NAT-P	*Pseudomonas fluorescence*	BioControl Products SA Ltd
N-Soy	*Bradyrhizobium japonicum*	BioControl Products SA Ltd
SoilFix	*Brevibacillus laterosporus, Paenibacillus chitinolyticus, Lysinibacillus sphaericus, Sporolactobacillus laevolacticus*	BioControl Products SA Ltd
Composter	*Bacillus* sp.	BioControl Products SA Ltd
N-Bean	*Rhizobium phaseolus*	BioControl Products SA Ltd
Histick	*Bradyrhizobium japonicum*	BASF South Africa Ltd, South Africa
Nodumax	*Bradyrhizobia*	IITA Business incubation platform, Nigeria
MycoApply endo	AMF (3 sp.) not specified	Mycorrhizal Applications Inc., Grants , US
MycoApply endonet	*Glomus. intraradi*	Mycorrhizal Applications Inc., Grants , US
MycoApply root dip gel	AMF (not listed)	Mycorrhizal Applications Inc., Grants , US
Soyflo	*Bradyrhizobium japonicum*	Soygro Ltd, South Africa
Rhizostim	*Azospirillum* sp.	Soygro Ltd, South Africa
Mazospirflo	*Azospirillum brasilense*	Soygro Ltd, South Africa
Legume fix	*Rhizobium* sp.	Legume Technology (UK)
Bio-N	*Azotobacter* sp.	Nutri-Tech Solution, Australia
Twin N	*Azorhizobium* sp., *Azoarcus* sp., *Azospirillum* sp.,	Mapleton Ltd., UK
Bac up	*Bacillus subtilis*	Biological control product Ltd, South Africa
BIOFIX	Not specified	MEA Fertiliser Ltd, Kenya
Vault NP	*Bradyrhizobium japonicum*	Becker Underwood, USA

Adapted from Herrmann et al. (2015)

The numerous strains of beneficial microbes have great potential in the development of biofertiliser products. As new beneficial strains with inoculum potential are isolated, characterised and stored in various laboratories in different countries around the world, new

products are also being developed. This has sustained the continuous production and improvement of biofertiliser products (Hayat et al., 2010; Rosen & Bierman, 2005). Biofertiliser strains can be obtained from various institutes or conservation centres where they are stored. In South Africa, biofertiliser strains can be obtained from the Plant Protection Research Institute (PPRI) at the Agricultural Research Council.

2.8 The economic importance of biofertiliser in smallholder agriculture

The benefit-cost analysis of biofertiliser is determined by calculating the proportion of the obtainable value of benefits compared to the actual cost of biofertiliser. For instance, legume-inoculant benefits are computed based on the amount of nitrogen fixed (Mulongoy et al., 1992). For instance, white clover crop was able to fix 200 kg of N /Ha and has a benefit-cost ratio of 416 while soybean fixed 100 kg of N/Ha and the benefit was calculated to be 17 when the cost of fixed nitrogen is considered as US$0.50 per kg (Mulongoy et al., 1992). The following are major contributions of biofertiliser to smallholder agriculture.

2.8.1 Increased yield and nutrient availability

Crop yield can be improved by using biofertilisers such as *Rhizobium* and *Bradyrhizobium*. *Rhizobium*'s symbiotic relationship with legumes replenishes soil nitrogen by fixing up to 50-200 kg N/Ha in the soil (Mishra & Dash, 2014). In addition, soybean inoculation causes an increase in yield, improves soil organic matter, while also fixing about 80% of crop nitrogen need (Chianu et al., 2011; Giller et al., 2011). Rose et al. (2014), reported that biofertiliser could replace about 52% nitrogen-fertiliser and cause an increase in rice yield over the control.

Therefore, the use of biofertilisers by resource-poor farmers can increase crop yield and reduce the cost of production through less application of chemical inputs, thereby increasing profitability (Suyal et al., 2016). *Azolla* soaked in 50 ppm of superphosphate when inoculated in a paddy field fixes about 40-55 kg N/Ha, 15-20 kg P/Ha and 20-25 kg K/Ha in a month per 1 kg of *Azolla* applied, bringing the yield of a flooded paddy to about 10-20% over the control (Mazid & Khan, 2014). Similarly, potassium-solubilising bacteria such as *Pseudomonas* and *Burkholderia* have been reported to cause an increase in growth and yield of wheat and pepper (Shanware et al., 2014), while *Bacillus* caused yield increase in cucumber and pepper. These

underscore the importance of biofertiliser to improve the yield of SHFs (García-Fraile et al., 2015).

2.8.2 Plant growth-promoting substances

Plant growth-promoting rhizobacteria (PGPR), produce phytohormones such as cytokinins, auxins and gibberellins that cause an increase in plant foliage, root elongation, fruit yield and plant-microbe symbiosis (Hassen et al., 2016; Vacheron et al., 2014). Indole acetic acid (IAA), also called auxin, affects plant root architecture, promoting increased root surface area and root-tip elongation (Ahmad et al., 2005), while gibberellic acid induces increased flowering, stems and internode elongation, fruit setting and growth in plants (Kumar et al., 2014; Zalewska & Antkowiak, 2013). Swain et al. (2007) reported in a study that yam (*Dioscorea rotundata)* inoculated with *Bacillus subtilis,* an IAA-producing strain, had an increased tuber length and an increased number of sprouts compared to uninoculated plants. Furthermore, rice and maize cultivated with gibberellic acid producing PGPR had a significant increase in growth and yield (Vacheron et al., 2014). Similarly, siderophores produced by PGPR stimulate the growth of maize in iron-poor soil (Egamberdiyeva, 2007), while an increase in yields of many cereal crops has been reported in the work of Pérez-Montaño et al. (2014) when the crops were grown with biofertilisers.

2.8.3 Low cost of nutrient supply

Different values for biological nitrogen fixation (BNF) in the soil have been reported. Galloway (1998) valued the annual BNF to be about 90-130 Tg N $year^{-1}$, while Boyer et al. (2004) approximated it to be roughly 107 Tg N $year^{-1}$. Similarly, Bhattacharyya (2014) estimated BNF on land to be 140 Tg N $year^{-1}$. It has also been reported that up to 300 kg N/Ha per season can be fixed on legume-cultivated land (Ngetich et al., 2012). Surprisingly, the energy bill for this process is fully paid for by nature. Furthermore, the quantity of biofertiliser required to achieve the same amount of nutrients supplied by inorganic fertiliser is relatively lower. The cost of *Rhizobium* biofertiliser sufficient for 1 Ha was reported to be US$5.20 in Zimbabwe and US$4.50 in Rwanda (Mulongoy et al., 1992). NoduMax costs only US$5/Ha in application as opposed to the US$100/Ha cost of urea fertiliser needed to supply the same quantity of nutrients (N2Africa, 2015). The required energy for manufacturing inorganic fertilisers is huge compared to that required in biofertiliser production. This has made mineral fertilisers more expensive. In

fact, about 60% of smallholders in Africa cannot afford the high-priced inorganic fertiliser (Chianu et al., 2011). However, the use of biofertilisers is cost-effective, economical and sustainable for SHFs.

2.8.4 Biocontrol ability of biofertilisers

The metabolic products or indirect competition of biofertiliser strains can inhibit pathogens, thereby preventing pests and disease attack (García-Fraile et al., 2015; Rudrappa et al., 2008). *Pseudomonas* and *Bacillus* produce some antibiotics that impede the growth of bacterial and fungal pathogens (Beneduzi et al., 2012; Figueiredo et al., 2010). Similarly, the nodule-forming symbiotic association between legumes and *Rhizobium* boosts the synthesis of cyanogenic defence substances, which increase plant resistance to herbivore attack (Mazid et al., 2011; Megali et al., 2015). It is a fact that bacterial and fungal attacks reduce crop productivity among SHFs (Sones, 2015). Therefore, using inoculants producing antifungal and antibacterial substances such as chitinases and β-glucanases is a good strategy to suppress plant pests and diseases. *Fusarium* wilt of pigeon pea and soft rot of potato caused by *Fusarium udum* Butler and *Erwinia Carotovora* can be controlled by *Pseudomonas fluorescens* and *sinorhizobium*, both producing chitinase and β-glucanases (Guo et al., 2013; Kumar et al., 2010). *Bacillus* sp. has also been found to inhibit important pathogens such as *Rhizoctonia solani* in tomatoes and *Phytophthora capsici* in pepper (Akgül & Mirik, 2008; Solanki et al., 2012).

Biofertilisers strains produce siderophore, an iron-chelating agent, which limits the available iron in the soil. This indirect competition for iron suppresses pathogens' ability to cause diseases (Solanki et al., 2014). *Bacillus* and *Pseudomona*s produce siderophores that attack Fusarium wilt of potato and maize. Likewise, *Burkholderia cepacia* has been used as a biocontrol of *Fusarium* sp. and *Pythium* sp., which affect mainly maize and wheat yield of SHFs in most parts of sub-Saharan Africa, especially in South Africa (Beneduzi et al., 2012).

2.8.5 Water stress resistance in plants

Drought seasons in South Africa have been one of the major challenges in increasing crop productivity among smallholders (Falkenmark & Rockström, 2008). Biofertilisers, being able to enhance water-stress tolerance in plants, is a viable technology to alleviate this challenge (Dimkpa et al., 2009). The production of cytokinins, auxins, gibberellins and 1-

aminocyclopropane-1-carboxylate (ACC) deaminase have been reported to cause an increase in plant water-stress tolerance (Khalil & El-Noemani, 2015; Mayak et al., 2004). For example, there was an increase in water-stress tolerance of potato plants when inoculated with *Bacillus* sp. that enhanced siderophore production, ACC deaminase activity and phosphate solubilisation (Gururani et al., 2013). Similarly, under drought conditions, AMF make available substantial amounts of ammonium and nitrate to the host plant by using the inorganic nitrogen released from organic sources or by taking up organic nitrogen in amino-acid form (Wu & Xia, 2006). Therefore, biofertiliser technology is important in improving the productivity of smallholders in dry regions and during drought seasons, especially in drought-prone sub-Saharan African countries such as South Africa (Kaushal & Wani, 2016).

2.8.6 Volatile organic compounds

Volatile organic compounds (VOCs) are part of normal metabolic activities of rhizosphere microbes and play an essential role as signals in plant-microbe interactions (Insam & Seewald, 2010; Santoro et al., 2011). Microbial-synthesized VOCs, which include jasmonates, terpenes acetone, isoprene and 3-butanediol can improve crop productivity. VOCs from rhizobacteria have been reported to cause an increase in growth parameters and biosynthesis of essential oil in *Mentha piperita* (peppermint) (Santoro et al., 2011).

2.8.7 Bioremediation

Recently, biofertilisers such as *Rhizobacteria* in consortium with AMF have found use in the clean-up of heavy metal-polluted soils (Khan, 2014; Singh et al., 2011). Similarly, the *Pseudomonas* sp. strain 10-1B has been reported as a viable remediating agent, especially in polycyclic aromatic hydrocarbon (PAH)-contaminated soils (Bello-Akinosho et al., 2015). Biofertilisers such as Cyanobacteria, *Azospirillum, Burkholderia, Bacillus, Enterobacter, Aspergillus and Penicillium* have also been found to be useful in bioremediation (Choudhary & Das, 2010; Jain & Khichi, 2014). The dual functions of some biofertilisers in bioremediation and fertilisation have made them an important technology in agroforestry (Bello-Akinosho et al., 2016). Bioremediation using biofertiliser strains for treating crude oil-contaminated soil has been successfully used in many sub-Saharan African countries, such as Ogoni land in Delta State, Nigeria (Zabbey et al., 2017) and creosote-contaminated soil in South Africa (Atagana, 2004). This highlight the importance of biofertiliser strains in sustainable agriculture.

2.9 Biofertiliser quality standards

Generally, quality standards are specific for different biofertiliser products and each country has adopted a particular standard in its biofertiliser statute, which is relevant for that country (Motsara & Roy, 2008; Roy et al., 2006). Quality is an important factor in the acceptance and use of biofertilisers among SHFs. Quality determines the potential efficiency of the product. When product quality is poor, inoculants cannot perform effectively when used in the field, and farmers' confidence in the technology wanes (Deaker et al., 2011). In most cases, the density of the viable microbial strains in a product is the major parameter that defines biofertiliser quality. However, other parameters such as pH, moisture content, odour and contaminant level should also measure within the acceptable standards (Table 2.4) (Yadav & Chandra, 2014). Many African countries, such as Kenya and Uganda, do not have a complete regulatory framework that defines biofertiliser standards. The majority have a "work in progress" framework, which can be referred to as an ordinary draft (Kenya standard, 2015; Uganda standard, 2014). Where regulatory guidelines are present, it has been made voluntary, such as is the case in South Africa (Lupwayi et al., 2000). The regulatory guidelines need to be legislated and must be compulsory in order to improve quality. Presently, the sub-Saharan African region has under-regulated the influx of biofertiliser products whose true qualities are rarely guaranteed (Simiyu et al., 2013). Therefore, there is a need for stringent national policy and regulatory strategies with effective monitoring management that will drive awareness on quality control, which consequently may lead to the production of quality biofertiliser products (Simiyu et al., 2013).

Table 2.4: Comparison between biofertiliser standards in India and Kenya

INDIAN BIOFERTILISER SPECIFICATIONS					KENYAN BIOFERTILISER SPECIFICATIONS			
Parameter	*Rhizobium*	*Azotobacter*	*Azospirillum*	PSB	*Rhizobium*	*Azotobacter*	*Azospirillum*	PSB
Base	Carrier base	Carrier base	Carrier base	Carrier base	Carrier base	Carrier base >106micron	Carrier lignite/ charcoal > 100 micron	Carrier lignite /charcoal >100micron
Viable cell (CFU)/g	10^7	10^6	10^7	10^8	10^8	10^7	10^7	10^7
Contamination level	Nil at 10^{-5}	Nil at 10^{-5}	Nil at 10^{-5}	Nil at 10^{-6}	Nil at 10^{-5}	Nil at 10^{-5}	Nil at 10^{-4}	Nil at 10^{-4}
Expiry date	6 months	6 months	6 months	6 months	6 months	6months	6months	6months
pH	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	7.0-8.0	6.5-7.5
Particle Size	212 micron	106 micron	106 micron	106 micron	0.15-212 mm	0.15-0212 mm	0.15-0212 mm	0.15-0212 mm
Pathogen	Shall be nil	Shall be nil	Shall be nil	Shall be nil	Shall be nil	Shall be nil	Shall be nil	Shall be nil
Moisture content %	30-40 %	30-40 %	30-40 %	30-40 %	30-40 %	35-40%	35-40 %	35-40 %
Efficiency character	positive nodulation ≥50% increase	N fixation ≥ 5mg/g sucrose	White pellicle in NFB at 10^{-7}	Phosphate mobilising zones ≥12mm at 10^{-5}	Positive nodulation 20mg/g glucose	N-Fixation >10mg/g sucrose / 1mm zone of P solubilisation	30-50% N-fixation and 1mm P-solubilisation zone	P-Solubilising 30-50% and 1mm zone.

Adapted from Kenya standard (2015); NCOF (2011). CFU: colony-forming units, PSB: Phosphate-solubilising bacteria

2.9.1 *Biofertiliser standard specifications*

The legal quality of biofertiliser products is set based on eight major parameters in China and India, and the same parameters apply to commercial biofertilisers sold in African countries such as South Africa and Kenya (Table 2.4). These parameters included the density of viable strains, carbon and moisture content, pH, particle size (solid products), appearance, contamination and shelf life (Malusá & Vassilev, 2014). These basic parameters are outlined below.

Carrier base: Materials such as peat, wood charcoal, lignite, humus or similar materials that favour the growth of inoculants are good carrier materials for solid products. They can be in the form of moist or dry powder, or granules (El-Fattah et al., 2013). However, broth cultures, mineral oil, water and oil-in-water emulsions are used in liquid products except for mycorrhiza, which is made with fine powder or granules, or root biomass carrier bases mixed with growing substrate (Bhattacharyya, 2014; Malusà & Ciesielska, 2014).

pH: The pH standard for most biofertiliser products is in the range 6.5-7.5 except for mycorrhiza and liquid phosphate solubilising biofertiliser (PSB), which is expected to be 6.0-7.5 and 5.0-7.5 respectively (FNCA, 2006). However, other countries may specify different pH standards. For example, the pH for *Azospirillum* products is specified to be 7.0-8.0 for Kenyan biofertiliser products (Kenya standard, 2015).

Viable cell count: The minimum density of viable cells should be 5×10^7 CFU/g or 1×10^8 CFU/ml for all bacterial biofertilisers while at least 100 viable spores per gram of mycorrhizal biofertiliser are required (Yadav & Chandra, 2014). Viable cells can be determined using the plate count technique. This involves serial dilution of the broth culture or carrier and spreading an aliquot on media to obtain viable count after a period of incubation (Lupwayi et al., 2000).

Particle size: The size and strength of a particle determine its dissolution ability. Most biofertiliser carrier materials have small particle sizes and are highly water-soluble. Hence, they dissolve quickly with soil moisture (Motsara & Roy, 2008). Particle-size estimation is an important parameter in determining the quality and potential efficiency of biofertiliser products. The acceptable standard stipulates that carrier base biofertilisers should pass through a 0.150-

0.212 mm IS sieve for bacteria, while for mycorrhizal powder biofertilisers, 90% should pass through a 0.250 mm IS sieve (60 BSS) (Yadav & Chandra, 2014).

Moisture content: The moisture percent by weight of a good biofertiliser product has been stipulated to be 30-40% maximum for bacteria biofertilisers, while for mycorrhizal products the maximum is 8-12%. Excessive drying of biofertiliser is prevented by keeping the package tightly closed after use and stored in a cool and dry place (Kaljeet et al., 2011).

Contamination level: According to biofertiliser standards, there should be no contamination at 10^5 dilutions. The product must be free of foreign elements that can reduce its efficiency. No pathogen should cohabit with the inoculants (NCOF, 2011).

Shelf life: This is the lifespan of the viable microorganisms in the biofertiliser when it is effective and free from deterioration. Though the standards vary from one country to another, the acceptable shelf life of carrier-based biofertilisers is six-months or more. However, it could be as long as two years for a liquid formulation (Brar et al., 2012). This is why liquid biofertiliser products are being developed and preferred over the carrier-based products. Liquid products contain special cell protectants that encourage spore or cyst formation, thereby improving products' shelf life (Ansari et al., 2015; Pindi & Satyanarayana, 2012).

Efficiency character: This is the expected character of the inoculants before being used on the field. Efficiency character of *Rhizobium,* for instance*,* is determined based on the formation of nodules on crops it can support. *Azotobacter* strains must have the capability to fix nitrogen, at least 10 mg of nitrogen per gram of sucrose consumed while the formation of a visible white pellicle in semisolid nitrogen-free bromothymol blue media defines the efficiency character for *Azospirillum* (Ghosh et al., 2001; Hardarson & Broughton, 2003). For PSB, the solubilisation ability should be a minimum of 30% when tested spectrophotometrically. However, a 5 mm minimum solubilisation zone is recommended in prescribed media with at least 3 mm thickness. The efficiency character of mycorrhizal biofertiliser is determined based on its infectivity potential. There should be at least 80 infection points in test roots per gram of mycorrhizal inoculum (Yadav & Chandra, 2014).

2.9.2 *Guidelines for buying and storage of biofertilisers*

Procurement and storage guidelines are critical for obtaining a quality biofertiliser product. The package's physiological properties should be intact and all informative labelling must be readable (El-Fattah et al., 2013). It is important that products be freshly produced, while expired products should not be sold or purchased. The products must be crop specific (e.g. *Rhizobium* and soybean) and should be stored in a cool place, preferably in a refrigerator since high temperatures can activate or damage the inoculants (Muraleedharan et al., 2010). Storage is an important factor in maintaining the stability and quality of biofertiliser products (Pindi & Satyanarayana, 2012). Different storage conditions, such as temperature and humidity, have been found to affect the carrier-material quality and the efficiency of an inoculant. This consequently affects the shelf life of the product. For better quality and longer shelf life, biofertilisers are to be stored at room temperature, except otherwise stated by the manufacturer on the product labels (Rajasekar & Elango, 2011). However, if the product is to be kept for a longer period, for example, 30-90 days before use, it is advisable to store the product in a refrigerator at 4-5 °C (Phiromtan et al., 2013).

2.10 Carrier material properties

Carrier materials allow for easy management, durability, improved shelf life and effectiveness of biofertilisers. They must be cheap, non-toxic, available in abundance and very easy to process with good moisture absorption and pH-buffering capacity. Carriers should have low levels of toxic or heavy metals that are dangerous to microorganisms, humans and the environment (Kaljeet et al., 2011). It is necessary that carrier materials be sterilised to support high numbers and different strains of beneficial microbes for a longer period and to prevent the proliferation of contaminants during storage. This also ensures that pathogens are not transferred to the agricultural field (Bazilah et al., 2011). The most commonly used methods of sterilisation are gamma irradiation and autoclaving methods. The autoclaving method has a lower cost of operation and the ability to produce a pure culture of inoculant. However, gamma irradiation is most suitable because it produces a better quality of final material without any effect on the physical and chemical properties (El-Fattah et al., 2013; Hung et al., 1998). In most cases, the granular form of (0.5-1.5 mm) peat, perlite, talcum powder, charcoal or soil aggregates is generally used as a carrier (Muraleedharan et al., 2010). Other carriers include

sterilised oxalic acid industrial waste, composted sawdust, kaolin, vermiculite, diatoms, wheat bran and sugarcane bagasse (Deepti & Mishra, 2014).

Chapter Three

Methodology

3. Methodology

3.1 Smallholder survey

Descriptive survey design, which involves the collection of data with the aim of answering questions related to the subject of study, was used to investigate the first objective (Appendix 1). This is also concerned with describing, analysing and reporting situations that exist or existed.

3.1.1 Study area

The study area is the Gauteng Province (latitude S25.93; longitude E28.05, Fig. 3.1), South Africa. Although efforts were made to include more provinces, time and budget constraints and other challenges in obtaining approval from the Department of Agriculture and Rural Development to engage smallholder farmers hindered the inclusion of other provinces in this study. This also affected the number of respondents interviewed in this study. However, since the Gauteng province is commercially viable and highly accessible for the introduction of new technologies and knowledge in agriculture, such as biofertiliser technology, it was envisaged that feedback obtained from smallholder farmers in the Gauteng province provided a countrywide estimate on biofertiliser perception and adoption.

According to StatsSA (2011), approximately 2.9 million households are involved in agriculture in South Africa. Among the provinces, the largest percentage of agricultural households were recorded in KwaZulu-Natal, Eastern Cape and Limpopo, with 25%, 21% and 16%, respectively. Gauteng comes after Limpopo with about 10% while the Western Cape (3%) and Northern Cape (2%) had the lowest percentage of agriculture households. Specific activities showed that vegetable production was high amongst agricultural households in Gauteng (13%) compared to all other provinces. In addition, Gauteng dominated in the production of other crops (29%), in all probability due to substantial courtyard cultivation (StatsSA, 2011).

The Province covers about 16 500 km^2 with an average annual rainfall below 800 mm and average annual maximum temperature of 25 °C in the north and 22 °C in the south. The province is about 1 500 m above sea level with an average relative humidity of 85% (Dyson, 2009), and it has a population of over 13 million, making it the most populated province in South Africa.

Gauteng is bordered in the south by the Vaal River, which separates it from the Free State Province. It also borders North-West Province to the west, Limpopo Province to the north and Mpumalanga Province to the east (https://En.Wikipedia.Org/Wiki/Gauteng; StatsSA, 2017).

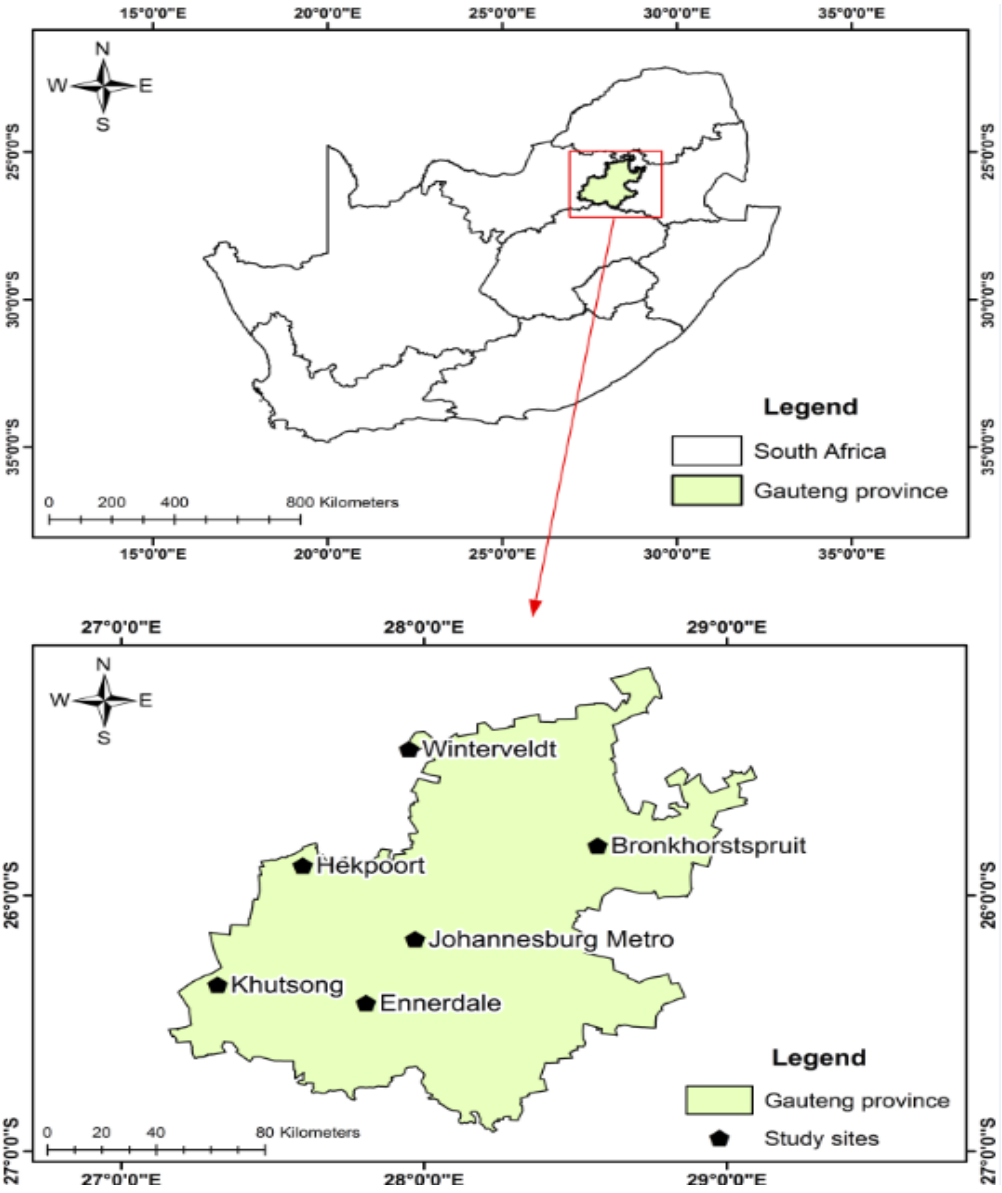

Figure 3.1: Location of the study area showing Gauteng Province and the municipalities.

3.1.2 Sampling procedure

The selection of a specified number of individuals called the 'sample', which is a representative of the target group, is essential in population analysis. This is because the main population is usually too large to be investigated, while the sample is smaller and can be more easily studied.

The sampling frame of this study consisted of SHFs who are involved in crop production. In drawing samples, probability and non-probability methods were used. In probability sampling, members have an equal chance of being chosen, while in non-probability sampling members are non-randomly selected to allow for the deliberate selection of some individuals. Non-probability sampling methods include judgment, convenience, quota and snowball (Kothari, 2004). In this study, the probability method was used to select the municipalities of interest while the non-probability method used for choosing the respondents was convenience sampling, which is based on ease of accessibility to the smallholders. A sample consisting of 67 SHFs was selected (Table 3.1).

Table 3.1: Site location and coordinates

Site	No of respondents	Coordinates
Randfontein Hekpoort	5	(26°10'47"S, 27°42'15"E)
Johannesburg Metro	10	(26°11'50"S, 28°2'31"E)
Winterveldt	24	(25°25'12"S, 27°56'56"E)
Bronkhorstspruit	13	(25°48'18"S, 28°44'47"E)
Khutsong	4	(26°20'1"S, 27°19'39"E)
Ennerdale	11	(26°24'35"S, 27°50'13"E)
Total	67	

3.1.2.1 Collection of data

A well-structured questionnaire was used to collect data. The questionnaire was designed using a series of closed-ended and open-ended questions. Closed-ended questions generate data that can easily be analysed, while open-ended questions allow for suggestions and further expression of the matter in question by the respondents. Data were collected through personal (face-to-face) interviews with the smallholders at their respective farms (Appendix 1).

3.1.2.2 Validity of the instruments

According to Gakuu and Kidombo (2010), validity is the appropriateness, meaningfulness and usefulness of the conclusion on the data obtained from a study. In order to ensure validity, expert judgment of the study supervisors and of statisticians was employed. The instrument was also pretested and problems observed were resolved, before the final questionnaire was formulated.

3.1.3 Ethical consideration

Ethical consideration is essential in order for the researcher to understand the standard of conduct in a particular field of study. It is also there to safeguard the rights of participants by ensuring the confidentiality of obtained information and identity protection, if need be (Artal & Rubenfeld, 2017). Approval to engage the SHFs was obtained from the Gauteng Department: Agriculture and Rural Development (Appendix 2) while UNISA CAES Research Ethics Review Committee granted ethical clearance (Appendix 3). The consent of the SHFs was obtained through a duly completed consent form (Appendix 19).

3.2 Laboratory experiment

Thirteen biofertiliser products that are commercially available in South Africa were analysed in this study. This constituted only the samples that were obtained at the time of sampling. Of the 13 samples, ten were liquid and three were carrier-based products. Codes assigned to the products were CBS and CBL to denote solid and liquid commercial biofertiliser respectively. The products were stored at 4 °C after sterilising the container with 70% (v/v) ethanol.

3.2.1 Physical and physicochemical properties

3.2.1.1 Water holding capacity (WHC)

Sterile distilled water was added to 100 g oven-dried biofertiliser product with continuous stirring until it was saturated. The mixture was allowed to stand for 20-25 min and thereafter filtered using a sieve of 0.05 mm. The quantity of water filtered was measured to determine the amount of water held by the carrier matter (Somasegaran & Hoben, 1994).

$$WHC\ \% = \frac{(amount\ of\ water\ added - amount\ of\ water\ filtered\ )}{(mass\ of\ dried\ weight\ of\ sample)} \times 100$$

3.2.1.2 Determination of pH

Twenty grams of biofertiliser product was added to sterile distilled water in a ratio of 1:3 and agitated to mix on a rotary shaker at 120 rpm for 20 min. The pH of the solution was measured with glass electrode AD1030 (Adwa, Hungary) pH meter.

3.2.1.3 Particle size determination

To measure the particles of carrier-based biofertilisers, sieves of prescribed mesh sizes were used. A 100 g biofertiliser sample was passed through 0.150 and 0.212 mm IS sieve. The entire biofertiliser must pass through the sieve, while for mycorrhizal biofertiliser, at least 90% should pass through 0.250 mm IS sieve (60 BSS) (Yadav & Chandra, 2014).

3.2.1.4 Determination of moisture content

A crucible containing 10 g of solid biofertiliser was placed in an oven at 105 °C for 48 h. The weight was measured intermittently until a constant weight was observed (Somasegaran & Hoben, 1994). The formula below was used to calculate the moisture content.

$$Moisture\ content\ (\%\ by\ weight) = \frac{B - C}{B - A} \times 100$$

$$A = weight\ of\ crucible$$

$$B = weight\ of\ crucible\ and\ biofertiliser\ before\ oven\ dry$$

$$C = weight\ of\ crucible\ and\ biofertiliser\ after\ oven\ dry$$

3.2.1.5 Estimation of electrical conductivity

The electrical conductivity (EC) was measured with a suitable conductivity electrode meter calibrated with 0.01 M potassium chloride. A 5 g biofertiliser mixed with 50 ml of deionised water in a bottle was centrifuged after shaking on a rotary shaker at 150 rpm for 30 min. The standard ratio 1:10 (mass/volume), which has been quite efficient in all sample analysis for compost and fertiliser samples, was used.

3.2.1.6 Storage temperature

Different temperatures were evaluated to ascertain efficient storage temperature for improved shelf life of biofertiliser products. Aliquots of biofertiliser products were stored at three different temperature conditions for three months. The 4 °C temperature condition was maintained in a refrigerator, 25 °C at room temperature, while 36 °C was maintained in an incubator. The temperatures were chosen based on various studies, which have confirmed these storage temperatures as efficient for increasing shelf life during storage (El-Fattah et al., 2013; Phiromtan et al., 2013). The microbial total viable count was determined after a 90-day storage period.

3.2.1.7 Estimation of total micronutrients and heavy metals

A multi-element detecting instrument called inductively coupled plasma optical emission spectrometry (ICP-OES) was used to estimate the macro and microelements in the samples. ICP-OES is a sequential instrument that detects elements almost immediately and simultaneously. Each element is detected and measured at an appropriate emission wavelength chosen for high sensitivity without spectral interferences. The wavelengths used were; magnesium 383.826 nm, calcium 422.673 and 317.933 nm, phosphorus 213.618 nm, potassium 769.896 nm, sodium 589.592 nm, iron 259.940 nm, manganese 257.61 nm, zinc 213.856 nm, aluminium 396.152 nm and copper 324.754 nm. This experiment was conducted at the analytical department of the Soil, Climate and Water business unit of the Agricultural Research Council.

3.2.1.8 Estimation of total nitrogen and carbon

Total carbon and nitrogen were determined on a Carlo Erba NA 1500 C/N/S analyser (Waltham, MA, USA), using an approximately 15 mg air-dried sample, which was weighed in tin-foil containers (Jimenez & Ladha, 1993). The container with the samples was ignited at 1 020 °C in oxygen (on a chromium oxide catalyst) to produce carbon dioxide, dinitrogen and oxides of nitrogen (plus other oxides). The gases produced passed through silvered cobalt oxide (to remove oxides of sulphur and halogens) and a column of copper at 540 °C to reduce the oxides of nitrogen to nitrogen gas (and to remove excess free oxygen). Subsequently, a trap of anhydrous magnesium perchlorate removed water vapour while the final separation of nitrogen gas and carbon dioxide was by gas chromatography using helium carrier gas. The elements were measured with a thermal conductivity detector.

3.2.1.9 Identity character, appearance and odour

Labelling details and manufacturer instructions, where available, were inspected and recorded. The colour and odour of the biofertiliser products were also observed and noted (NCOF, 2011). It was observed that not all the products had complete labelling information. In some cases, manufacture date, expiry date, shelf life, microbial densities or product functions were not stated. The claimed microbial strains in the products were used to categorise the products as Rhizobia, free-living N-fixing, and PGPR products. These products could be made of single or consortium strains. Where the products consisted of two or more of the above group they are referred to as mixed products.

3.2.2 Quantitative analysis of microbial content of biofertilisers

3.2.2.1 Total viable count

The total viable count was estimated by the dilution plate technique (Motsara & Roy, 2008). A tenfold serial dilution was made by adding 10 g of solid or 10 ml of liquid biofertiliser product to 90 ml of saline solution (0.85% (w/v) sodium chloride) in sterile glass bottles. The solution was agitated on a rotary shaker at 150 rpm for 25 min prior to further dilution up to 10^{-9}. Subsequently, 0.1 ml from dilution 10^{-5} to 10^{-9} was spread on different culture media plates in triplicate (Table 3.2). Colonies below 300 were enumerated after incubating for two to five days and microbial density was expressed as colony-forming units (CFU g^{-1} or ml^{-1}).

$$No\ of\ cells\ (CFU\ per\ ml) = \frac{(no\ of\ colonies \times dilution\ factor)}{(volume\ of\ inoculum)}$$

Table 3.2: Media used in microbial isolation

Media	Organisms	Incubation °C	References
Congo red yeast extract Mannitol agar	Rhizobia	30 °C ± 2 °C	Datta et al. (2015)
N-free semi-solid bromothymol blue Nfb	*Azospirillum*	30 °C ± 2 °C	Baldani et al. (2014)
Burks N-free medium	*Azotobacter*	30 °C ± 2 °C	Revillas et al. (2000)
Potato Dextrose Rose Bengal agar	Fungal strains	25 °C ± 2 °C	Rao et al. (2007)
Nutrient agar	*Bacillus, Pseudomonas* and others	36 °C ± 2 °C	Sigma-Aldrich, India

(The chemical composition is in appendices 12, 13, 14 and 15)

3.2.2.2 Most probable number

The most probable number (MPN) technique estimates viable cell numbers based on the likelihood and assumption that a cell or group of cells will show a positive response in a tube (Alexander, 1965; Herbert, 1990). This was used to enumerate the *Azospirillum* in products CBL6 and CBL7. The results are usually estimated using already published tables (Cochran, 1950) or software such as Most Probable Number Enumeration Systems (MPNes, by NifTAL) or an MPN calculator (by the United States Environmental Protection Agency). A 0.1 ml aliquot of dilutions 10^{-5} to 10^{-9} were placed in 25 ml McCartney bottles containing 10 ml of nitrogen-free (Nfb) media and incubated for four days. The experiment was conducted in five replicates.

3.2.3 Molecular analysis of biofertiliser products

3.2.3.1 DNA extraction

A pure colony of an overnight bacterial culture on nutrient agar was suspended in 50 µl of sterile polymerase chain reaction-grade water and heated for 2 min in a microwave (Defy, model DMO351, China) to lyse the cells and release the nucleic materials. Subsequently, the microwaved cell suspension was centrifuged (JP Selecta Centrifuge, Barcelona, Spain) at 10 000 rpm for 1 min and 2 ml of the supernatant of the lysed cell was taken as a template for the polymerase chain reaction (PCR). For fungal isolates, a five-day-old fungal growth on PDA was used for DNA extraction using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc, California, USA), according to the manufacturer's instructions. A 0.25 g or ml of sample was placed into a PowerBead tube containing lysis solution, homogenised and vortexed at maximum speed for 10 min and was centrifuged at 10 000 × g for 30 sec at 25 °C. The supernatant obtained was purified twice by adding an inhibitor removal and centrifuge at 10 000 × g for 60 sec after incubating at 4 °C for 5 min. This is to precipitate non-DNA organic and inorganic materials such as humic substances, cell debris and proteins. Subsequently, the supernatant was mixed with a binding solution in a collection tube, briefly vortexed to mix and the resulting solution was loaded onto a Spin Filter and centrifuge at 10 000 × g for 60 sec. The DNA is selectively bound to the filter membrane in the Spin Filter under the high salt concentration of the binding solution. The bound DNA on the silica filter membrane was further cleaned by the addition of an ethanol-based wash solution and centrifuged at 10 000 × g for 30 sec at room temperature. This solution removes residual salts, humic acids and other contaminants. The Spin Filter is centrifuged again at 10 000 × g for 1 minute at room temperature to remove any residual solution of ethanol wash reagent. The DNA is then released from the silica Spin Filter membrane into a 1.5 ml collection tube with sterile elution buffer by centrifuging at 10 000 × g for 30 sec. The DNA extracted was stored at -20 °C for downstream application.

Similarly, the PowerSoil DNA Isolation Kit was also used to extract total genomic DNA of microorganisms in the biofertiliser products. The extracted DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen, California, USA) and the integrity was verified on 1% (w/v) agarose gel electrophoresis stained with ethidium bromide. The gel was run at 80 V for 45 min.

3.2.3.2 Amplification of bacterial 16S rRNA gene and fungal ITS regions 1 and 2 of isolates.

The V3-V4 region of the 16S rRNA gene of bacterial isolates and the internal transcribed spacer (ITS) regions 1 and 2 of fungal isolates were amplified using universal primers (Table 3.3). Polymerase chain reaction was performed in a T100^{TM} (Bio-Rad, USA) thermal cycler. Each PCR-reaction mixture contained 12.5 µl of one *Taq* 2× Master Mix with Standard Buffer (New England, Biolabs Inc, USA), 2 µl of DNA template and 0.2 µM each of forward and reverse primers and nuclease-free water to a final volume of 25 µl. The thermocycling conditions are as follows: initial denaturation was at 94 ^{o}C for 30 sec, 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 55 ^{o}C for 55 sec and extension at 68 ^{o}C for 60 sec. The final extension was at 68 ^{o}C for 5 min. An aliquot of 2 µl PCR-product was run on a 1% (w/v) agarose gel at 80 V for 45 min to verify the integrity and the size of the PCR amplicons.

Table 3.3: Primers for bacterial 16S rRNA gene and fungal ITS regions 1 and 2 amplification.

Organism	Primers	Primer sequence (5´-3´)	Reference
Bacteria	27F	AGAGTTTGATCCTGGCTCAG	Frank et al. (2008)
(16S rRNA gene)	1492R	TACGGYTACCTTGTTACGACTT	
Fungi	ITS 1 F	TCCGTAGGTGAACCTGCGG	White et al. (1990)
(ITS regions 1 & 2)	ITS 4 R	TCCTCCGCTTATTGATATGC	

3.2.3.3 Sequencing and taxonomic assignment

The PCR amplicon samples were sequenced at Stellenbosch University's Central Analytical Facility, using universal primers (Table 3.3). Sequences obtained were manually inspected, edited and bidirectional sequences were merged using the BioEdit Sequence Alignment Editor to generate contiguous consensus sequences. For the taxonomic assignment, contiguous sequences were matched against available sequences in the National Centre for Biotechnology Information (NCBI) database using the basic logical alignment search tool (BLAST). A phylogenetic tree with relatives above 97% similarity was constructed using MEGA 7.0.25 (Kumar et al., 2016).

3.2.4 Arbuscular mycorrhizal fungal biofertiliser spore count and viability determination

3.2.4.1 Spore count

Arbuscular mycorrhizal fungi spores were extracted using the wet-sieving and decanting method as described by Habte and Osorio (2001). Enough distilled water was added to 10 g biofertiliser product (CBS13) in a beaker and thoroughly agitated for 30 min to release the spores from the dispersed biofertiliser aggregates. The resultant solution was passed through nested sieves with mesh sizes of 0.75, 0.50, 0.25, 0.10 and 0.05 mm arranged in descending order of size. The trapped spores in 0.050-, 0.100- and 0.250-mm sieves were centrifuged at 2 00 × g for 3 min after being suspended in distilled water. The sediment was centrifuged again after being re-suspended in 50 ml of 50% (w/v) sucrose solution. The supernatant fluid was washed with distilled water to release the spores from the sucrose. Subsequently, the spores were transferred to a Petri dish and were examined and counted under a light microscope (Motic BA210, Spain). The results were expressed in the number of counted spores per 10 g of biofertiliser product.

3.2.4.2 Spore viability determination

The procedure of Habte and Osorio (2001) was used in assessing the viability of the AMF spores. Sterilised soil contained in a Petri dish was moistened to its maximum water-holding capacity with a solution of 0.1% (w/v) Trypan blue. This solution increases the visibility of hyphal growth. Pieces of membrane filter about 10 × 10 mm (pore size 0.45 µm) were placed on a nylon mesh of pore size 50 µm positioned on the soil surface. The membrane filters were pre-sterilised in 70% (v/v) alcohol for 5 min and rinsed with distilled water. The Petri dish was covered and incubated in the dark at 20 °C after a spore has been placed on each piece of filter square. After 14 days of incubation, the filter membrane was removed, and the spores examined under a light microscope (Motic BA210, Spain). Viability is indicated by the growth of the spores.

3.2.5 **Analysis of bacteria in biofertiliser products using Illumina MiSeq system**

3.2.5.1 16S rRNA gene library construction

The Illumina MiSeq 16S library preparation guide (Illumina Inc.) was followed in building the gene library. The hypervariable V3-V4 region (approximately 460 base pair) of the 16S rRNA gene was amplified using region-specific primers 341F (5′-CCTACGGGNGGCWGCAG-3′)

and 805R (5′-GACTACHVGGGTATCTAATCC-3′) in a PCR that was performed in a SimpliAmp™ Thermal Cycler (Thermo Fisher, USA). Both the forward and the reverse Illumina overhang adapters (Illumina Inc., California, USA) were clamped to the 5′ end of the primer-pair sequences for compatibility with Illumina index and sequencing adapters. In summary, the library preparation workflow involved a first-stage of PCR amplicon, intended to amplify the target region through a PCR using 2.5 µl DNA template (5 ng/µl in 10 Mm Tris, pH 8.5), 12.5 µl of 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Massachusetts, USA) and 5 µl of each of the forward and reverse primers (1 µM) to a final volume of 25 µl. The thermocycling conditions were an initial denaturation at 95 °C for 3 min, 25 cycles of initial denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec, and a final extension of 72 °C for 5 min.

PCR amplicons were cleaned using Agencourt A MPure XP magnetic beads (Beckman Coulter Inc, CA, USA). After PCR cleanup, the Illumina indexes and sequence adapters (Illumina Inc., CA, USA) were clamped to the amplicon using the Nextera XT Index Kit (Illumina Inc, CA, USA). The indexed PCR products were subjected to another clean-up process and afterwards quantified using a Qubit fluorometer (Qubit 3.0, Life Technologies, Malaysia). The partial 16S rRNA were normalised and denatured in 0.2 N NaOH before loading on the MiSeq V3 reagent cartridge (Illumina Inc., CA, USA). De-multiplexing and downstream analyses of the obtained sequences were performed using MiSeq reporter software (Illumina Inc., CA, USA) after the 2× 300 bp paired-end sequencing run had been executed (Mashiane et al., 2017).

3.2.5.2 NGS processing, operational taxonomic unit assembly and diversity analyses

The quality of obtained sequence reads was checked with FastQC and AfterQC software (version 0.11.5, Babraham Bioinformatics, UK). Following quality check, PANDAseq (Masella et al., 2012) was used for assembling the forward and the reverse reads. Thereafter, merged reads were clustered into operational taxonomic units (OTUs) using the open-reference OUT-picking strategy in QIIME (Caporaso et al., 2010). For the OTU picking, sequences were aligned against the SILVA rRNA database using SILVA 123 QIIME release (Quast et al., 2012) with usearch61. The PyNast aligner was further used to align sequences for phylogenetic tree-building alignment (Caporaso et al., 2010). The generated OTUs were subsampled (rarefied) and then taxonomically summarised with the computation of the alpha (α) and beta (β) diversity

using R software (R Core Team, 2013). Different R packages such as vegan, ape, labdsv, heatmap.plus and gplot were employed in the statistical analyses and plot construction.

3.2.5.3 16S rRNA metagenomics' prediction of community functional profiles

Community functional abilities were predicted using the Tax4Fun package in R. The Tax4Fun package predicts functional profiles of 16S rRNA gene diversity by transforming the SILVA assignment counts to functional profiles in three major steps (Aßhauer et al., 2015). Firstly, the 16S rRNA profile obtained from the SILVA rRNA database alignment is transformed to a taxonomic profile of prokaryotic KEGG organisms, with the aid of a precomputed association matrix (Kanehisa et al., 2013). Subsequently, estimated abundance of KEGG organisms was normalised by the 16S rRNA copy number obtained from the NCBI genome annotations. The prediction of the functional profile of the microbial community was conducted after the precomputed functional profiles of the KEGG organisms has been linearly combined using the normalised taxonomic abundances. The UProC and PAUDA were used for fast computation of microbe-specific and metagenomics functional KEGG Orthology (KO) (Aßhauer et al., 2015).

3.2.6 Biochemical tests

3.2.6.1 Carbohydrate utilisation

The ability of isolates to ferment various carbohydrate sources such as sucrose, glucose and lactose was tested using Triple Sugar Iron (TSI) agar. During carbohydrate digestion, gas or acid may be produced, which lowers the pH of the medium and causes the indicator (phenol red) to change colour to yellow. If the microorganisms do not ferment the sugar, the medium remains red. A black precipitate at the butt of the tube indicates the production of hydrogen sulphide from ferrous ammonium sulfate (Appendix 18).

TSI agar (Appendix 4) slant in a 25 ml McCartney bottles was inoculated with the test bacterial strain at the bottom by stabbing the butt of the tube and then streaking the strain onto slant's surface. The tubes were incubated at 35 °C ± 2 °C for 18-24 h (Singh et al., 2008). An uninoculated TSI agar slant served as a negative control. This is to confirm that the carbohydrate was utilised by the tested bacterial isolates.

3.2.6.2 Citrate utilization test

A citrate test is used to determine the ability of bacteria to utilise inorganic ammonium dihydrogen phosphate and sodium citrate as the sole nitrogen and carbon source, respectively.

When the cell absorbs exogenous citrate, it is metabolised to oxaloacetate and acetate by citrate lyase in the presence of a permease. The oxaloacetate is further broken down into pyruvate and carbon dioxide. This test is commonly used to differentiate the Enterobacteriaceae.

Aseptically prepared slants of Simmons Citrate agar (Appendix 5) in a test tube were inoculated with 18-24 h cultures and incubated at 35 °C. Tubes were monitored for 7 days for slow-growing microbes. The intense prussian blue colour observed indicated a positive result that confirms the production of alkaline carbonates and bicarbonates as by-products of citrate metabolism, raising the pH of the medium to above 7.6 (Appendix 18). A negative reaction results in the retention of the dark-forest green colour of the medium (Faidy & Ali-Shtayeh, 2000).

3.2.6.3 Ammonia production

The production of ammonia by the tested bacterial isolates was determined using Nessler's reagent (Appendix 6). Bacteria growing in a urea-exposed environment may decompose their substrate using urease. The occurrence of this enzyme is confirmed when ammonia is produced from the breakdown of urea. Ammonia production by isolates was tested by adding 0.5 ml of Nessler's reagent to a culture broth of isolates grown in 10 ml of peptone water for 48 to 72 h at 36 °C. The development of brown to yellow colouration after three to five minutes is positive for ammonia production (Appendix 18). Uninoculated peptone water served as the control to confirm ammonia production was by the tested isolates

3.2.6.4 Production of hydrogen cyanide

Bacterial isolates were screened for hydrogen cyanide (HCN) production using the method of Lorck (1948). Some bacteria are able to produce HCN and carbon dioxide from decarboxylation of glycine. The enzyme HCN synthase catalyses this oxidation reaction. HCN is toxic to organisms; however, HCN-producing microbes have developed mechanisms such as cyanide-tolerant respiratory systems and cyanide-detoxification mechanisms that protect them from HCN toxicity (Knowles, 1976). This is why HCN-producing microbes have found use in biocontrol of soil-borne pathogens.

A 0.1 ml bacterial suspension was inoculated on modified nutrient agar (Sigma-Aldrich, India) supplemented with 4.4 g of glycine/l. Whatman filter paper no. 1 (Whatman International Ltd., Maidstone, England) was saturated with a solution of 2% (w/v) sodium carbonate in 0.5% picric

acid solution and placed in the upper lid of the plate. Incubation followed at 30 °C for 4 days after sealing the plates with parafilm to prevent volatilisation. Uninoculated plate containing nutrient agar supplemented with glycine was used as a control. The development of an orange to red colour indicated HCN production.

3.2.6.5 Oxidase test

The oxidase test is based on the oxidation of N, N, N, N-tetramethyl-1, 4-phenylenediamine by oxidase, to produce the coloured compound, indophenol blue. A number of microorganisms are characterised by cytochrome oxidase. The cytochrome oxidase is an iron porphyrin that oxidises reduced cytochrome diatomic carbon and reconverts it to an active form by the transfer of electrons to molecular oxygen (Steel, 1961).

Oxidase activity was detected by making a smear of the tested bacterial isolates on filter paper already moistened with 1% tetramethyl-p-phenylenediamine dihydrochloride. A change of colour to dark purple within 30 sec is positive, while the absence of colour is a negative result.

3.2.6.6 Lipase production

Lipolytic organisms have a lipase that catalyses the hydrolysis of lipids. Lipases are a subclass of esterases and perform significant roles in the digestion, transport and breakdown of dietary lipids in most organisms (Gurung et al., 2013). Lipase production was tested by growing test isolates on Tween 20 agar plate (Appendix 7) and incubation was at 30 °C for 48 h (Ghodsalavi et al., 2013). Bacterial colonies with depositions around their edges showed lipase activity. The ability of bacterial strains to produce lipase was rated -ve = no ability or +ve = ability.

3.2.6.7 Catalase test

During the aerobic breakdown of sugars, some bacteria produce oxidative products such as hydrogen peroxide, which is highly toxic causing cell death upon accumulation. Microbes, which produce catalase, are able to decompose hydrogen peroxide either to nascent oxygen or secondary substrates that have no effect on the organisms. Catalase is only present in viable cultures; therefore, more than 24-hour-old cultures may give false negative results during testing (Hemraj et al., 2013). To test for catalase, a colony of pure bacterial isolate grown on nutrient agar overnight was transferred to a sterile microscopic slide and 2-3 drops of 3% (v/v) hydrogen peroxide was added. A positive result showed a rapid effervescence of oxygen with bubbles while negative results do not evolve bubbles (Appendix 18).

3.2.6.8 Urease test

Urease is an enzyme that catalyses the breakdown of urea to ammonia by attacking the amide link. The ammonia produced increases the pH of the medium. This test is used to distinguish members of the genus *Proteus* from other lactose non-fermenting enteric microbes. Urea agar-based solution was autoclaved at 121 °C for 15 min and cooled to 50 °C. A 50 ml of 40% urea solution, filtered with a 0.45 mm pore-size filter was aseptically added. The urease activity was detected by growing the tested bacterial isolates on the urea agar slants (Appendix 8). The bright-pink colouration is positive, while otherwise, it is a negative result (Faidy & Ali-Shtayeh, 2000).

$$(NH_2)_2CO_{(s)} + 2H_2O_{(l)} \xrightarrow{\text{Urease}} CO_{2(g)} + H_2O_{(l)} + 2NH_{3(g)}$$ Boman and Obreza (2002)

3.2.6.9 Methyl Red and Voges-Proskauer test

Microorganisms ferment glucose to produce stable acids that lower the pH of growth medium and cause the change in colour of indicators. The methyl Red and Voges-Proskauer (MR-VP) is used to identify enteric bacteria based on their pathway of glucose metabolism. Pyruvic acid is initially produced by glucose metabolism while a mixed-acid pathway is used by enteric bacteria to further metabolise pyruvic acid to products such as lactic, formic and acetic acids (Goldman & Green, 2015). The tested isolates were incubated in the MR-VP broth (Appendix 9) for 48 h at 35 °C and 0.2 ml of Methyl Red indicator was added to 5 ml of the broth culture. The formation of a red colour is positive while yellow colouration is a negative reaction. For VP, 0.6 ml of Barritt's reagent A and 0.2 ml of Barrit's reagent B was added (Appendix 10). A red colouration observed at the top of the culture is positive, while a yellow colour is negative (Mcdevitt, 2009).

3.2.7 Functional attributes

3.2.7.1 Indole-3-acetic acid production

The method of Patten and Glick (2002), using Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml 35% (v/v) $HClO_4$ solution) was used for screening isolates for Indole-3-acetic acid (IAA) production. Twenty microliters of cultivated culture were transferred into a sterilised bottle containing 10 ml of Tryptophan broth (Sigma-Aldrich, Canada) and incubated at 30 °C for 48 h with shaking (200 rpm). One and a half millilitres of the culture was placed in 2 ml

microcentrifuge tube and centrifuged (JP Selecta Centrifuge, Barcelona, Spain) at 10 000 rpm for 15 min at 4 °C. One millilitre of the supernatant was placed in a tube containing 2 ml of Salkowski's reagent and was incubated in the dark at room temperature for 25 min. A red to pinkish colouration confirms IAA production. The colour intensity from yellow, pink (pale or deep) to red (dark to reddish) shows no-IAA, low-IAA and high-IAA production, respectively. The absorbance was measured at 540 nm and the concentration was calculated by comparing with the plotted standard curve (Appendix 20 & 21).

3.2.7.2 Phosphate solubilising activities

Isolates' ability to solubilise phosphate was investigated by estimating the halo zone formed on the phosphate growth medium. The National Botanical Research Institute's phosphate medium (NBRIP) supplemented with 15 g Bacto-agar (Difco Laboratories, Detroit, MI, USA) containing insoluble tricalcium phosphate was used as the single phosphorous source (Appendix 11). Triplicate wells of known diameter were bored with a sterile glass Pasteur pipette on each agar plate. The plates were incubated at 30 °C for 14 days after placing 0.01 ml of pure culture in each well. The clear halo zone around the bacteria colonies signifies the ability to solubilise phosphate. The inorganic phosphate (Pi) solubilisation index was calculated by the formula below (Nautiyal, 1999).

$$Pi\ Solubilisation\ index\ (PSI) = \frac{diameter\ of\ halo\ zone + well\ (mm)}{diameter\ of\ well\ (mm)}$$

3.2.7.3 Acid phosphatase assay

This method is based on the principle of the quantity of ρ-nitrophenol released when isolates are incubated with ρ-nitrophenyl phosphate (ρNPP) (Tabatabai & Bremner, 1969). The reagents included modified universal buffer (MUB) stock solution and buffer, ρ-Nitrophenyl phosphate solution (ρNPP) (Sigma-Aldrich, India), calcium chloride solution, sodium hydroxide solution and ρ-nitrophenol standard solution (Appendix 17).

A 0.5 ml of culture supernatant in NBRIP broth was added to a McCartney bottle containing 4 ml of MUB working solution and 1 ml of 0.05 M ρ-nitrophenyl phosphate solution. The bottle was tightly closed, vortexed to mix and incubated at 37 °C for 1 h. One millilitre of 0.5 M calcium chloride and 4 ml of 0.5 M sodium hydroxide was added and mixed thoroughly before

filtering through a folded Whatman filter paper no 2v. (Whatman International Ltd., Maidstone, England). A control containing culture supernatant was made the same way but the 1 ml ρNPP was added after adding calcium chloride and sodium hydroxide. The absorbance of the yellow-coloured filtrate was measured at 405 nm after calibration (Appendix 22) and the concentration was calculated from the standard curve (Appendix 23) (Behera et al., 2017).

3.2.7.4 Nitrogen-fixing potential

The ability of isolates to fix nitrogen was examined on a nitrogen-free media (Burk's medium) (Appendix 12) and nitrogen-free bromothymol blue medium supplemented with 15 g agar (Appendix 13). Pure isolates were streaked on already prepared sterile Burk's medium and incubated at 30 °C for 24 to 48 h. Bacteria that grew in the medium were categorised as positive (+) for growth and negative (-) if no growth was observed.

3.2.7.5 Siderophore production

The method described by Louden et al. (2011), which employs the use of chrome azurol S (CAS) blue agar and hexadecyltrimethylammonium bromide (HDTMA) as indicators, was used to evaluate siderophore production. A pure isolate was spot inoculated onto the blue agar and incubated at 30 °C for 24-72 h. A yellow to orange halo zone around the colonies is a positive result for siderophore production. Steps involved in CAS agar preparation are described in Appendix 16.

3.2.8 Statistical analysis

Descriptive statistics such as frequencies and percentages were generated for the analysis of the survey experiment and the results were presented in the form of standard tables. The scores were subjected to a one-to-one frequency table and a chi-Square test (χ^2) was performed to test for equal proportions. Contingency RxC frequency tables were performed for the association between farmers' characteristics such as gender or age groups and tested parameters such as biofertiliser awareness and institutional factors (Snedecor & Cochran, 1967). Chi-Square (χ^2) tests were performed for the association. For the laboratory experiments, mean and standard deviations were computed for replicate measurements.

Chapter Four

Results

4. Results

The descriptive statistical analyses of the general characteristics of the respondents as well as the results of the laboratory experiments are presented in this chapter. The characteristics of SHFs such as age, gender, level of education and farming experience as well as types of fertilisers applied, probably influencing awareness and adoption of biofertiliser, were presented using frequencies and percentages.

4.1 Characteristics of smallholder farmers

4.1.1 Gender, age and level of education

Sixty per cent of the respondents interviewed were females while 40% were males. The majority of the respondents, about 69%, were in the age group 50 years and above. Approximately 22% of the respondents were between the age range 40-49 years while the youthful age groups, 20-29 years and 30-39 years, accounted for the lowest percentages, at 3% and 6%, respectively. The mean age of SHFs was found to be 50 years (Table 4.1). The results revealed that many of the smallholders are not very literate, with 43% having primary education only and 11% having no formal education. However, 15% of the farmers had some tertiary education (diploma, degree and above) while 31% had only secondary education. The chi-square test of independence showed a close proportion of males and females were not aware of biofertiliser, with no significant difference in the proportion, χ^2 (1, N = 67) = 0.0633, $p_{0.05}$ = 0.8013. Similarly, the differences in the proportion of biofertiliser knowledge amongst the farmers' age groups were not significant χ^2 (4, N = 67) = 4.7827, $p_{0.05}$ = 0.3103.

4.1.2 Farming experience, application and types of fertiliser

There was a low level of farming experience among the interviewed respondents. Forty-five per cent (45%) of the respondents had less than five years of experience while only 17% had more than 15 years of cultivation experience. Nineteen per cent of respondents had between 5-10 years and 10-15 years of experience. The results also revealed that about 84% of the respondents applied fertilisers for cultivation while 16% did not use any fertiliser. While 37% of the respondents were reported to be using organic fertilisers, 24% were using inorganic

fertilisers. Another 24% of the respondents used a combination of organic and inorganic fertilisers. However, none of the respondents used biofertiliser.

Table 4.1: Gender, age, educational level, farming experience and fertiliser application among smallholder farmers in Gauteng Province.

Variable	Frequency	Percentage	Chi square value	Probability $p = 0.05$
Gender				
Male	27	40		
Female	40	60	2.5224	0.1122
Age group (years)				
20-29	3	3		
30-39	4	6		
40-49	14	22	Mean = 50	
50- above	46	69	100.6866	< 0.0001
Educational qualification				
No- formal education	7	11		
Primary (Grade 7) education	29	43		
Secondary (Grade 12) education	21	31		
Tertiary education (diploma, degree above)	10	15	18.4328	0.0004
Farming experience (years)				
Below 5 years	30	45		
5 -10 years	13	19		
10-15 years	13	19	14.1343	0.0027
15- above years	11	17		
Application of fertilisers				
Yes	56	84		
No	11	16	30.2239	< 0.0001
Types of fertilisers used				
Inorganic fertiliser	16	24		
Organic fertiliser	25	37		
Combination	16	24		
Biofertiliser	0	0		
None	10	15	6.8507	0.0768

Source: Field Work, November 2017

4.2 Challenges in increasing crop productivity and awareness of biofertiliser

4.2.1 Reasons for using fertilisers and crop-productivity challenges

Respondents had put forth many reasons for using fertilisers. Sixty-one per cent (61%) of respondents emphasised yield increase as the major reason for using fertilisers, while 31% and 6% indicated soil-nutrient improvement and crop health respectively as their reasons. About 2% did not state any reason for using fertilisers. In addition, 43% of respondents reported their major challenge in crop production as plant pests and diseases, whereas 30% of the respondents indicated crop yield increase and 12% indicated soil nutrient (nitrogen, phosphorous, and potassium) deficiency. However, 25% reported all of these challenges as issues affecting their productivity while 4.5% reported no challenges (Table 4.2).

4.2.2 Biofertiliser knowledge and application, and product awareness

Biofertiliser knowledge was very poor amongst the respondents interviewed, with over 95% not having any knowledge of biofertiliser and its application. Only about 5% of the respondents claimed to have some knowledge of biofertiliser and its application. In addition, about 97% of the respondents did not have any idea on seasonal usage of biofertiliser products (Table 4.2).

Awareness of biofertiliser was further assessed by asking respondents to choose products they had come across in the past amongst the listed commercial biofertiliser products in the questionnaire (Appendix 1). About 97% of the respondents had not come across any of the listed biofertiliser products. However, a respondent stated a brand of biofertiliser, Amgrow, when asked to list any other commercial products not listed in Appendix 1.

Table 4.2: Reasons for using fertilisers, challenges in farming and, biofertiliser knowledge and awareness among smallholder farmers in Gauteng Province.

Variable	Frequency	percentage	Chi square value	Probability $p = 0.05$
Reasons for using fertilisers				
Yield increase	41	61		
Soil nutrient improvement	21	31		
Crop health improvement	4	6		
No reason	1	2	60.7015	< 0.0001
Challenges in farming				
Nitrogen supply	3	4.5		
Phosphorous supply	4	6		
Potassium supply	1	1.5		
Crop yield increase	10	15		
Plant pest and disease incidence	29	43.3		
All of the above	17	25.2		
None	3	4.5	65.1642	< 0.0001
Biofertiliser knowledge				
Yes	3	4.5		
No	64	95.5	55.5373	< 0.0001
How often do you use biofertiliser				
Every season	-			
Every other season	-			
When necessary	2	3		
Not at all	65	97	59.2388	< 0.0001
Biofertiliser product awareness				
Yes	2	3		
No	65	97	59.2388	< 0.0001

Source: Field Work, November 2017

4.3 Types of crop cultivated

Major crops cultivated by the respondents included spinach, onion, cabbage, beetroot, tomato, maize, pumpkin, beans, potatoes, lettuce and carrot (Table 4.3).

Table 4.3: Major crops grown by smallholder farmers in selected municipalities in Gauteng Province.

Crops	Frequency	Percentage (%)
Spinach	47	70
Onion	24	36
Cabbage	23	34
Beetroot	22	33
Tomato	21	31
Vegetable (others)	21	31
Maize	16	24
Pumpkin	16	24
Bean	15	22
Potato	14	21
Lettuce	11	16
Carrot	10	15

Source: Field Work, November 2017

4.4 Individual perception and institutional support

When respondents were asked to rate their knowledge on biofertiliser, about 90% reported their knowledge to be poor, 7% said they do not know, while 3% described their knowledge as being fair. Similarly, over 95% of the respondents had no perception of the quality of biofertiliser nor the performance of biofertiliser with respect to other fertilisers. This was expected as almost all the respondents did not know what biofertiliser is (Table 4.4).

Extension services on biofertiliser were reported by 36% of respondents to be poor while 16% and 15% agreed that they are good and excellent respectively. Some respondents (18%) agreed that extension services are fair while another 15% responded: “don’t know”. About 76% of respondents stated that accessibility of biofertiliser is poor while 22% indicated “don’t know”.

Furthermore, more than 75% of the respondents interviewed considered the support and policies of government on biofertiliser to be poor. However, 4% indicated that the government support was fair while 18% indicated they do not know (Table 4.4).

In addition, the majority of respondents suggested training and on-field trials would improve their knowledge and, possibly, the adoption of biofertiliser. It was also proposed that product samples should be given to farmers for use before they can commit to a purchase. Respondents also recommended that extension programmes and financial support be intensified to promote awareness and the use of biofertiliser.

Table 4.4: Individual perception of and institutional support for biofertiliser application.

Questions	QA		QB		QC		QD		QE		QF	
Response	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Poor	60	90,0	2	3,0	1	1,5	24	36,0	51	76,0	50	75,0
Fair	2	3,0	1	1,5	-	-	12	18,0	-	-	3	4,0
Average	-	-	-	-	1	1,5	-	-	1	2,0	-	-
Good	-	-	-	-	-	-	11	16,0	-	-	1	1,5
Excellent	-	-	-	-	1	1,5	10	15,0	-	-	1	1,5
Don't know	5	7,0	64	95,5	64	95,5	10	15,0	15	22,0	12	18,0

Question keys

QA: How would you describe your knowledge about biofertiliser?

QB: Overall, what is your perception of the quality of biofertilisers you know of?

QC: How would you rate the performance of biofertiliser with respect to other fertilisers?

QD: How would you rate extension services on biofertilisers?

QE: Describe the accessibility of commercial biofertiliser products

QF: Rate the support/policy of the government on biofertilisers

4.5 Physicochemical properties of biofertiliser products

4.5.1 Total carbon and nitrogen

Total carbon and nitrogen were measured in percentage of the total sample weight. The results showed that samples CB5S and CB2S had a high carbon content of 33.6% and 23.9%, respectively. Other samples, CB1S, CB10S, CB3S and CB4S, had low carbon content while the lowest carbon content, below 1% was found in samples CB13S, CB9L and CB11L. For the total nitrogen, samples CB5S (2.13%), CB10L (0.87%) and CB2S (0.64%) had relatively high nitrogen content. Other samples with a total nitrogen content below 0.2% were CB9L, CB11L and CB13S (Fig. 4.1).

The ratio of carbon to nitrogen content (C/N) of the products showed that samples CB13S, CB2S CB1L and CB5S had high C/N ratio of 42, 37, 25 and 16, respectively. Samples CB3L and CB4L had fairly average ratios of approximately 7%. However, other samples had a very low C/N ratio below 4.

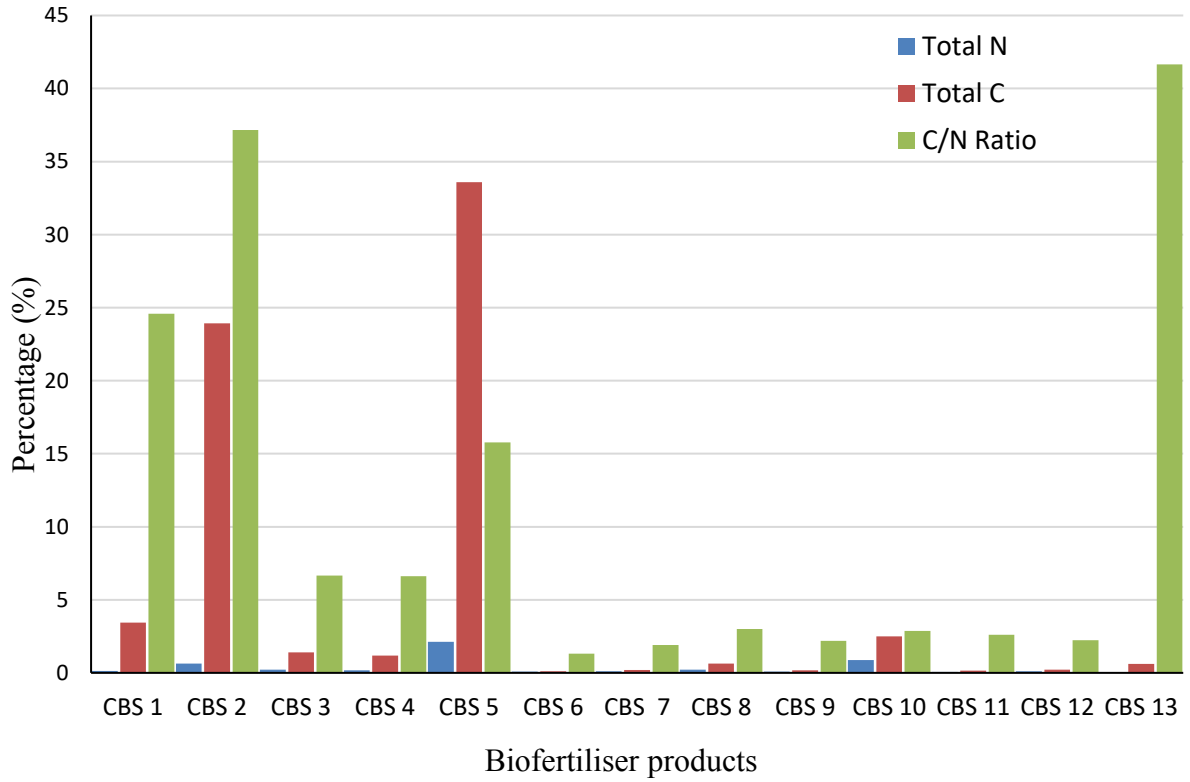

Figure 4.1: Total carbon and nitrogen in biofertiliser products.

4.5.2 Electrical conductivity

The electrical conductivity (EC) results showed that samples CB10L, CB1L, CB8L and CB5S had high EC of 1 234, 1 076, 1 052 and 923 mS/m, respectively. Other samples with relatively high EC above 600 mS/m included CB7L, CB9S, CB3S and CB6L. Samples CB13S, CB2S and CB12L had the lowest EC below 220 mS/m (Table 4.8).

4.5.3 Total micronutrients and heavy metals in biofertiliser products

The results of the analysis of macro and micronutrients showed that the element with the highest quantity was potassium with 5 128 mg/kg in product CB1L (Table 4.5). Similarly, at 965.6 and 849 mg/kg, respectively sulphur and calcium were high in CB3L. Another important element, phosphorous was present in relatively high amounts in CB2S, CB5S and CB13S. Micronutrients such as copper, manganese, boron and zinc occurred at lower amounts below 23 mg/kg, except manganese which had 68.4 mg/kg in CB13S and 41.8 mg/kg in CB5S. The heavy metals analysed included mercury, arsenic, cadmium, lead and cobalt. The results showed that cobalt occurred in high quantities amongst other metals. Product CB13S had 4.26 mg/kg of cobalt while the maximum amount of arsenic was observed in CB2S and CB5S with an amount less than 6 mg/kg. Overall, the quantities of each of the heavy metals were less than 20 mg/kg in the biofertiliser products.

Table 4.5: Metal content of biofertiliser products and the maximum levels of potentially harmful element permitted in fertiliser products

Metals (mg/kg)	Standard (mg/kg)	CB1L	CB2S	CB3L	CB4L	CB5S	CB6L	CB7L	CB8L	CB9L	CB10L	CB11L	CB12L	CB13S
Total C%	na	3.44	23.9	1.40	1.19	33.6	0.119	0.19	0.63	0.18	2.49	0.16	0.23	0.61
C/N Ratio	na	30.3	37.2	6.7	6.6	15.8	1.4	1.9	3.1	2.3	2.9	2.9	2.2	41.7
Total N%	na	0.11	0.64	0.21	0.18	2.13	0.087	0.10	0.21	0.08	0.87	0.06	0.10	0.02
P	na	37.5	426	206	164	292	17.3	10.3	38.9	13.5	142.2	9.20	27.8	275
K	na	5128	0.71	1272	838	1.22	47.5	38.0	138.7	49.0	447	24.8	76.0	4.98
Ca	na	598	19.3	849	60.38	22.6	3.85	3.04	6.95	4.04	24.30	3.07	4.41	1.98
Mg	na	88.2	5.12	205	31.7	2.4	1.87	0.92	4.69	1.29	14.64	0.799	1.86	0.862
Na	na	34.78	527	435.4	426.4	180	4.79	7.16	4.09	4.21	3.85	5.19	6.56	63
Fe	na	8.92	178	40.71	45.78	3045	13.53	11.38	5.47	5.50	5.43	6.48	11.51	243
S	na	503.9	1.03	965.6	118.3	4.93	20.29	16.43	36.81	17.13	154.85	7.31	14.80	0.530
Cu	750	0.39	3.84	1.50	0.28	6.5	0.30	0.11	0.15	0.08	21.06	0.093	0.13	7.58
Mn	na	9.69	35.9	3.90	1.14	41.8	0.033	0.038	0.058	0.066	0.176	0.069	0.030	68.4
Zn	2750	1.03	15.3	2.95	2.93	22.7	0.65	0.39	0.79	0.46	13.46	0.39	0.52	28.8
B	80	6.32	7.98	2.24	0.39	13.70	0.39	0.54	0.33	0.54	0.57	0.43	0.76	7.99
Hg	10	<0.4	<7.0	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<7.0
As	15	<0.4	<6.0	<0.4	<0.4	3.3	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<6.0
Cd	20	<0.06	<1.0	<0.06	<0.06	0.12	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<1.0
Pb	400	<1.3	<20	<1.3	<1.3	0.75	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<20
Co	100	<0.2	<3	<0.2	<0.2	0.90	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	4.26

*The permissible standards for the heavy metals in fertilisers was obtained from SAFL (1977), na = not applicable

4.5.4 pH of biofertiliser products

A neutral pH is essential for the growth and survival of inoculant; hence, biofertiliser products must not be highly acidic or basic. The pH results showed that products CB3L, CB4L and CB12L were acidic while product CB9L was basic (Fig. 4.2). However, other biofertiliser products fall within the acceptable pH standard of 6.0-7.5.

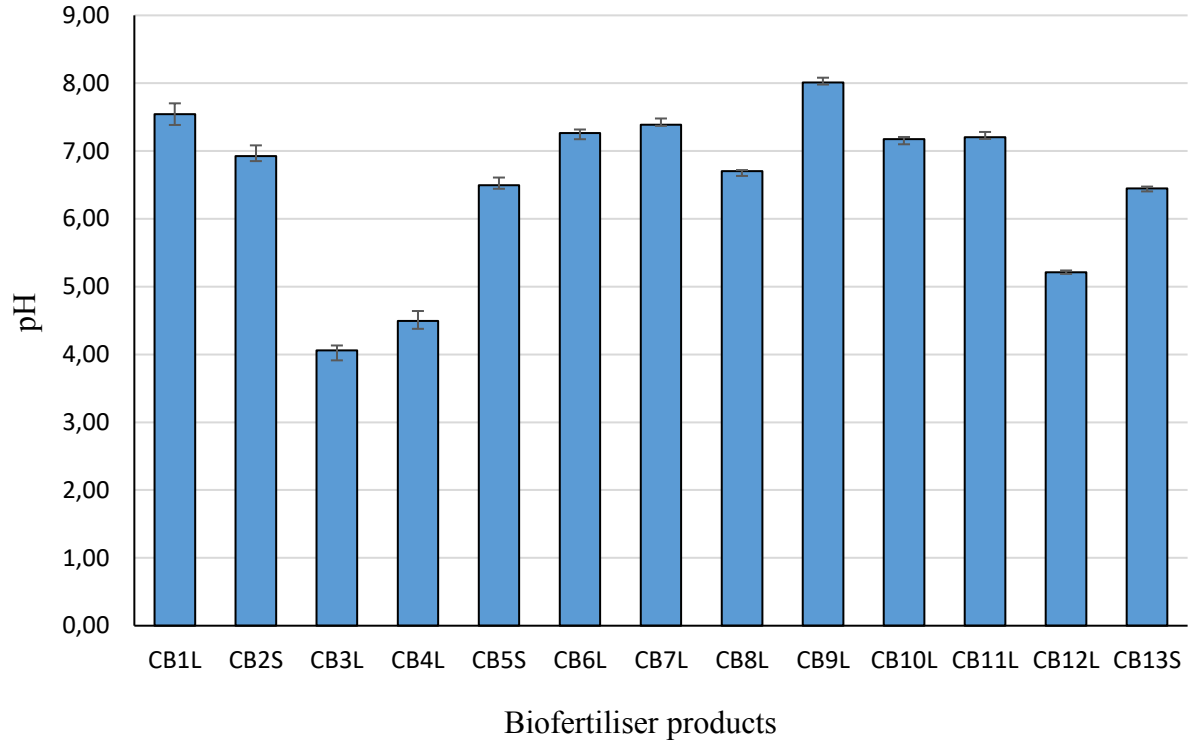

Figure 4.2: pH readings of biofertiliser products showing error bars representing standard deviation (n=3).

4.5.5 Particle sizes, water holding capacity and moisture content

The particle sizes, water-holding capacity and moisture content of the carrier-based biofertiliser products (CB2S, CB5S and CB13S) were investigated. The results showed that all the particles of samples CBS2 and CBS5 passed through a sieve of mesh size 0.15 mm whereas sample CB13S had 85% of its particles pass through 0.25 mm mesh size. In addition, the water-holding capacity and moisture content were high for products CB2S and CB5S but very low for product CB13S (Table 4.6).

Table 4.6: Particle sizes, water-holding capacity and moisture content of biofertilisers

Samples	Particle sizes	WHC(%) ±SD	MC(%) ±SD
CB2S	< 0.150 mm	68 ± 0.5	62 ± 0.5
CB5S	<0.150 mm	54 ± 0.5	48 ± –
CB13S	85% passed <0.212 mm	20 ± 0.9	1 ± –

4.5.6 Storage temperature

The results of the microbial count of products after three months of incubation under different temperatures showed that microbial densities varied for different temperature conditions (Table 4.7). In samples CB5S, CB7L, CB8L and CB11L, the higher the storage temperature, the higher the microbial population density. At room temperature, the cell counts were lower in samples CB1L and CB2S and higher in CB3L, CB4L, CB6L, CB9L, CB10L and CB11L when compared to the other storage temperatures. All the products maintained optimum viable cell count above 10^8 $CFUg^{-1}$. In general, the total viable cell count for the different temperature conditions increased after the three months of storage time for all biofertiliser products but was more stable for CB5S and CB2S which are carrier-based products.

Table 4.7: Microbial count at different storage temperatures

Sample Code	@ 4 °C × 10^9 ±SD	@ 25 °C × 10^9 ±SD	@ 36 °C × 10^9 ±SD
CB1L	5.17 ± 8.39	8.23 ± 16.20	7.07 ± 9.02
CB2S	5.40 ± 4.36	6.93 ± 10.02	5.70 ± 25.24
CB3L	3.87 ± 3.06	10.10 ± 4.93	7.87 ± 2.52
CB4L	4.40 ± 4.58	7.00 ± 12.77	4.93 ± 1.53
CB5S	4.20 ± 3.61	5.37 ± 10.12	5.53 ± 6.11
CB6L	3.50 ± 2.65	5.47 ± 9.45	5.00 ± 10.0
CB7L	7.73 ± 5.69	9.57 ± 12.58	9.73 ± 9.45
CB8L	3.37 ± 5.51	4.83 ± 7.37	5.47 ± 5.86
CB9L	6.30 ± 4.58	7.97 ± 8.62	5.67 ± 5.86
CB10L	4.87 ± 5.03	7.70 ± 2.68	5.90 ± 16.70
CB11L	7.43 ± 6.66	8.53 ± 5.69	10.40 ± 2.52
CB12L	6.13 ± 2.52	8.87 ± 9.02	6.77 ± 7.09
CB13L	ND	ND	ND

*ND - not determined

Table 4.8: Characteristic of commercial biofertiliser products.

Parameters	Unit / Code	CB1L	CB2S	CB3L	CB4L	CB5S	CB6L	CB7L	CB8L	CB9L	CB10L	CB11L	CB12L	CB13S
pH @ 25 °C		7.54	6.92	4.06	4.49	6.49	7.27	7.39	6.70	8.01	7.21	7.20	5.21	6.45
Product Type	Solid/liquid	L	S	L	L	S	L	L	L	L	L	L	L	S
Moisture Content	%	na	62%	na	na	48%	na	na	na	na	na	na	na	1.0%
Shelf life	months	Ns	Ns	Ns	Ns	Ns	3	3	3	3	3	3	3	Ns
Manuf/Expiry date		Ns / Ns	St / Ns	Ns / Ns	Ns / Ns	St / St	Ns / Ns	Ns / Ns	Ns / Ns	Ns / Ns	Ns / Ns	Ns / Ns	Ns / Ns	St / Ns
Physical appearance	Colour	black	black	black	milky	black	brown	brown	brown	brown	brown	brown	brown	brown
	odour	+ve	-ve	++ve	+ve	-ve	++ve	++ve	++ve	++ve	++ve	++ve	++ve	-ve
Composition		Cs	SS	Cs	Cs	SS	Cs	Cs	SS	Ss	Cs	Cs	SS	SS
Microorganisms		bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	AMF
Particle size	<220 µm sieve	na	Passed	na	na	Passed	na	na	na	na	na	na	na	passed
EC	mS/m	1076	303	736	499	923	655	814	1052	798	1234	319	221	311

L= liquid, S= solid, Ns= not stated, Cs= consortium of strains, Ss= single strain, na= not applicable, -ve= odourless, +ve= odour, ++ve= pungent odour

4.6 Biochemical properties and functional attributes of the tested bacterial isolates

4.6.1 Biochemical properties

The biochemical test results showed that most of the isolates obtained could utilise various carbohydrates (Table 4.9). About 82% of the test isolates were able to ferment glucose and 69% could ferment both lactose and sucrose whereas only 3% produced hydrogen sulphide from the ferrous ammonium sulfate in the medium. In addition, about 44% of the tested isolates were positive for citrate utilisation and oxidase test while 80% were positive for ammonia production. Hydrogen cyanide and lipase production, as well as methyl Red and Voges-Proskauer tests, were positive in over 21% of the isolates. Most of the isolates were catalase positive while approximately 52% were able to produce urease, an enzyme that catalyses the breakdown of urea to ammonia.

Table 4.9: Biochemical characterisation of tested presumptive isolates.

Test	NH_3	HCN	Oxidase	Lipase	Catalase	Urease	MR	VP	H_2S	Glucose	Lactose	Sucrose	Citrate
O2	-	-	-	-	+	-	-	-	-	+	+	+	-
O3	+	-	-	-	+	+	-	-	-	+	+	+	+
O4	+	-	+	-	+	+	-	-	-	+	+	+	-
O5	+	-	+	+	+	-	-	-	-	+	+	+	+
O7	+	-	+	+	+	+	-	-	-	-	-	-	-
O10	+	-	+	+	+	+	-	-	-	+	+	+	+
O12	-	-	+	+	+	+	-	-	-	+	+	+	+
O14	+	-	-	-	+	-	-	-	-	+	+	+	-
O15	+	-	+	-	-	-	-	-	+	+	+	+	+
O17	+	-	-	-	+	+	-	-	-	+	+	+	+
O18	+	-	+	-	+	+	-	-	-	-	-	-	-
O19	+	-	-	-	+	-	-	-	-	-	-	-	+
O23	-	-	+	-	+	-	-	-	-	+	+	+	-
RO3	+	-	+	-	+	-	+	+	-	-	-	-	-
SO	-	-	+	-	+	-	+	-	-	-	-	-	+
TO	+	-	+	-	+	+	-	-	-	-	-	-	-
TOF	-	-	+	-	+	+	-	-		-	-	-	-
NB1	+	+	+	-	+	-	-	-	-	+	+	+	+
NB2	+	-	-	+	+	+	-	+	-	+	+	+	+
NB4	+	+	+	-	+	-	-	+	-	+	-	-	-
RNB1	+	-	+	+	+	+	-	-	-	+	+	+	+
ANP1	+	+	+	+	+	-	+	-	-	+	+	+	-
ANP3	+	-	+	+	+	+	-	-	-	+	+	+	+
AN1	+	-	-	-	-	+	-	-	-	+	+	+	-
AN2	+	+	-	-	+	+	-	-	-	-	-	-	-
AA	+	-	+	+	+	+	-	-	-	+	-	-	+
SF2	+	-	-	-	-	-	-	-	-	+	+	+	-
SF3	+	-	-	-	+	-	+	-	-	+	+	+	-
NS1	+	+	+	+	+	-	-	-	-	-	-	-	-
NS3	+	+	-	+	-	+	-	-	-	+	+	+	-
BN	-	-	+		+	-	-	-	-	+	-	-	+
NP1	+	-	+	+	+	-	-	-	-	+	+	+	-
NP2	+	-	-	-	+	-	-	+	-	+	-	-	+
NP3	+	+	-	-	-	+	+	-	-	+	+	+	-
NP4	-	+	+	-	+	+	-	-	-	-	-	-	+
CP1	+	-	-	-	+	+	+	-	-	+	+	+	+
CP3	+	+	-	-	+	+	+	-	-	+	-	-	-
VQ2	-	-	-	-	+	+	-	-	-	+	+	+	-
VQ3	-	+	-	-	-	-	-	+	-	+	+	+	+
VQ4	+	+	-	-	+	-	-	+	-	+	+	+	-
LV	+	-	+	+	+	-	-	-	-	+	+	+	-
SV1	-	-	+	-	+	+	-	-		-	-	-	-
SV2	+	-	+	+	+	+	-	-	-	+	+	+	+
NT1	+	-	-	-	+	-	-	-	-	+	-	-	-
NT3	+	-	-	-	+	+	-	-	-	+	+	+	+
NT4	+	-	-	-	+	-	+	+	-	+	+	+	+
NT5	+	-	-	-	+	-	-	-	-	+	+	+	-
NT6	+	+	-	-	+	+	+	+	-	+	-	-	-
RN1	+	-	+		+	+	-	+	+	+	+	+	+
RN2	+	-	-	+	-	-	+	-	-	+	+	+	-
LF2	+	-	+	-	+	+	-	+	-	+	+	+	-
LF4	+	+	-	-	+	-	+	+	-	+	+	+	-
LF5	+	+	-	-	-	+	-	+	+	+	+	+	+
BC1	-	-	-	-	+	-	-	+	-	+	+	+	+
BC3	-	-	-	-	+	+	-	-	-	+	+	+	+
BC5	+	-	-	-	+	+	-	-	-	+	-	-	+
BC7	+	-	-	+	-	-	-	-	-	+	+	+	-
HS2	+	-	-	-	+	-	+	-	-	+	+	+	-
HS3	+	-	-	-	+	+	-	-	-	+	+	+	+
HS4	+	+	-	-	+	-	+	-	-	+	+	+	-
HS5	+	-	-	-	+	+	-	-	-	+	+	+	-

4.6.2 Indole-3-acetic acid production

The results of indole-3-acetic acid (IAA) production amongst isolates showed that approximately 87% of the isolates tested positive with isolate BC3 from sample CB13L producing the highest amount of IAA, about 115.4 µg/ml while isolate NP2 from sample CB9L produced a relatively smaller quantity, approximately 1.1 µg/ml. The average concentration of IAA produced in each product is presented in Fig. 4.3. Products CB3L and CB13S had the highest concentration of IAA produced and the lowest concentration was produced in CB9.

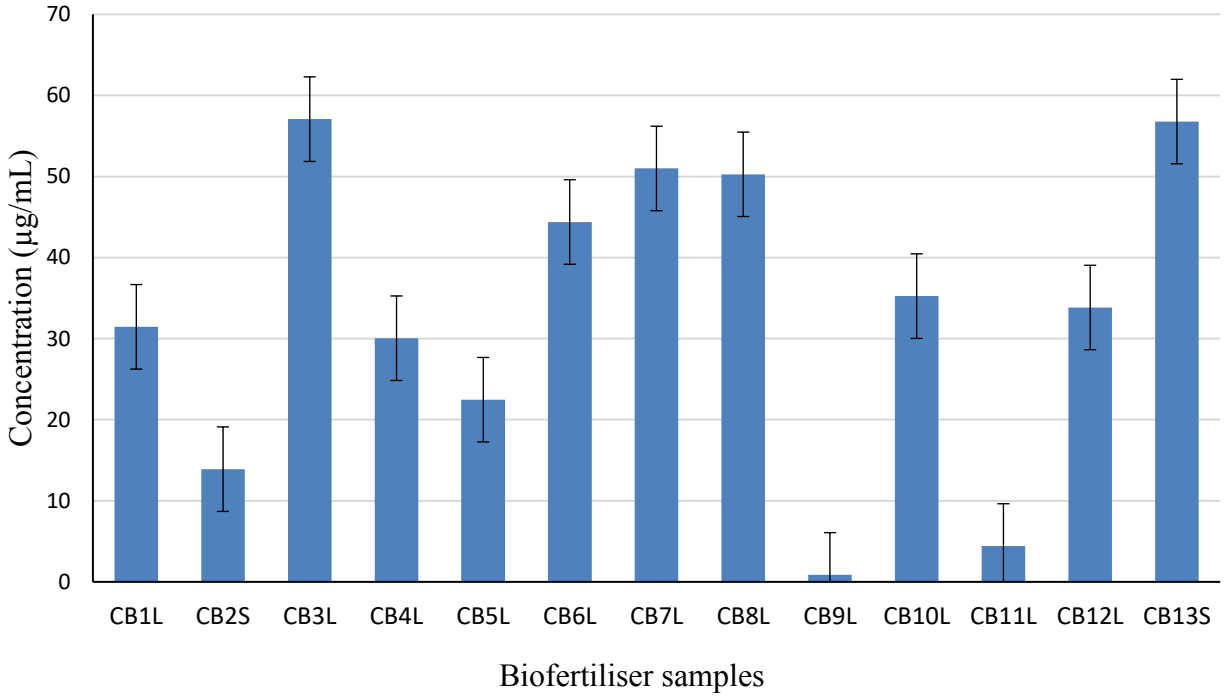

Figure 4.3: Indole acetic acid production in biofertiliser products

4.6.3 Phosphate solubilising ability

The inorganic phosphate solubilisation ability depicted by the calculated phosphate solubilisation index (PSI) varied among the different isolates (Table 4.10). Of all the isolates tested, approximately 60% exhibited phosphate-solubilisation ability as indicated by the halo-zone formation (Fig. 4.4). The halo zone ranges from an average minimum of 1.5 mm occurring in isolate NT5 to a maximum of 18.3 mm in strain NB4. Some of the isolates did not solubilise inorganic phosphate and they included O10, O19, LF3, HS6, VQ1, VQ2 and NS2.

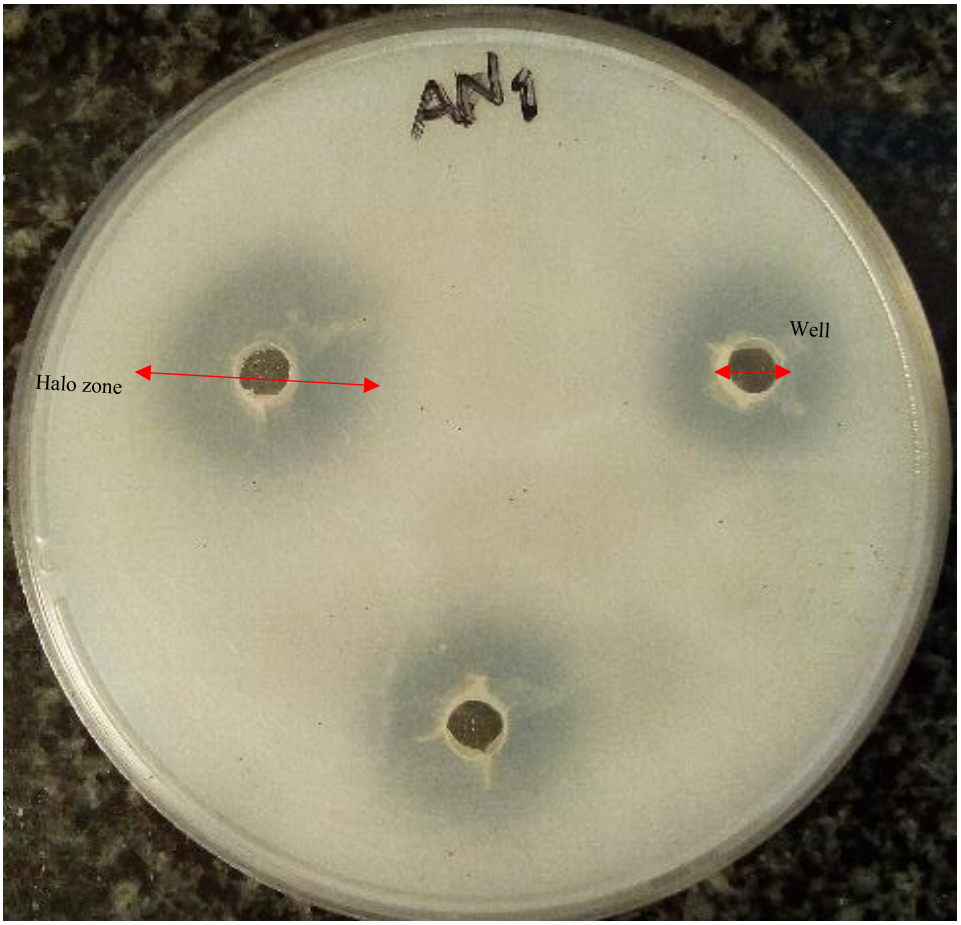

Figure 4.4: Phosphates solubilisation on NBRIP agar showing halo zone formation.

Table 4.10: Phosphate-solubilisation index (PSI) and siderophore production of isolates.

Isolate ID	PSI ± SD	Siderophore (mm) ± SD
AN1	4,07 ± 0.8	-
ANP1	3,07 ± 0.1	47,33 ± 6.11
BC1	-	16,67 ± 1.53
BC3	1,67 ± 0.3	-
BC5	1,80 ± –	15,67 ± 0.58
BC7	-	16,33 ± 1.53
BN	1,93 ± –	-
CP1	4,13 ± 0.3	16,00 ± 2.00
CP3	3,00 ± 0.1	14,00 ± 1.00
HS2	1,47 ± 0.1	16,67 ± 3.21
HS3	-	16,33 ± 1.53
HS5	-	16,00 ± 2.00
LF2	1,40 ± –	27,67 ± 2.52
LF4	2,00 ± 0.3	-
LF5	-	24,67 ± 2.52
LV	1,93 ± –	-
NB1	3,93 ± 0.1	28,33 ± 2.08
NB2	4,00 ± 0.3	16,33 ± 2.52
NB4	4,67 ± 0.8	48,00 ± 2.65
NP1	-	-
NP2	3,67 ± 0.1	51,67 ± 4.04
NP3	4,27 ± 0.1	-
NP4	-	-
NS1	3,60 ± 0.3	56,33 ± 3.79
NS3	-	30,67 ± 3.06
NT3	1,47 ± –	-
NT4	1,33 ± 0.1	-
NT5	1,20 ± 0.1	-
NT6	1,67 ± 0.1	-
O12	2.87 ± 0.1	-
O15	1,40 ± –	-
O17	1,33 ± 0.1	-
O18	1,47 ± 0.1	25,67 ± 2.08
O2	1,87 ± 0.1	25,00 ± 2.65
O23	1,47 ± 0.1	-
O3	1,67 ± 0.3	-
O5	1,47 ± 0.1	59,33 ± 3.79
O7	1,67 ± 0.0	50,33 ± 3.51
RN1	2,40 ± 0.1	-
RNB1	4,40 ± 0.1	-
SF2	3,47 ± 0.6	-
SF3	3,20 ± 0.7	21,67 ± 2.52
VQ3	1,40 ± –	57,67 ± 4.04
VQ4	1,40 ± –	59,00 ± 2.65

4.6.4 Acid phosphatase assay

Isolates that solubilise phosphate on NBRIP agar medium were further screened for acid-phosphatase production. Some of the isolates produced acid phosphatase, with the highest enzyme production observed in isolates from samples CB11L (36.96 µg/ml), followed by CB8L (33.75 µg/ml) and CB6L (31.38 µg/ml), while the least enzyme production was observed in isolates from samples CB5L and CB13S with 7.33 and 7.74 µg/ml, respectively (Fig. 4.5).

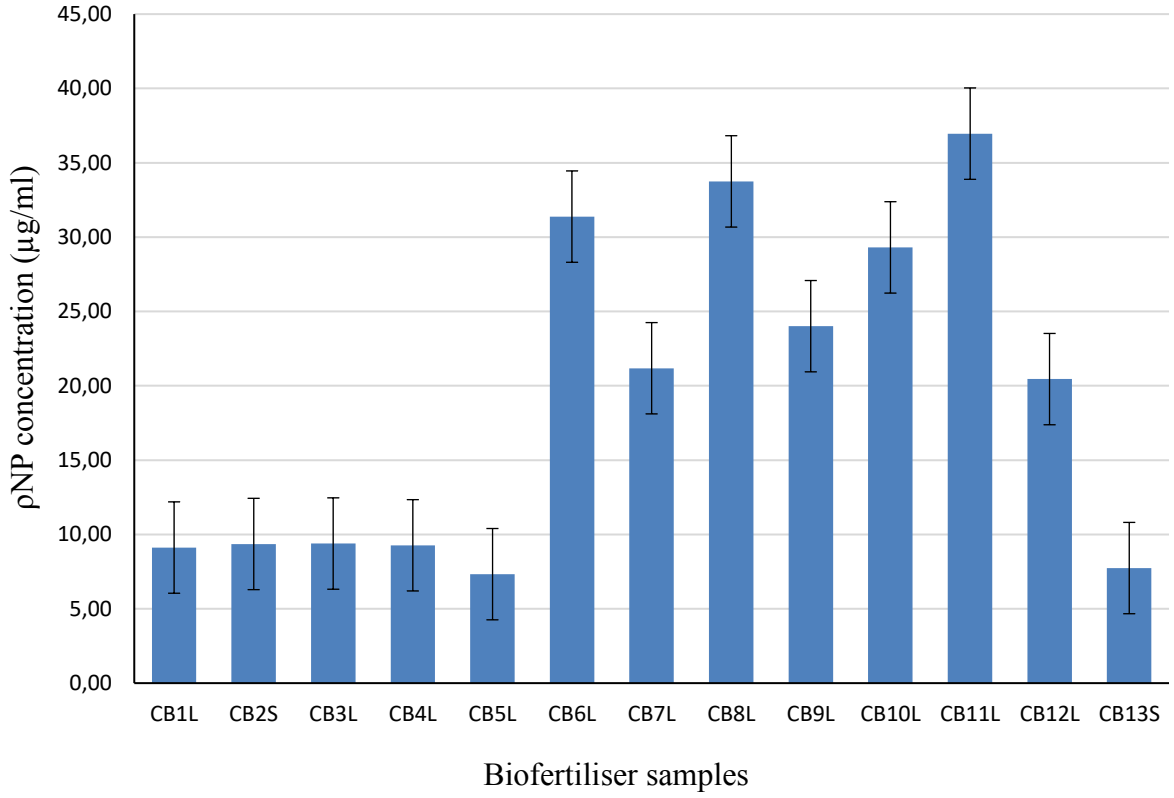

Figure 4.5: Acid phosphatase production from the biofertiliser products.

4.6.5 Nitrogen-fixing potential

The results of the nitrogen-fixing potential of isolates showed that 41% of bacterial isolates grew on Burk's nitrogen-free medium, while 38% grew on Nfb medium (supplemented with 15 g agar). The Nfb medium confirmed the nitrogen-fixing ability of isolates by the change in colour from green to blue due to ammonia production (Fig. 4.6). Sample CB12L had the highest percentage of isolates that can fix nitrogen, while CB1L had the lowest percentage (Fig. 4.7).

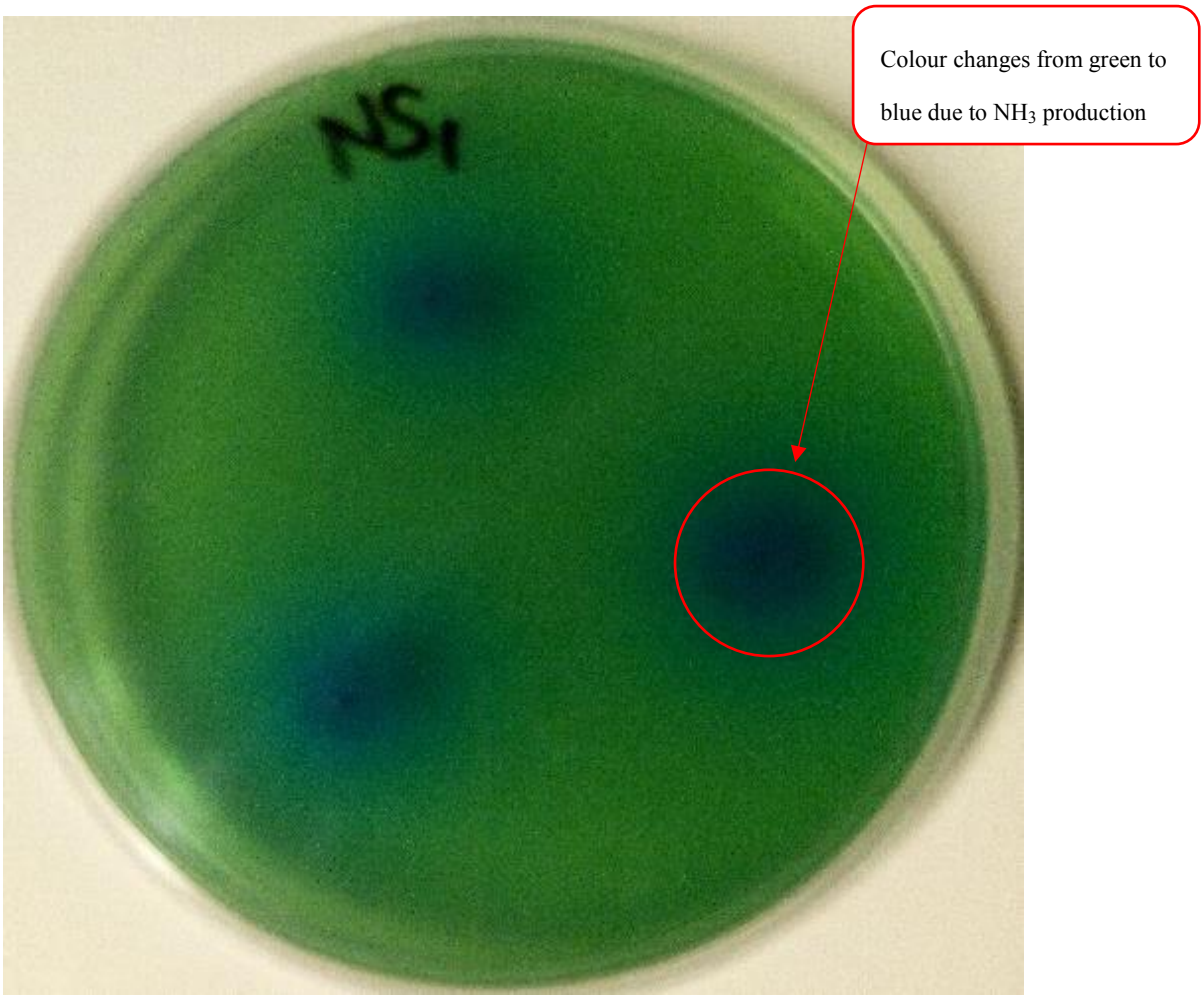

Figure 4.6: Ammonia production by isolates on nitrogen-free bromothymol blue agar.

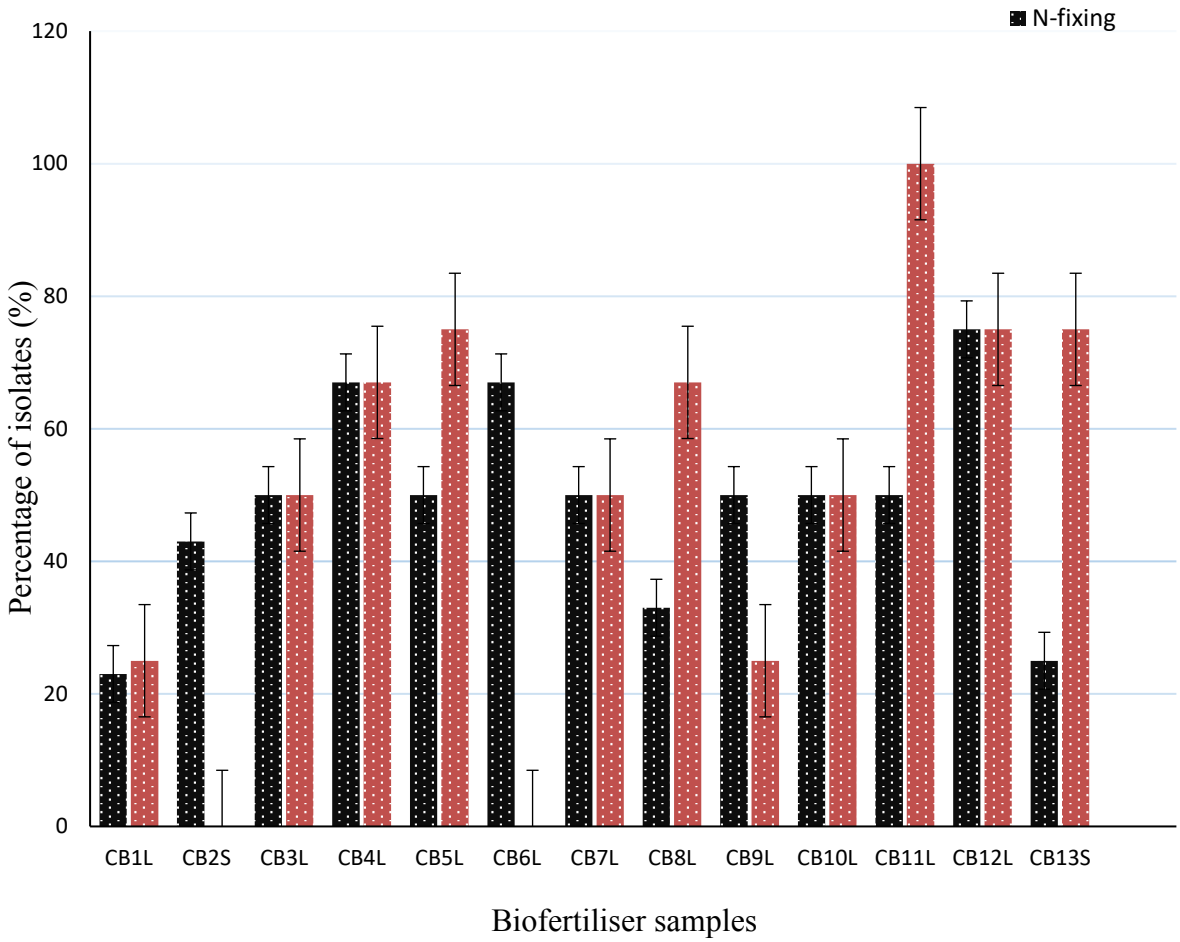

Figure 4.7: Nitrogen fixation and siderophore production potential in biofertiliser products

4.6.6 Siderophore production

Approximately 40% of the tested isolates were able to produce siderophore. Isolates that formed yellow to orange halos around the colonies were considered positive for siderophore production (Fig. 4.8). All the isolates in sample CB11L had the ability to produce the iron-chelating agent, siderophore, while only 25% of isolates in samples CB1L and CB9L produced siderophore. However, sample CB2S had no siderophore producing isolates (Fig. 4.7).

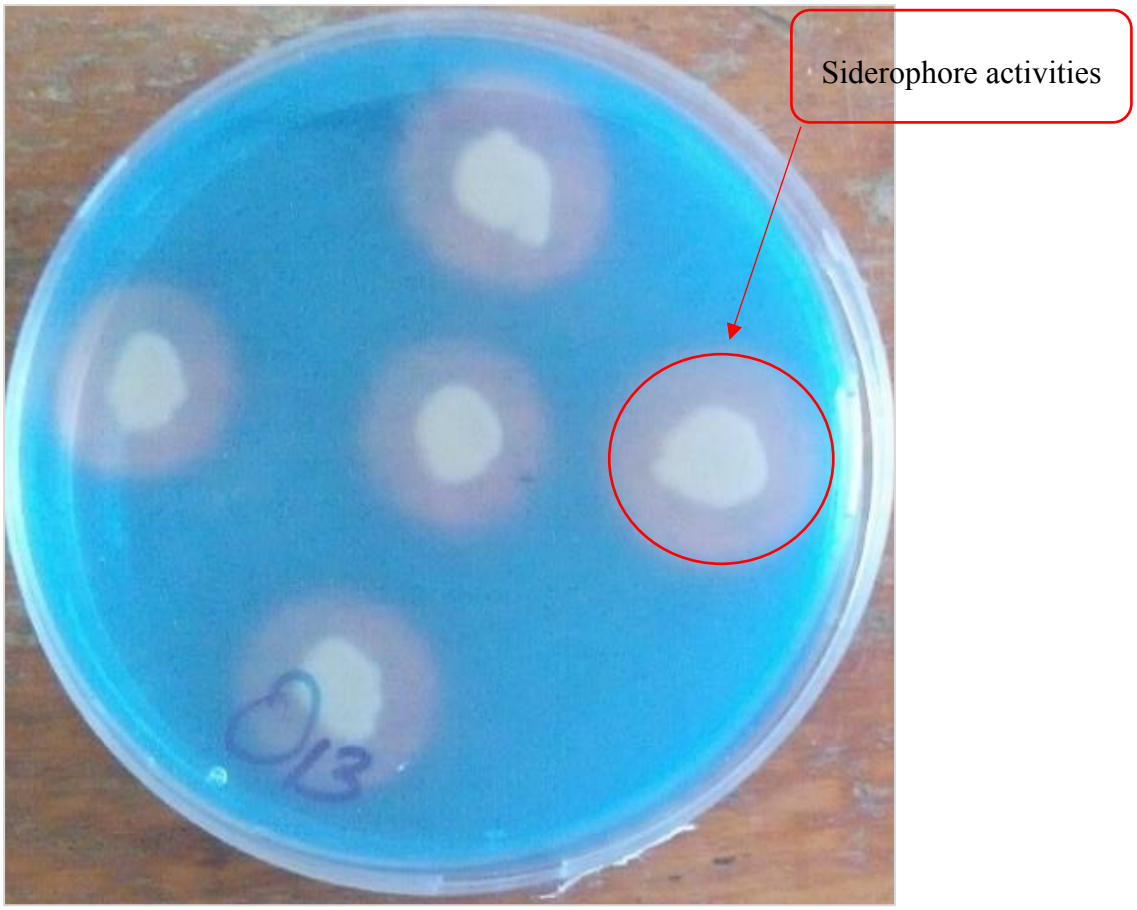

Figure 4.8: CAS agar plate showing halos zones indicating siderophore production.

4.7 Culture-dependent microbial identification

4.7.1 Microbial isolation, and Sanger sequencing of the 16S rRNA gene and ITS regions 1 and 2.

A total of 58 bacterial and three fungal isolates were obtained from all the culture media types. The partial 16S rRNA gene sequences of bacterial isolates were clustered into 28 OTUs while the ITS regions 1 and 2 sequences of fungal isolates were clustered into two OTUs. The phylogenetic association of sequences from the isolates with close relatives in the GenBank were depicted in the phylogenetic tree (Fig. 4.9). Some of the closest matches in the GenBank included *Bacillus subtilis, Acinetobacter junii, Pseudomonas japonica, Brevibacillus laterosporus, Alcaligenes aquatilis* and *Enterococcus ratti,* while the fungal strains are *Aspergillus fumigatus* and *Candida ethanolica* (Table 4.12).

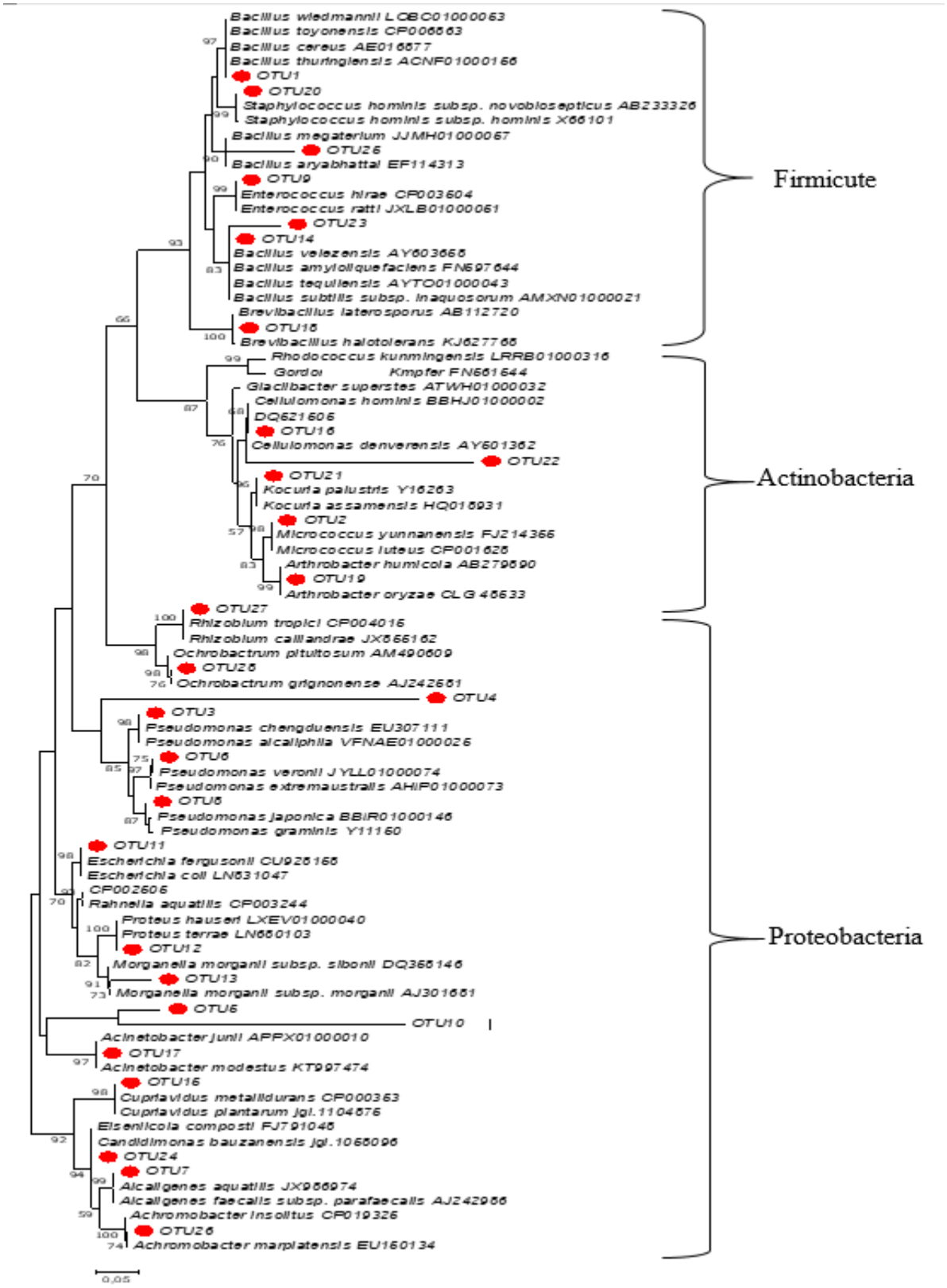

Figure 4.9: Phylogenetic tree of 16S rRNA gene sequences with their closest relative sequences. The maximum likelihood method based on the Tamura 3-parameter model was used

to infer the evolutionary history of the sequences. The analysis involved 84 nucleotide and evolutionary analyses were conducted in MEGA7.

4.7.2 Biofertiliser product quality and level of contamination using Sanger sequences

In this study, isolated strains not reported by the manufacturer but present in the products were considered contaminants. The Sanger sequences obtained were used to analyse the microbial component of biofertiliser products and the nucleotide sequences showed different target bacteria and levels of contamination. On this basis, the products were categorised as high, medium, low or of poor quality (Fig. 4.10).

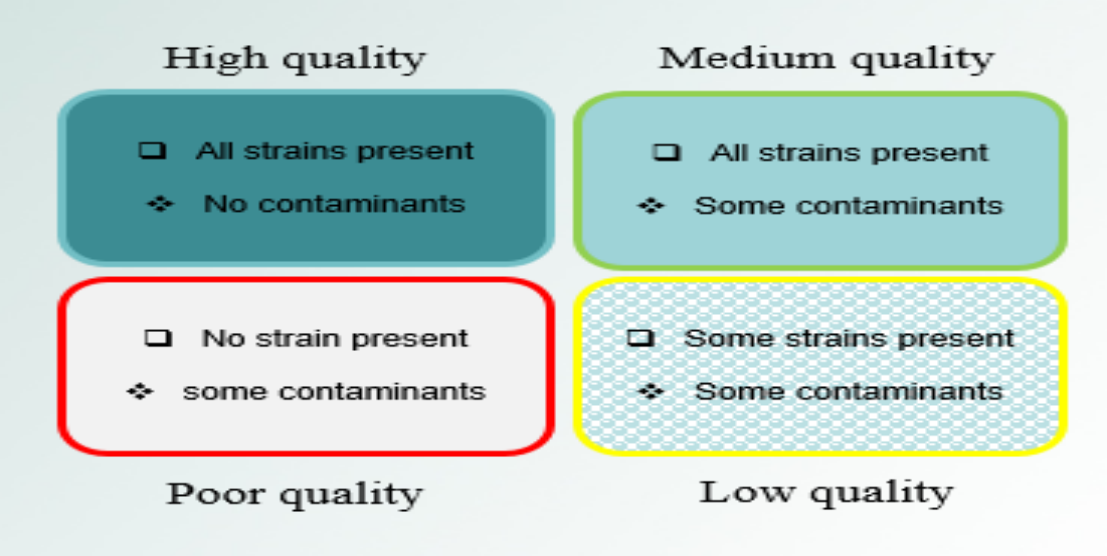

Figure 4.10: Biofertiliser quality as determined by microbial strains and level of contamination.

All the biofertiliser products analysed had more strains than claimed by the manufacturers, implying high levels of contamination (Table 4.11). Generally, products CB1L, CB2S, CB3L and CB13S had higher levels of contaminants when compared to other products. Products CB1L and CB3L had 12 and 5 contaminants, respectively. Based on the claimed strains and the obtained isolates, none of the products qualifies as high- or medium-quality products. However, five of the products were categorised as low-quality products and these included 25% of rhizobia, 40% of PGPR and the mixed-strains products. Eight products were of poor quality. These products included all the free-living nitrogen-fixing products, 75% of rhizobia and 60% of PGPR products.

Table 4.11: Quality categories of biofertiliser products using Sanger sequences

Category	Rhizobia		Free N-fixing		PGPR		Mixed product*		Total	
	Frequency	(%)	Frequency	(%)	Frequency	(%)	Frequency	(%)	Frequency	(%)
High quality product	0	-	-	-	-	-	-	-	-	
Medium quality product	-	-	-	-	-	-	-	-	-	
Low-quality product	1	25	-	-	2	40	2	100	5	38
Poor-quality product	3	75	2	100	3	60	-	-	8	62
Total	4	100	2	100	5	100	2	100	13	100
Less than expected strains	-	-	-	-	-	-	-	-	-	
Same as expected strains	-	-	-	-	-	-	-	-	-	
More than expected strains	4	100	2	100	5	100	2	100	13	100
Total	4	100	2	100	5	100	2	100	13	100

*Mixed products (PGPRs and either rhizobia or free-living nitrogen-fixing), F- frequency.

4.7.2 Total viable count

The total viable count (TVC) showed that samples CB1L, CB2S, CB3L and CB4L had one or more of the claimed microbial strains while other samples had none of the claimed microbial strains, therefore had no TVC (Table 4.12). This observation included the result of the MPN count, which showed that the products tested had no *Azospirillum* strains in them contrary to the manufacturers' claim. Sample CB4L had a TVC of 3.6×10^9 $CFUg^{-1}$. On the other hand, samples CB1L, CB2S and CB3L had TVCs of 1.53×10^7 $CFUml^{-1}$, 5.4×10^7 $CFUg^{-1}$ and 1.68×10^8 $CFUml^{-1}$, respectively.

The results of the spore count for AMF showed that 194 spores were obtained per gram of biofertiliser product, while the viability test revealed that 152 spores germinated (developed a germ tube). This is within the acceptable standard for AMF viable spore count, which is stipulated to be at least 100 viable spores per gram of biofertiliser.

Table 4.12: Microbial community of biofertiliser products obtained from Illumina and Sanger sequences

Sample code	Claimed microbial strains	No	NGS obtained sequences (OTU)	No	Sanger sequences	No	Viable cell count
CB1L (Mixed)	*Enterobacter, Stenotromonas, Bacillus, Rhizobium, Pseudomonas, Trichoderma,*	30	*Pseudomonas*, Devosia, Chryseobacterium, Pertimonas, Pigmentiphaga, Proteiniphilum, Pusillimonas, Dysgonomonas, Salinihabitans, Flavobacterium, Paucimonas Sphigomonas, Methylocystis, Thioalkalispira, Parvibaculum, Pelagibacterium*	15	*Bacillus wiedmannii*, Pseudomonas* xanthomarina, P. oleovorans* subsp. *Lubricantis, P. chengduensis P. alcaliphila*, P. seleniipraecipitans, Micrococcus yunnanensis, Rahnella aquatilis, Lysinibacillus fusiformis, L. mangiferihumi, Micrococcus aloeverae, Candidimonas bauzanensis, Achromobacter marplatensi, Candida ethanolica*	12	1.53×10^7
CB2S Rhizobia	*Rhizobium tropica*	1	*Rhizobium*, Cellulosimicrobium, Nocardioides, Promicromonospora*	3	*Rhizobium tropici*, Bacillus velezensis, Lysinibacillus fusiformis, Acinetobacter juni Cellulomonas denverensis, C. pakistanensis, ,*	5	5.40×10^7
CB3L (Mixed)	*Bacillus thuringiensis, B. subtilis Lactobacillus* sp., *Trichoderma harzianum, Saccharomyces cerevisiae, Pseudomonas florescense, Azotobacter chrococcum*	7	*Lactobacillus*, Nosocomiicoccus, Pediococcus*	2	*Bacillus velezensis*, B. siamensis Cupriavidus metallidurans, , Bacillus megaterium*, Aspergillus fumigatus, Candida ethanolica*	4	1.68×10^8
CB4L (PGPR)	*Bacillus* sp.	1	*Bacillus*, Brevibacillus, Lactobacillus, Stenotromonas*	4	*Acinetobacter junii, Bacillus* paralicheniformis, Bacillus velezensis*	3	3.69×10^9

CB5S (Rhizobia)	*Bradyrhizobium japonicum*	1	*Bradyrhizobium**, *Nosocomiicoccus*	1	*Brevibacillus laterosporus, Arthrobacter oryzae, Staphylococcus hominis* subsp. *novobiosepticus, Kocuria palustris*	4	Nil
CB6S (N-free living)	*Azospirillum brasilense, Azospirillum lipoferum*	2	*Macellibacteroides Alcaligenes, Pseudomonas, Hafnia, Clostridium sensu stricto* 1, 12 & 5, *Proteus, Dysgonomonas, Microvirgula, Morganella*	11	*Enterococcus ratti, Alcaligenes faecalis* subsp. *faecalis,*	2	Nil
CB7S (N-free living)	*Azospirillum brasilense, Azospirillum lipoferum, Azotobacter chrococcum*	3	*Microvirgula, Clostridium sensu stricto* 1, 12, 2 & 5, *Proteus, Dysgonomonas, Macellibacteroides, Morganella Phascolarctobacterium, Alcaligenes, Ruminiclostridium* 5, *Pseudomonas,*	13	*Enterococcus ratti, Pseudomonas gessardii*	2	Nil
CB8S (Rhizobia)	*Bradyrhizobium japonicum*	1	*Alcaligenes, Pseudomonas, Enterobacter, Enterococcus Proteus, Lactobacillus, Lactococcus, Microvirgula,*	8	*Pseudomonas japonica, Proteus hauser, Ochrobactrum pituitosum*	3	Nil
CB9L (PGPR)	*Pseudomonas florescense*	1	*Alcaligenes, Clostridium sensu stricto* 1, 12, 2 & 5, *Dysgonomonas, Macellibacteroides, Microvirgula, Morganella, Enterobacter, Ruminiclostridium, Hafnia, Escherichia-Shigella, Desulfovibrio, Proteus*	15	*Enterococcus ratti, Hafnia paralvei, Alcaligenes faecalis* subsp. *faecalis*	3	Nil

CB10L (PGPR)	*Brevibacillus laterosporous, Paenibacillus chitinolyticus, Sporolactobacillus laevolacticus, Lysinibacillus sphaericus,*	4	*Citrobacter, Clostridium sensu stricto* 2 & 5, *Desulfovibrio, Kluyvera, Dysgonomonas, Escherichia-Shigella, Proteus, Enterobacter, Macellibacteroides, Morganella, Microvirgula Phascolartctobacterium, Ruminiclostridium*	14	*Enterococcus rattii, Escherichia coli,*	2	Nil
CBS11 (PGPR)	*Bacillus* sp.	1	*Cronobacter , Clostridium sensu stricto* 1&5, *Alcaligenes, Pantoea, Ruminiclostridium* 5, *Microvirgula, Dysgonomonas, Enterobacter, Tyzzerella, Escherichia-Shigella, Citrobacter, Morganella, Desulfovibrio, Desulfovibrio Proteus, Macellibacteroides*	16	*Morganella morganii* subsp. *sibonii, Citrobacter werkmanii*	2	Nil
CBS12 (Rhizobia)	*Rhizobium phaseolus*	1	*Enterobacter, Ewingella, Providencia, Morganella, Proteus, Pseudomonas, Serratia, Rahnella, Hafnia,*	9	*Pseudomonas japonica, P. veronii, Bacillus tequilensi, Alcaligenes faecalis* subsp. *parafaecalis*	4	Nil
CBS13 (PGPB)	*Rhizophagus clarus, Gigaspora gigantea, Funneliformis mosseae, Claroideoglomus etunicatum, Paraglomus occulum* (AMF)	5	*Amycolatopsis, Arthrobacter, Nocardioides, Marininema Pseudonocardia, Atopostipes, Fictibacillus, Streptomyces, Nosocomiicoccus, Promicromonospora, Micromonospora Psychrobacillus, Saccharopolyspora, Bacillus*	14	*Aspergillus fumigatus, Candida ethanolica, Bacillus subtilis* subsp. *Subtilis, Arthrobacter oryzae, Pseudomonas alcaliphila, , Acinetobacter modestus, Enterococcus ratti*	7	152 spores/g

*Expected strains observed.

4.8 Culture-independent microbial identification

4.8.1 High throughput sequencing of the 16 rRNA gene nucleotides

A total of 2 886 464 reads were generated with the highest reads (431 646) and the lowest reads (106,807) obtained in samples CB1L and CB4L, respectively (Table 4.13). However, after quality trimming 2 186 464 high-quality reads were obtained and subsequently assigned to OTUs. In total, 5 791 OTUs were generated after performing read rarefaction at a depth of 17900 sequences per sample. Among the biofertiliser samples, CB13S had the highest number of observed OTUs while CB2S had the least number of OTUs. The OTU richness rarefaction curve in each of the biofertiliser samples signified that bacterial communities were sufficiently sampled (Fig. 4.12). From the Alpha diversity indices, it was observed that liquid samples had higher Simpson and Shannon indices than the solid biofertiliser samples. Similarly, OTUs/species evenness and Choa1 values were higher in liquid products compared to the solid products, except for product CB13S (Fig. 4.11). In general, the OTU diversity obtained in all the biofertiliser samples was a close estimation of the true OTU diversity as indicated by the calculated Goods coverage, which was approximately one in all the biofertiliser products.

Table 4.13: Illumina generated reads and operational taxonomic unit with diversity indices.

Diversity indices	Biofertiliser products												
	CB1L	CB2S	CB3L	CB4L	CB5S	CB6L	CB7L	CB8L	CB9L	CB10L	CB11L	CB12L	CB13L
Observed OTUs	502	154	209	181	265	210	201	234	249	281	313	240	2,049
Shannon index (H)	5,50	2,43	2,60	2,65	1,40	3,90	4,60	4,07	4,39	4,88	4,80	4,07	7,64
Simpson index (D)	0,93	0,70	0,68	0,67	0,29	0,88	0,93	0,86	0,90	0,93	0,92	0,88	0,97
Chao1	847,29	238,29	468,29	363,40	547,39	349,33	319,75	471,39	504,91	411,63	538,78	450,52	3 765,85
Goods coverage	0,99	1,00	0,99	0,99	0,99	1,00	1,00	0,99	0,99	0,99	0,99	0,99	0,94
Simpson Reciprocal index	13,34	3,29	3,16	2,99	1,41	8,34	14,59	7,30	10,05	15,38	12,25	8,51	38,61
Equitability (evenness)	0,61	0,34	0,34	0,35	0,17	0,51	0,60	0,52	0,55	0,66	0,58	0,51	0,69
Total Reads	431 646	273 544	166 802	106 807	208 900	178 363	169 842	182 082	246 315	386 353	114 853	181 707	239 250
Quality filtered Reads	280 166	186 906	108 642	70 028	137 940	116 088	112 733	120 507	166 517	251 340	77 446	121 367	162 898

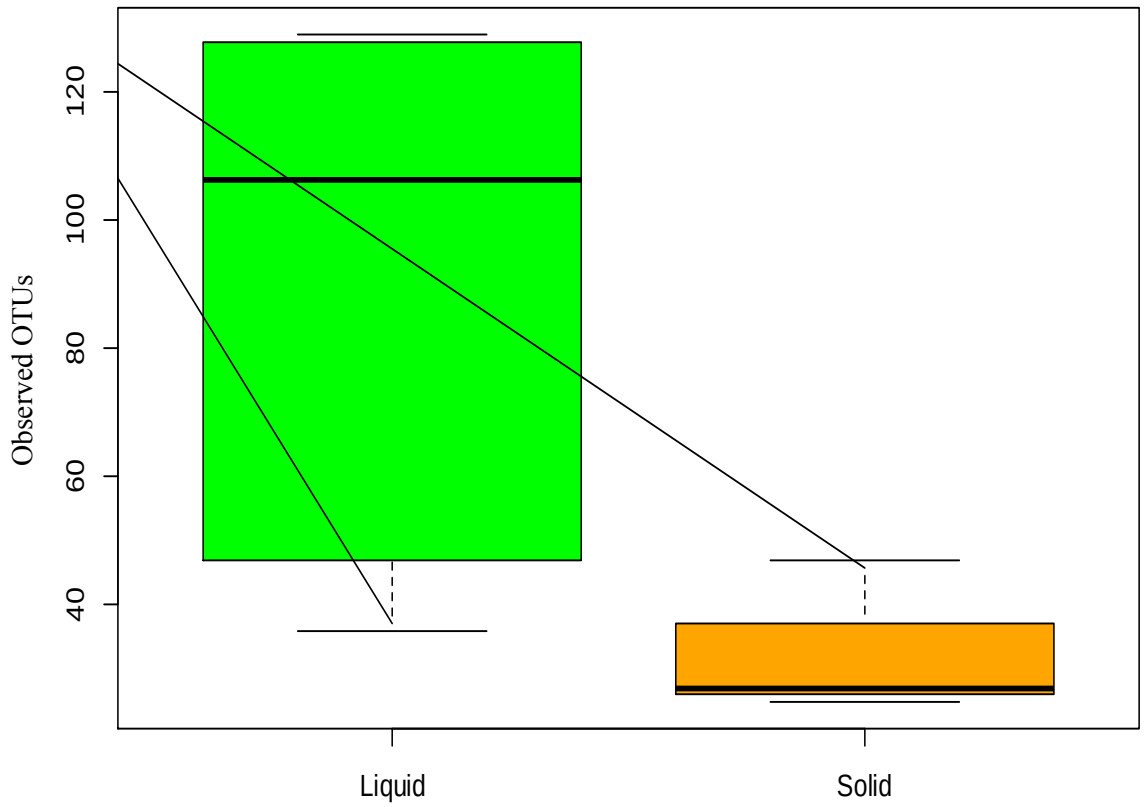

Figure 4.11: Box plot showing observed OTUs in liquid and solid products.

The Wilcoxon rank sum test with continuity correction showed that the potential differences were significant (W = 27.5, *P*-value = 0.04196). Alternative hypothesis: true location shift is not equal to 0. Therefore, there is an evidence to conclude that there are differences in the richness of the biofertiliser product microbiome between the liquid and the carrier-based products.

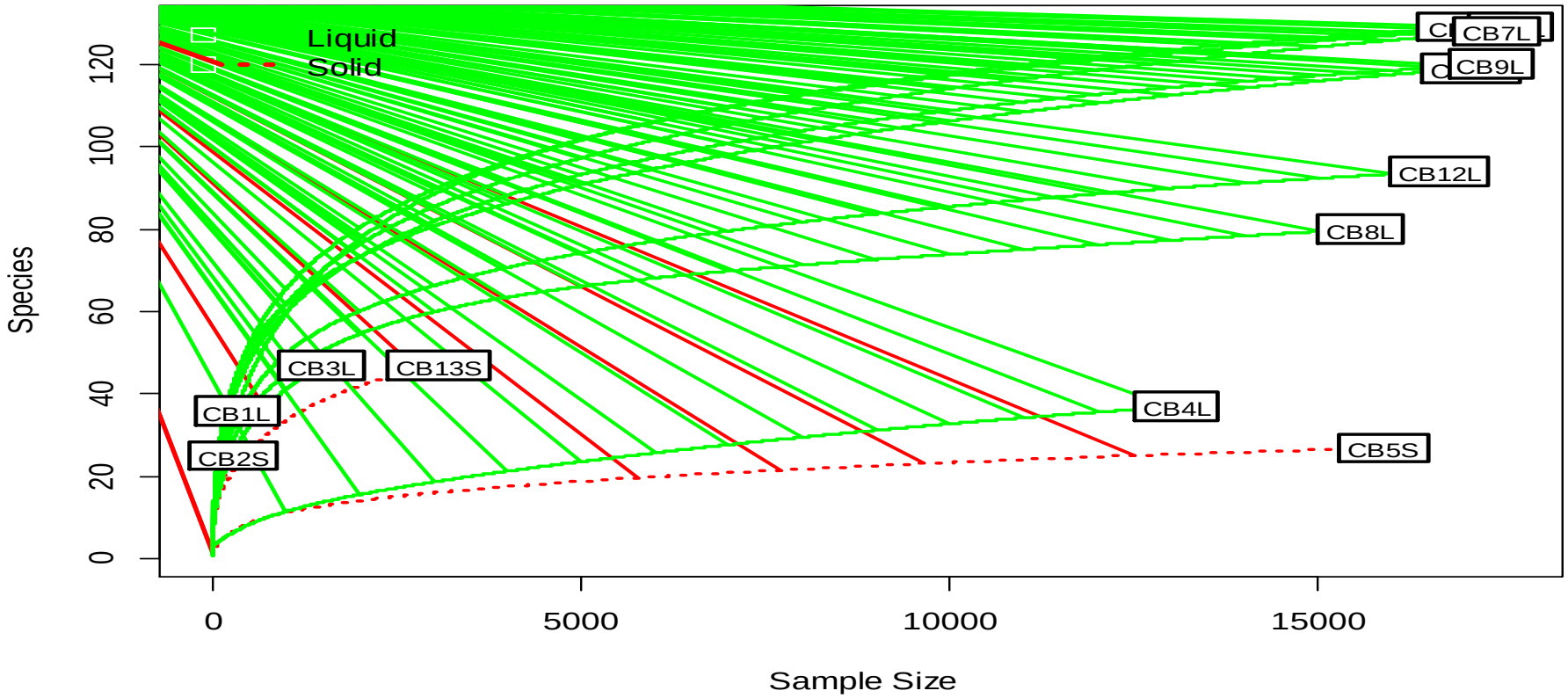

Figure 4.12: Rarefaction curve.

4.8.2 Operational taxonomic units diversity in biofertiliser products

The observed bacterial OTU diversity from Illumina sequences were taxonomically spread across 35 phyla (Fig. 4.13), 92 classes (Fig. 4.14), 222 orders (Fig. 4.15), 453 families (Fig. 4.16), and 1030 genera (Fig. 4.17) with classified reads of 100%, 88%, 79%, 69% and 66%, respectively. A number of OTUs occurring below 1% at different taxa levels were grouped as "others". From the relative abundance of phyla (Fig. 4.13), Proteobacteria was the major phylum, which is dominant in all the products except in [1]CB3L and CB4L, where it occurred below 3%. Other dominant phyla included Firmicutes, Bacteroides and Actinobacteria. In addition, the phyla occurring above 1% but below 10% were Chloroflexi, Planctomycetes, Acidobacteria, Cyanobacteria and Verrucomicrobia. Firmicutes was more dominant in the liquid than in the carrier-based biofertiliser products while Proteobacteria was predominant in the single-strain products except in CB4L.

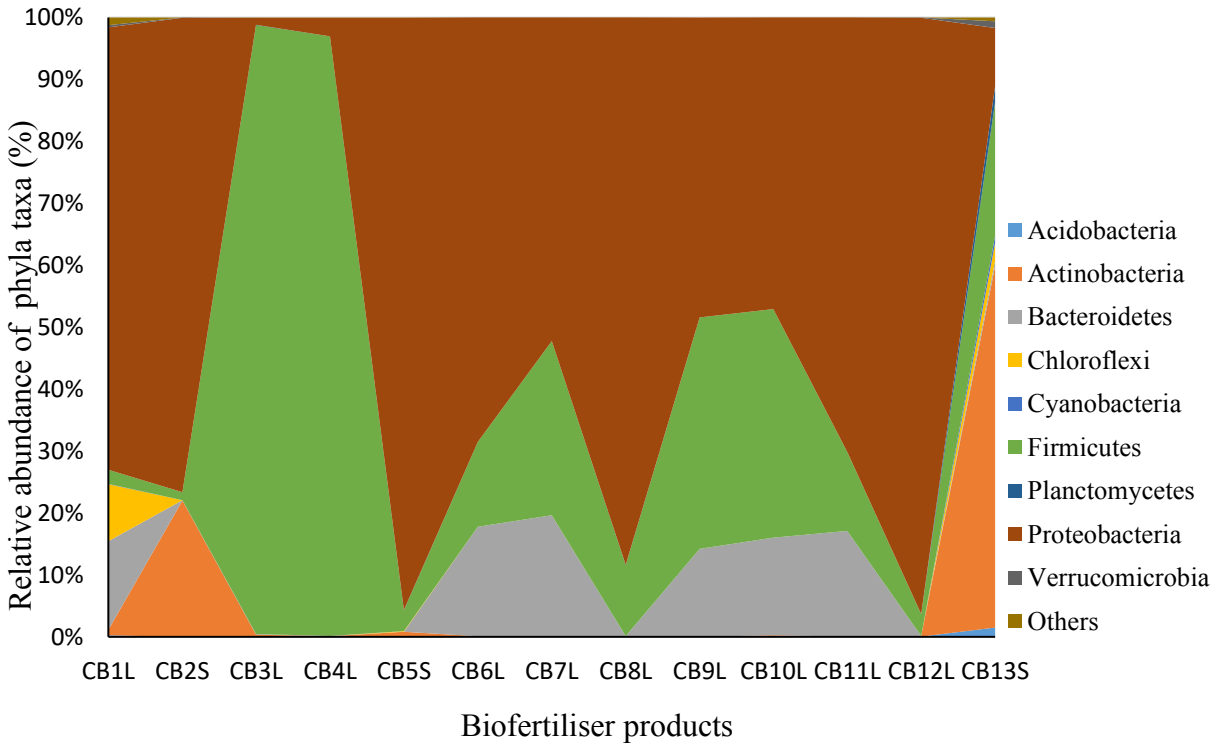

Figure 4.13: Relative abundance of bacterial OTU phyla taxa in biofertiliser products.

[1] Sample codes (CB1- CB13) were obtained by a combination of CB -commercial biofertiliser, numeric- for sample numbers 1-13 and either S- for solid (carrier-based) or L- for liquid products

At the class taxa level, OTUs having above 1% abundance in the biofertiliser products included (in order of highest to least abundant) Gammaproteobacteria, Bacilli, Alphaproteobacteria, Betaproteobacteria, Clostridia, Bacteroidia, Actinobacteria, JG30-KF-CM66, Negativicutes, Deltaproteobacteria, Flavobacteria, Sphingobacteria, Planctomycetacia, Cytophagia, Acidobacteria and Cyanobacteria (Fig. 4.14). Gammaproteobacteria, which was the most dominant class, occurred in ten of the products but had higher abundance in CB12L and CB8L. The class Alphaproteobacteria occurred in only four products (CB5S, CB2S CB1L and CB13S) while Bacilli was predominant in products CB3L and CB4L with relative abundance of 97% and 98%, respectively. Betaproteobacteria and Bacteroidia were present in eight and six biofertiliser products, respectively. The class taxa abundance OTUs are more diverse in the liquid biofertiliser products (Fig. 4.11). On the other hand, the carrier-based products had a maximum of three major classes, which were Alphaproteobacteria, Bacilli and Actinobacteria. The classes Cyanobacteria, Acidobacteria, and Planctomycetacia occurred only in CB13S while Cytophagia, Sphingobacteriia, Flavobacteria and JG 30-KF-CM66 occurred only in CB1L. The classes occurring only in two products were Actinobacteria (CB2SL and CB13S) and Negativicutes (CB7L and CB10L) while Deltaproteobacteria occurred only in three products (CB9L, CB10L and CB11L).

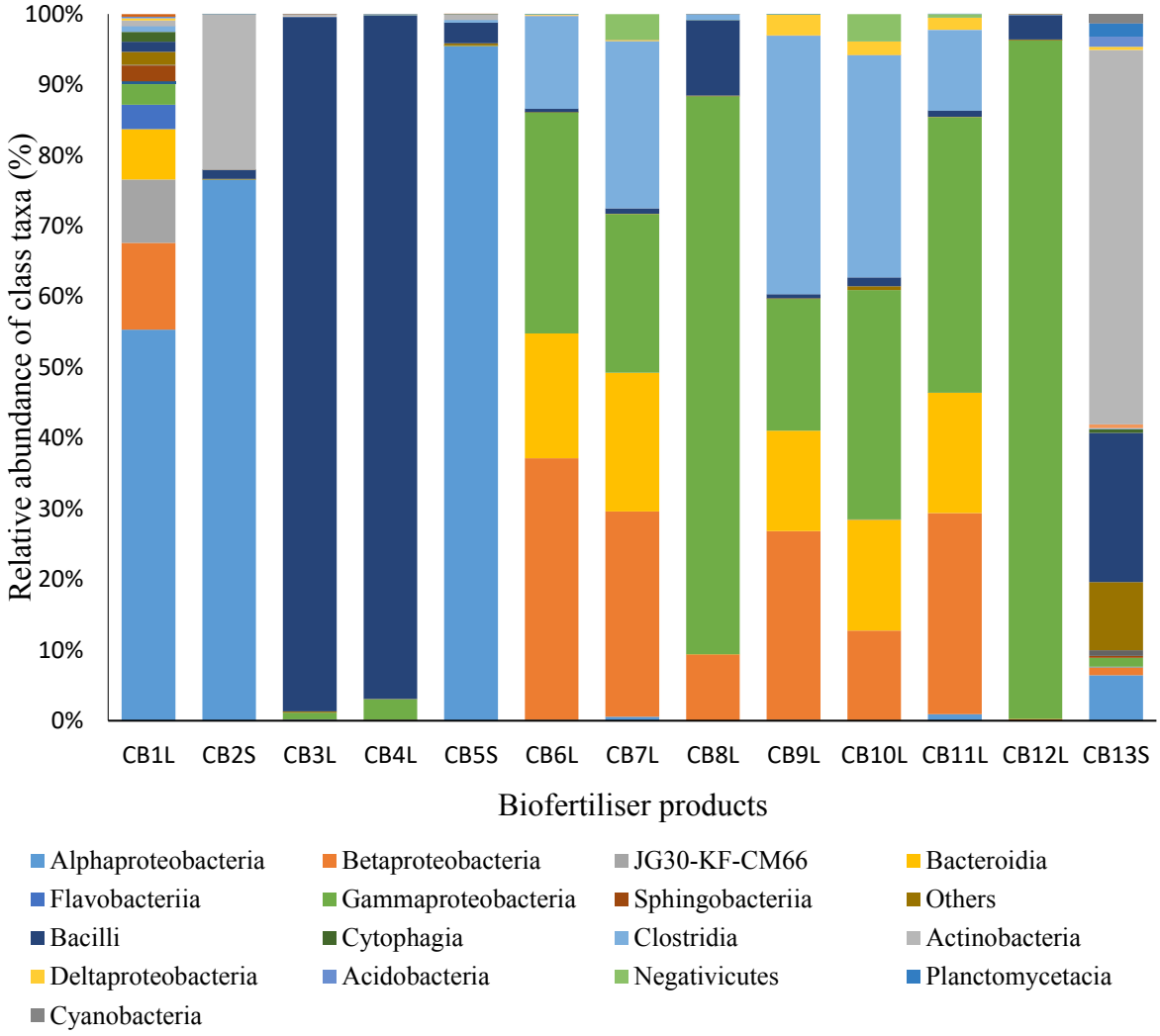

Figure 4.14: Relative abundance of bacterial OTU class taxa in biofertiliser products.

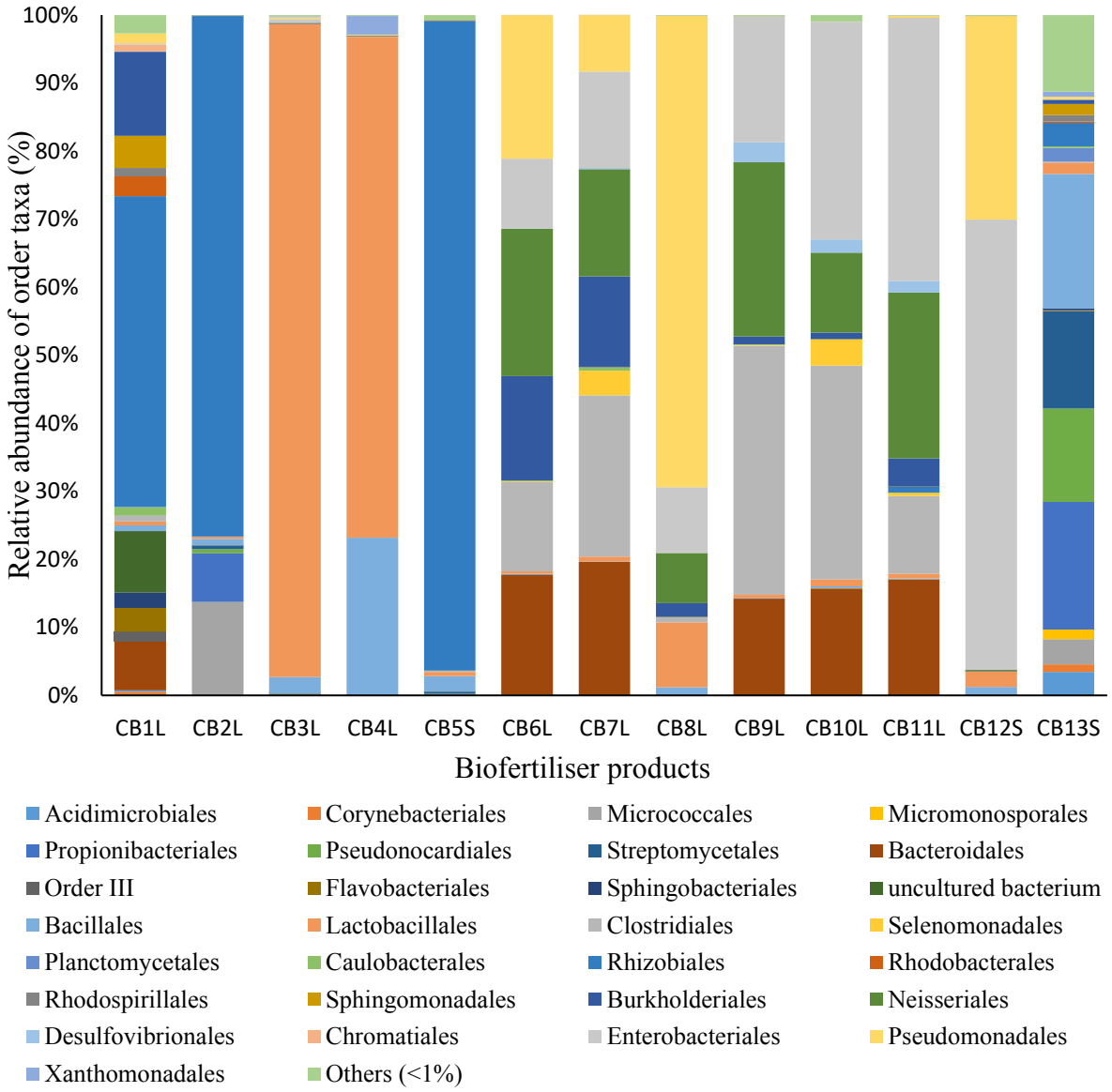

Figure 4.15: Relative abundance of bacterial OTU order taxa in biofertiliser products.

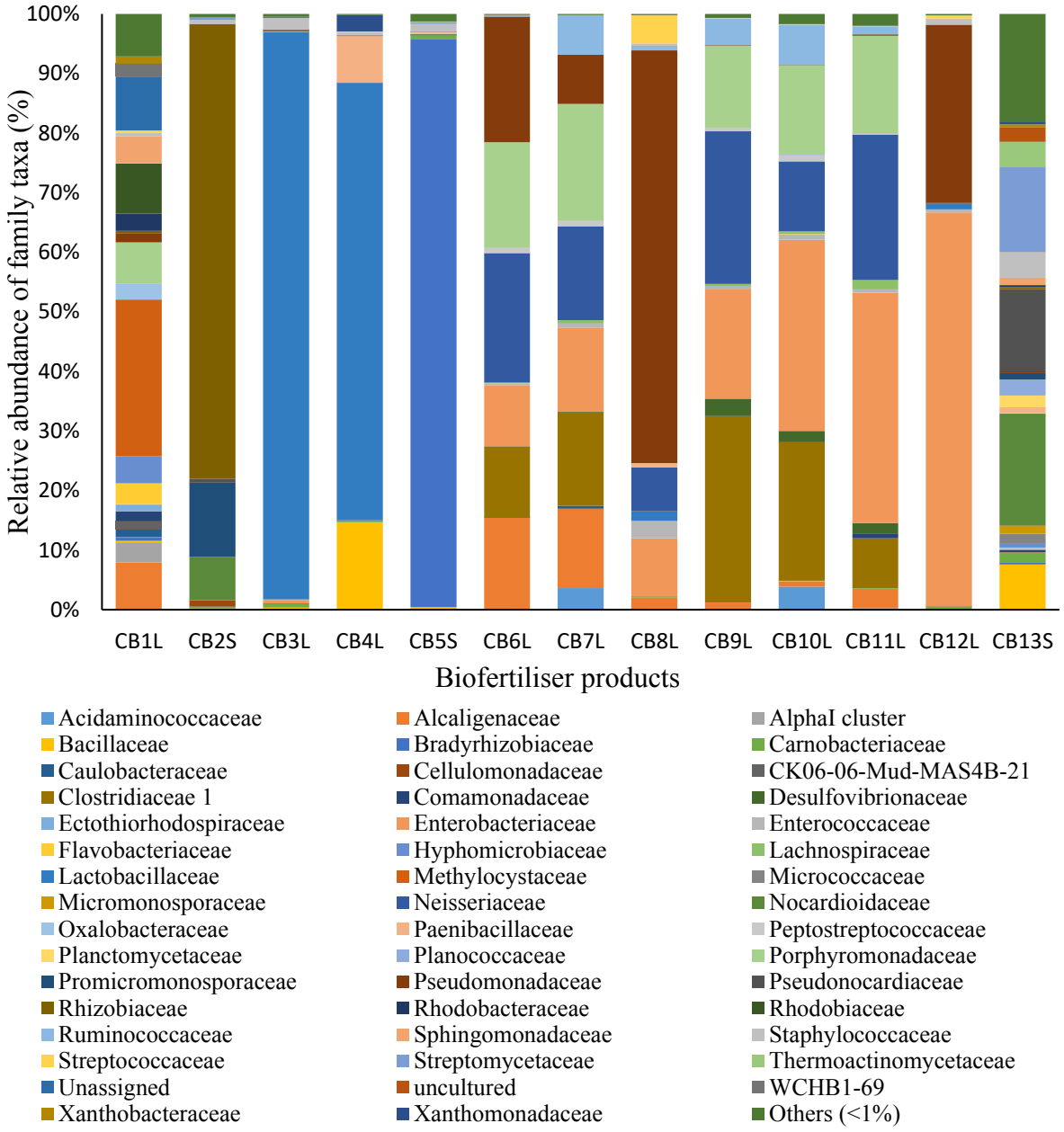

Figure 4.16: Relative abundance of bacterial OTU family taxa in biofertiliser products.

The relative abundance at the genus level is significantly diverse across the various biofertiliser products. Of the total genera obtained, only 67 genera occurred at a minimum of 1% abundance in at least one biofertiliser product (Fig. 4.17). The genera *Methylocystis, Parvibaculum, Pusillimonas, Pelagibacterium, Paucimonas, Salinihabitans, Pigmentiphaga, Thioalkalispira, Proteiniphilum, Flavobacterium, Devosia, Sphingomonas, Chryseobacterium* and *Petrimonas* occurred only in sample CB1L, *Cellulosimicrobium* and *Rhizobium* occurred only in CB2S, *Brevibacillus* and *Stenotrophomonas* were observed only in CB4L while *Providencia, Serratia,*

Rahnella and *Ewingella* occurred only in CB12L. Other genera found only in a single product included *Pediococcus* (CB3L), *Bradyrhizobium* (CB5S), *Lactococcus* (CB8L), *Kluyvera* (CB10L) and *Enterococcus* (CB8L). Similarly, *Amycolatopsis, Arthrobacter, Fictibacillus, Marininema, Micromonospora, Pseudonocardia, Atopostipes* and *Streptomyces* were only present in CB13S while *Cronobacter, Pontoea* and *Tyzzerella* 3 were observed only in product CB11L. Of all the genera, *Lactobacillus* had the highest abundance occurring in products CB3L, CB4L and CB8L with 91%, 73% and 2% abundance respectively, followed by *Pseudomonas*, which was found in five different products with abundance of 1.4% in CB1L, 21% in CB6L, 8.3% in CB7L, 69% in CB8L and 30% in CB12L. Other genera of high relative abundance included *Microvirgular*, which occurred in samples CB6L to CB11L. With respect to the types of the products, OTUs abundance observed at the genus taxa were more in the liquid products than in solid products. The liquid products had as high as 16 dominant genera in samples CB1L and CB11L and as low as three genera in CB3L. However, apart from CB13S with 14 dominant genera, other carrier-based products CB2S and CB5S had four and two dominant genera, respectively.

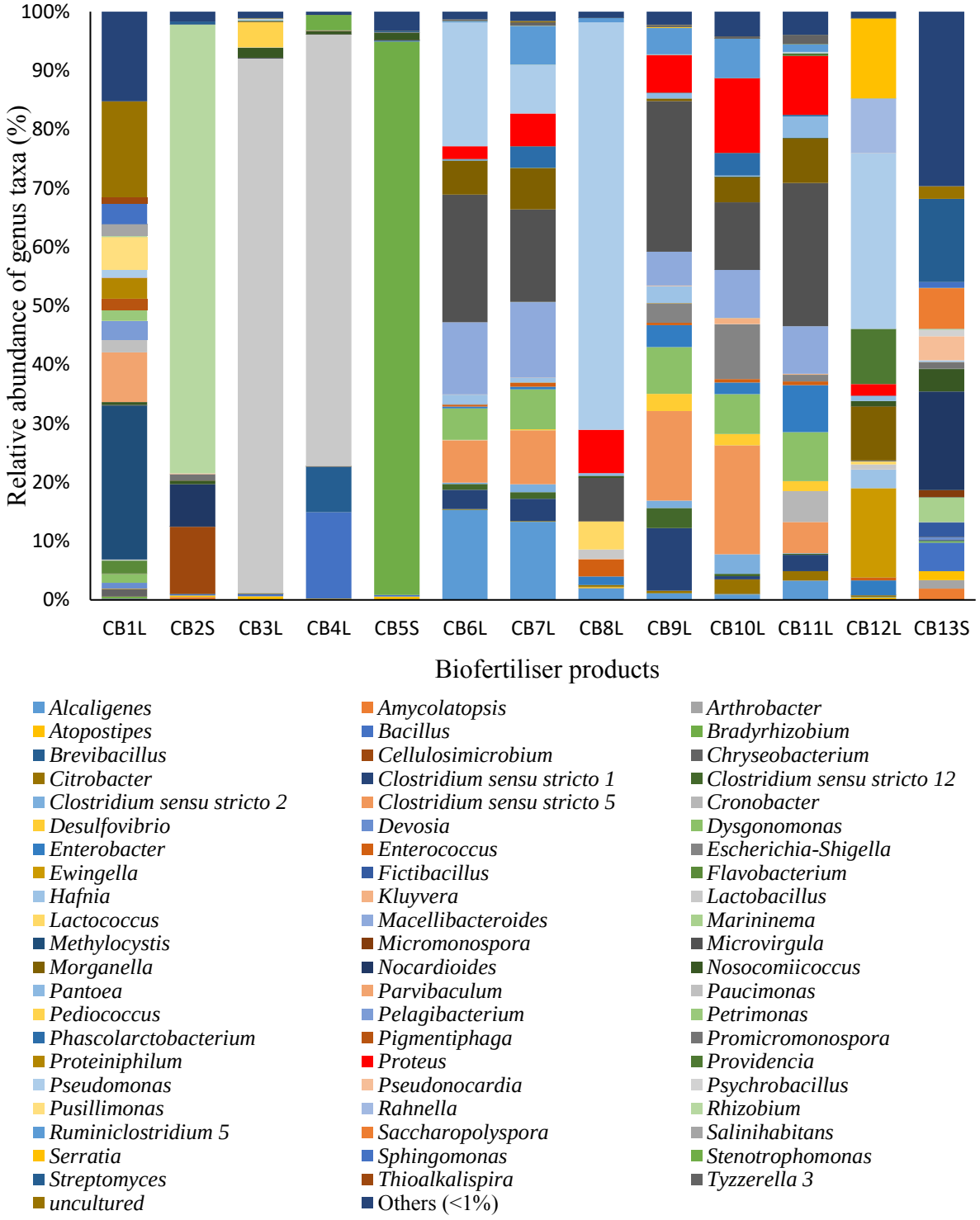

Figure 4.17: Relative abundance of bacterial OTU genus taxa in biofertiliser products.

4.8.3 Biofertiliser product quality and level of contamination using Illumina sequences

The Illumina sequences showed that products CB1L and CB3L contained only one of the expected microbial strains while samples CB2S, CB4L, CB5S and CB13S contained the claimed microbes as stipulated by the manufacturers. However, 54% of the biofertiliser products did not have any of the claimed microbes represented by the observed genera at above 1% OTUs relative abundance. In addition, the observed sequences showed that all the samples contained one or more contaminants. Sample CB5S had the claimed microbe, *Bradyrhizobium* at 94% abundance and only one major contaminant, *Nosocomiicoccus* at 1.2% abundance (Fig. 4.17). Similarly, CB2S had the expected strain, *Rhizobium* at approximately 76% abundance with contaminants such as *Cellulosimicrobium, Nocardioides* and *Promicromonospora* at 11.4%, 7.2% and 1.0% abundance respectively. Therefore, CB5S and CB2S could be regarded as medium quality products because it contained the claimed microbial strain but with other microbial strains at a relatively lower abundance (as discussed earlier Fig. 4.10). Furthermore, sample CB4L was regarded as a low-quality product because it had the specified microbes, *Bacillus* at a lower abundance (14.7%) than the unclaimed genera, which occurred at a much higher abundance: *Lactobacillus* (73.4%), *Brevibacillus* (7.8%) and *Stenotromonas* (2.6%). Other samples, CB1L, CB3L and CB13S, were also categorised as low-quality biofertilisers. For instance, the beneficial strains in CB1L were stated to be 30, according to the manufacturer's labelling information. Unfortunately, the observed OTUs showed *Pseudomonas* to be the only strain present at 1.4% among the claimed strains while other unspecified strains were observed in the sample (Fig. 4.17). Similarly, of the seven specified organisms in CB3L, *Lactobacillus* at 91% abundance was the only observed microbes while *Nosocomiicoccus* (1.7%) and *Pediococcus* (4.3%) were unspecified strains observed. For sample CB13S, 14 bacterial OTU genera not specified by the manufacturer were observed. Other products lacking the specified strains but containing other microbes were regarded as poor-quality products. None of the products had the specified strains without any contaminant, therefore cannot be categorised as high-quality products.

The medium-quality products were mainly the rhizobia products (CB5S and CB2S) while the poor-quality products were the free-living nitrogen-fixing bacteria products (CB6L and CB7L). Of all the 125 contaminants recorded at ≥ 1% OTU abundance, an average of nine and 11 occurred in single strain and consortia strains products, respectively.

4.8.4 Community functional profiles predictions from metagenomics of 16S rRNA data

The computation of Taxa4Fun metagenome specific functions for all biofertiliser products was based on KEGG Orthology (KO) expressions. A table of specific OTUs benefits to each KEGG Orthology description was generated and a total of 6 524 KO terms were obtained from the imputed metagenomes of all the biofertiliser products. Various gene relatives of concern were examined based on the conditions that they are present and of environmental importance, or with the potential for product-quality damage (as contaminants or toxins). The investigated genes included genes encoding for nitrogen fixation (K02586, K02588, K02591, and K01426), denitrification (K04561, K02305, and K00376), phosphate solubilisation (K01077, K01078, K01085), glucose degradation (K01187, K01190, K01179), iron uptake (K07229, K03711), sulphur uptake (K01130, K01133) and toxin production (K11006, K11007, K11038, K03558). The nitrate reductase (K00370, K00371, K00373, and K00374) and nitrite reductase (K00362, K00363, K00366, and K00367) genes were also investigated.

Generally, the nitrogen-fixing genes; K02586, K02588, K02591 and K00531 were present in most of the products but had a high presence in products containing rhizobia except CB12L (Fig. 4.18). The microbial community profiling also showed the dominance of alkaline phosphatase to be predicted across all the products. In addition, glucose-1-phosphatase (K01085) was abundant in products CB12L, CB11L, CB10L CB9L and CB7L while 4-nitrophenylphosphatase (K01101) was dominant in CB3L, CB13S, CB12L, CB4L and CB2L. Of the five sulphur-degrading enzymes assessed, arylsulphatase (K01130) was the most prevalent across all the products while other sulphur genes (K01133, K01134, K01135 and K01137) occurred in low absolute richness. Other essential genes investigated included the iron-uptake and carbohydrate-degrading genes. Ferric uptake regulator (K03711) was predicted to be dominant amongst the products, with the highest prediction in CB13S. Similarly, ferric-chelate reductase (K07229) was also observed to be dominant in products CB10L, CB11L and CB12L but in lesser amounts in CB2S and CB13S. Genes encoding for galactosidase (K01190) and glucosidase (K01187) were widely predicted in high abundance amongst the products, except in products CB1L and CB5S, where they occurred in low abundance.

Toxin genes such as Shiga, leucocidin and haemolysin toxins were also predicted. While the leucocidin and haemolysin toxin (K11038) gene had higher prevalence amongst all the products, Shiga toxin genes (K11006 and K11007) were only abundant in products CB3L,

CB8L, CB11L and CB12L. In addition, genes encoding for denitrification enzymes and amidase were also investigated. The nitric oxide reductase subunit B (K04561), nitrous-oxide reductase (K00376) and amidase (K01426) had high predicted occurrence amongst the products while the nitric oxide subunit C (K02305) had a low predicted prevalence. Other important genes predicted in the products included the nitrate and nitrite reductase gene. The nitrite genes, nitrite reductase large subunit (K00362) had high prevalence in all the products while nitrite reductase small subunit (K00363) and ferredoxin-nitrite reductases (K00366, K00367) were relatively predicted in low abundance. Similarly, the nitrate reductases (K00370, K00371, K00373, and K00374) were predicted to be abundant in all the products. However, nitrate reductase 1, alpha subunit (K00370) and beta subunit (K00371) occurred in higher

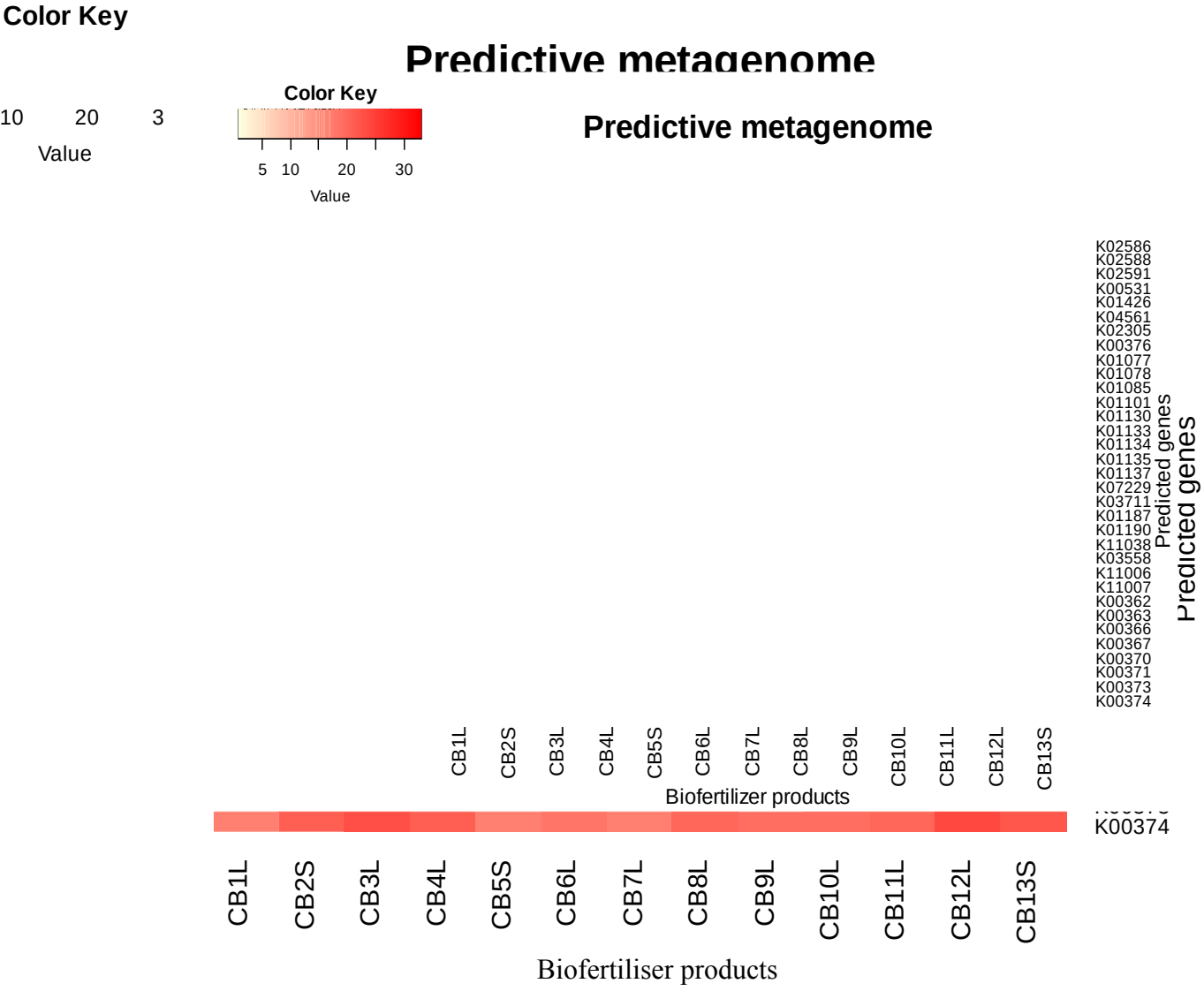

Figure 4.18: Heat map showing the metagenomics contributions of some important genes.

4.9 Bacterial communities obtained from the Sanger and Illumina MiSeq sequences

Different types of bacterial communities were reported in the biofertiliser products, with 23 and 63 bacterial genera obtained from Sanger and Illumina MiSeq sequences, respectively. Twelve of the bacterial genera were present for both techniques and these included *Acaligenes, Arthrobacter, Bacillus, Brevibacillus, Citrobacter, Escherichia, Hafnia, Morganella, Proteus, Pseudomonas, Rhanella* and *Rhizobium* (Fig. 4.19). In addition, two major fungi genera, *Aspergillus* and *Candida* were isolated from the culture-dependent Sanger sequences.

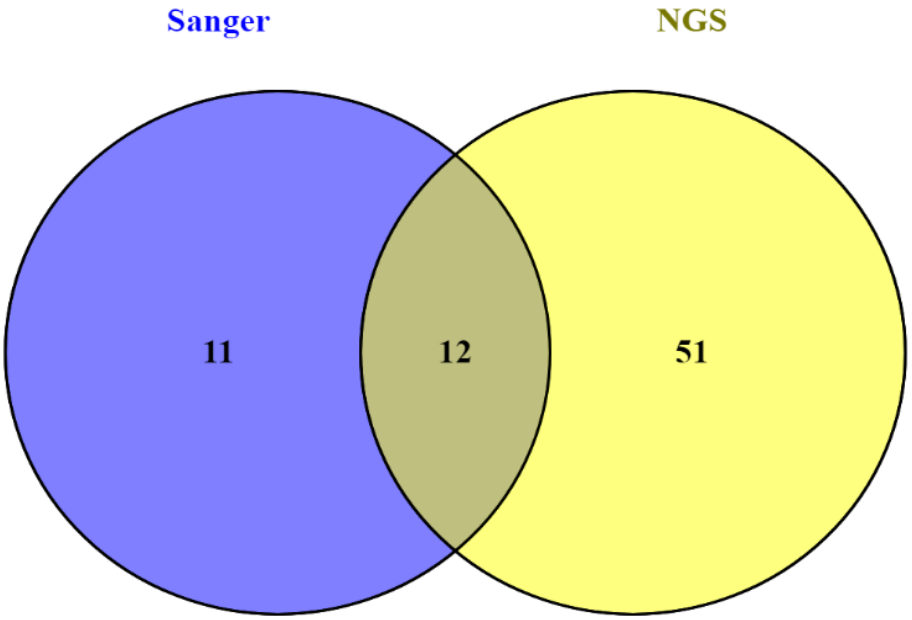

Figure 4.19: Overlapping genera between Sanger and Illumina sequences obtained.

Chapter Five

Discussion

5. Discussion

The new age-agriculture is focused on sustainable practices that are aimed at improving crop productivity through profitable, energy-conserving and eco-friendly farming practices (Lichtfouse et al., 2009). Over a century, microbial products such as biofertiliser have been widely used for increasing crop productivity because of the huge benefits in soil nutrient management and sustainable agriculture. These benefits have caused tremendous attention in the development and application of biofertilisers considering the negative environmental impact of the excessive and continuous application of inorganic fertilisers (Parnell et al., 2016). In South Africa, a large proportion of marginal land cultivated by SHF is nutrient deficient (Goldblatt et al., 2010; Morris et al., 2007). While it is imperative to improve soil nutrient content to increase crop yield and alleviate food security challenges (Nwanze, 2011), the resource-poor farmers lack financial capability to afford the expensive inorganic fertilisers. This has necessitated cheaper and sustainable soil-fertility management such as biofertiliser. Consequently, awareness and application of biofertiliser are key to realising the economic importance of SHFs in South Africa (Raimi et al., 2017).

5.1 Awareness and application of biofertilisers

Awareness and application of biofertiliser could be influenced by farmers' characteristics such as age, gender, level of education and years of farming experience (Doss & Morris, 2000). The increasing population of women in smallholder farming, especially in Africa was attested to in this study. The present study reported more female than the male SHFs. Nowadays, it is essential for women to support the household economy by providing extra food and income for the family, especially in the era of South African economic decline. Women in the agricultural labour force are over 43% in developing countries and approximately 50% in sub-Saharan Africa (Nelson et al., 2012). However, the influence of gender in awareness and application of biofertiliser was not significant ($p_{0.05}$ = 0.80). On the contrary, Doss and Morris (2000), concluded in their study that female SHFs are less likely than male counterparts to adopt new farming practices. Furthermore, the mean age of 50 years observed amongst SHFs suggests an aged group of farmers. Age has been positively correlated with the adoption of improved technologies. This may be that the older the farmers get, the more experienced they become in their choice of efficient farm practices. On the other hand, younger farmers may be better adapted to use new technology than the older risk-averse farmers. In addition, the low level of

education amongst SHFs may be a reason for the low adoption of biofertiliser, though education was not significant in this study ($p_{0.05} = 0.3$). It is agreed that learning new techniques and extension programmes involve a rigorous process and a higher level of reasoning. Hence, farmers with a higher level of education are always at an advantage. Recent agricultural research and practices are privately driven, focusing on knowledge-intensive technology that does not consider the economic class of many SHFs (Rapsomanikis, 2014). The observation in this study is in agreement with Van Eeden and Korsten (2013), who correlated age and level of education to the adoption of technology among smallholders.

Farmers' experience in basic farm practices, its successes and challenges, is principal in making present and future decisions. The majority of the SHFs examined had few years of experience, less than five years, and this perhaps may be responsible for the low level of awareness and application of biofertiliser. Farmers experience was significant ($P_{0.05} = 0.03$) in the present study. This observation was corroborated by (Isabirye et al., 2010) who reported that high levels of experience caused SHFs to intensify adoption of innovation. In addition, organic and inorganic fertilisers were predominantly used amongst SHFs to increase crop productivity (Rowell & Hadad, 2004). However, none of the farmers apply biofertiliser, and this may be as a result of the low awareness of biofertilisers among African SHFs (Masso et al., 2015; Simiyu et al., 2013).

A major reason for applying fertilisers was reported as yield increase, while plant pests and diseases were reported as major challenges in increasing crop productivity. The application of biofertiliser was not considered an option in addressing these challenges due to the poor knowledge of biofertiliser amongst the SHFs (Ochieng, 2015). The majority of SHFs lacked understanding of what biofertiliser is. Thus, it is important that farmers gain full knowledge of biofertiliser and its benefits for complete uptake (Srinivas & Bhalekar, 2013). The perception of the benefits of biofertiliser may influence application among SHFs (Mutuma et al., 2014). In sub-Saharan Africa, low demand of *Rhizobium* inoculum among farmers was attributed to lack of awareness and knowledge on the benefits of inoculum (Khonje, 1989). Several biofertilisers have been found useful as biocontrol agents. For example, *Rhizobium* can increase plant resistance to herbivore attack (Kawalekar, 2013; Raja, 2013). Corn yield increased when biofertiliser caused a reduction in the population of predatory insect (Megali et al., 2015). In

addition, bacterial and fungal diseases have also been suppressed by the use of biofertilisers such as *Pseudomonas, Sinorhizobium* and *Bacillus* (Arora et al., 2008; Guo et al., 2013).

Crops grown by the SHFs interviewed were mostly stable crops. Maize, an important food for South African households was commonly cultivated (Kumwenda et al., 1997). Interestingly, biofertilisers have been widely used on staple crops for increased yield. *Rhizobium trifolii, Azospirillum brasilense* and *Glomus mossea* have been used to enhance the productivity of many stable crops (Egamberdiyeva, 2007; Riggs et al., 2001). Therefore, if awareness and adoption of biofertiliser improve, there is hope for better productivity amongst SHFs. Furthermore, growing cash crops may increase farmers' income, which could possibly increase investment capability in new technology such as biofertilisers (Santos Ordóñez, 2011).

Extension management, government policies and financial support, accessibility and biofertiliser quality have been reported to affect awareness and application of biofertiliser (Santos Ordóñez, 2011). The SHFs interviewed had no perception of biofertiliser quality and could not attest to its performance or efficiency with respect to other fertilisers. This is expected, considering the poor knowledge and the fact that farmers do not use biofertiliser. The challenges of extension services may have also affected the adoption of biofertilisers. This was corroborated by Khapayi and Celliers (2016) who reported that low extension activities impacted negatively on farmers' access to biofertiliser knowledge. Effective extension management can generate immense awareness through a strategy of on-field trial, training, workshops and seminars that expose the farmers to biofertiliser benefits. This will cause a transfer of knowledge and agricultural inputs from the source (research centres, manufacturers, government institutions) to the farmers (Ochieng, 2015). Farmers contact with biofertiliser promoting institutions and group members may also influence the use of biofertilisers (Mutuma et al., 2014).

The accessibility of agro-inputs is key to increasing biofertiliser awareness. The SHFs in the study area reported poor access to commercial biofertilisers. This may suggest that products are not readily available at different agronomic shops. Therefore, an efficient biofertiliser distribution system must be in place to improve the accessibility of products (Masso et al., 2015). The governmental support for biofertiliser technology was also reported poor by the SHFs interviewed. Specifically, almost all the farmers reported a lack of financial and policy

support from the government as a major constraint. The use of biofertiliser has been constrained by lack of policy support in sub-Saharan African countries (Odame, 1997). Therefore, adequate regulations with an effective monitoring system and efficient extension management will improve awareness and usage of biofertilisers amongst SHFs (Masso et al., 2015).

5.2 Biofertiliser physicochemical properties

5.2.1 *Total carbon, nitrogen contents and C/N ratio*

The total carbon and nitrogen content of the biofertiliser products analysed were relatively lower for liquid products but higher for carrier-based products. The same results were obtained for the C/N ratio. It is important that biofertilisers have adequate nutrient contents and C/N ratios to support microbial growth and survival during production and storage. Low carbon and nitrogen contents adversely affect microbial strain, in the biofertiliser products, thereby affecting the efficiency of inoculum when used on the field (Balume et al., 2015). Carriers with high organic carbon contents have been suggested for biofertiliser formulation for improved efficiency of inoculum (Hung & Sylvia, 1988). However, the nutrient content of biofertilisers should not be too high to interfere with the biofertilisation activities of beneficial microbes (Vessey, 2003). Different ranges of carbon and nitrogen content have been reported for different biofertiliser carriers. Tabassam et al. (2015) reported a range of 0.65-0.89% nitrogen in five different carriers while Bocchi and Malgioglio (2010) reported a range of 37-41% carbon in *Azolla* biofertilisers. In addition, peat, which has been widely used as a carrier due to its good physicochemical characteristics, has high carbon and nitrogen contents, an average of 29% and 2%, respectively. This is similar to the observation in this study. The higher C/N ratio in some of the products, especially the carrier-based products is an indication that the products can support a high density of inoculum. These observations are similar to that of Bocchi and Malgioglio (2010), who observed a higher C/N ratio amongst the tested biofertilisers. However, other samples with lower C/N ratio may not support optimal growth of inoculum for a long period of storage and therefore may not be good-quality products. A similar report of low C/N ratio of carrier materials has been made by Deepti and Mishra (2014).

5.2.2 Electrical conductivity

The biofertiliser products assessed had high electrical conductivity (EC) values, signifying high amounts of water-soluble nutrients in the products. The amount of available nutrients in the products usually impacts microbial activities. At high EC, microbial activities such as growth, respiration and organic-matter decomposition are adversely affected due to osmotic stress caused by the large concentration of cations (Shah & Shah, 2011). Electrical conductivity correlates with properties such as organic matter, cation-exchange capacity as well as salinity (Nanda & Abraham, 2011). For instance, most soils with $EC_{1:1}$ readings less than 100 mS/m are considered non-saline and the salinity of this soil may not impact microbial activities. However, at $EC_{1:1}$ readings above 100 mS/m, the soils are considered saline and microbial processes are affected (Smith & Doran, 1996). Low EC values in the range 45– 312 mS/m have been reported in different biofertiliser products. This is contrary to the results in this study where high ECs were observed in the products (Datta et al., 2015; Phiromtan et al., 2013).

5.2.3 Total micronutrients and heavy metals

The analysis of micronutrients showed a relatively low level of the elements in the biofertiliser products. Micronutrients such as manganese, molybdenum, zinc and copper usually act as co-factors in enzymatic reactions in microbial cells. The metals are required in small quantities and where they occur in high quantities, they become toxic to microbial cells. The level of micronutrients observed in the biofertiliser products evaluated is adequate for maintaining the microbial quality of the products. The metal ions and elements requirements are unique and probably vary amongst different microorganisms (Merchant & Helmann, 2012). High amounts of trace elements (Cu > 16 mg/kg, Mn > 127mg/kg, Zn > 42 mg/kg) have been reported in different biofertiliser carriers (Tabassam et al., 2015). This is, however, contrary to the obtained results in this study.

Additionally, the heavy-metal content of biofertiliser products analysed was within the acceptable standards stated in the South African Fertiliser legislation (SAFL, 1977). It is imperative that biofertiliser products contain low levels of heavy metals to protect the microbial community from the toxic effects of heavy metals (mercury, arsenic, chromium, lead, cobalt and cadmium). This will also reduce heavy metal carcinogenic potential and bioaccumulation in the food chain, as well as the pollution of agricultural soils when biofertilisers are used in the field (Carvajal-Muñoz & Carmona-Garcia, 2012; Yabe et al., 2010). Similar observations of low levels of heavy metal contents in biofertilisers have been reported (Negreanu- Pirjol et

al., 2011). According to Arif et al. (2016), heavy metals reduce crop productivity where their concentration increases beyond the required threshold in the soil. It is important to control heavy-metal pollution to avoid environmental degradation, especially where industrial effluent or municipal waste is used as carriers for biofertilisers. Carrier materials must be non-toxic and should not have materials that pollute the soil or hinder the growth of crops or beneficial rhizosphere microorganisms (El-Fattah et al., 2013; Malusá et al., 2012).

5.2.4 pH, particle sizes, water-holding capacity and moisture content

A pH range of 6.0-7.5 has been considered acceptable for quality biofertiliser products (NCOF, 2011). Approximately 80% of the biofertiliser products investigated had pH within the stipulated standards. If pH is too acidic or basic, microbial cell growth and development are hindered. Generally, extreme pH affects the structure of microbial molecules (Jordan & Jacobs, 1948). At high pH, the hydrogen bonds holding DNA molecules collapse, modifying the ionisation of the amino-acid functional group, which causes changes in the folding of the molecules, promoting denaturation and destroying activities (Sinden, 2012). For instance, pH values lower than 6.5 create an unstable growth condition for rhizobia, causing an increase in the death rate of the viable cells (Kaljeet et al., 2011). Therefore, poor quality biofertiliser may result from inappropriate pH conditions. Biofertiliser carriers must have a good pH buffering capacity to maintain a relatively constant pH during production and storage (Malusá et al., 2012). The observation in this study is similar to that reported by Stella and Sivasakthivelan (2009) where the biofertiliser evaluated had a neutral or slightly above-neutral pH.

Further examination of the biofertiliser products revealed that the carrier-based bacterial biofertiliser products investigated had particle sizes < 0.150 mm; while the mycorrhizal product fell short of the acceptable standard, with less than 90% of the particles < 0.250 mm (Kenya standard, 2015; Yadav & Chandra, 2014). Carrier materials with particle sizes within the specified standards support the easy application of products. It also prevents lump formation in the products during storage (Tabassam et al., 2015). The influence of particle sizes in biofertiliser formulation is related to the greater surface area of carriers that are exposed to microbial activity when smaller particles are used. In addition, smaller particle sizes create a high-adhesive capacity with microporous quality that can offer a near-natural habitat for the beneficial microbes and enhance high moisture content as well (Malusà & Ciesielska, 2014).

The carrier-based biofertiliser products assessed had the water-holding capacity as well as moisture content within the acceptable standards except for one product. The amount of moisture a carrier can absorb and hold is a measure of biofertiliser quality. This is because moisture affects microbial cell growth and survival, and consequently the shelf life of the products (Deaker et al., 2011). The water-holding capacity of a good carrier has been stated to be above 50%, while the moisture content should be a minimum of 35-40% (Yadav & Chandra, 2014). Generally, if water-holding capacity is below 50%, moisture content is reduced and organisms may experience lack of moisture, which impacts negatively on biofertiliser quality (Griffith & Roughley, 1992). Moreso, the amount of bacterial broth (containing viable cells) that can be added to the carrier is also affected. Therefore, it is recommended that manufacturers maintain the acceptable standard for particle sizes, water-holding capacity, pH and moisture content in order to have a quality product that can perform optimally on the field (Feng et al., 2002).

5.2.5 Identity character and appearance

Some of the products evaluated had incomplete labelling information and approximately 29% of the products had different forms of odours (Table 4.8). Complete labelling information is an essential component of biofertiliser quality to guide end users on the correct purchase and effective application of the products (Malusá & Vassilev, 2014). Hence, lack thereof suggests that the products may not be effective because of the shortcomings of the manufacturers not meeting up with the acceptable quality standards or perhaps, the wrong purchase and/or misapplication by end users, the smallholders (Bala et al., 2011; Masso et al., 2015). This buttresses the fact that stringent regulations on commercial biofertilisers are basic measures that will protect farmers from losses associated with low-quality biofertiliser products (Balume, 2013; Herrmann & Lesueur, 2013; Lupwayi et al., 2000). Similar observations of noncompliance on product labelling amongst manufacturers have been reported (Herrmann et al., 2010).

5.2.6 Storage temperature

The obtained viable cell density after 12 weeks of storage at different temperatures was higher at 25 °C compared to 4 °C with an average of 54% variation across the products. In addition, products stored at 36 °C had a higher viable density compared to 4 °C but a much lower density than at 25 °C, with a few exceptions. Storage temperatures influence the viability and density of microbial cells in biofertiliser products. Hence, the overall efficiency and reliability of

biofertiliser products to improve crop yield are at risk without proper storage conditions. It is essential to emphasise appropriate storage temperatures that will support a longer shelf life of biofertiliser products (El-Fattah et al., 2013; Kaljeet et al., 2011; Phiromtan et al., 2013). Different studies have reported 28 °C as the optimal storage temperature that supports the viability of bacterial cells (El-Fattah et al., 2013; Kaljeet et al., 2011). This is in agreement with the observation at 25 °C ± 2 °C in this study. The microbial density over three months of storage remained within the acceptable standard for biofertiliser products. However, at lower storage temperatures, microbial physiological and metabolic activities are reduced. According to Roughley (1968), continuous storage of peat rhizobia at 4 °C caused a restricted multiplication with reduced viable cell numbers and maximum microbial density was achieved after 26 weeks of storage. It is important to note that the impact of storage temperature on biofertiliser products may also depend on culture purity, moisture content and types of microbial strains (Phiromtan et al., 2013; Roughley, 1968).

5.3 Molecular analysis of biofertiliser products

The Sanger sequencing technique is widely used to analyse microbial components of many environmental samples such as food, water and soil. However, the Illumina high-throughput sequencing technique, which is more efficient and cost-effective, is now widely used in microbial community analysis of environmental samples (Sun et al., 2017; Tyx et al., 2016; Zhang et al., 2017). This study is one of the few that have used both techniques to analyse microbial communities in biofertiliser products.

5.3.1 Microbial isolates from biofertiliser products

The microbial communities observed with Sanger sequences in the biofertiliser products were diverse in products CB1L and CB13S, having 11 and eight strains respectively. Similarly, Illumina sequences revealed that microbial diversity as measured by the Shannon and Simpson Diversity Index was considerably higher in the same biofertiliser products. Product CB1L is made of consortia of strains, containing PGPR (such as *Bacillus* and *Pseudomonas*) and rhizobia (*Rhizobium*). This observation is in agreement with the work of (Herrmann et al., 2015) who observed high microbial diversity in biofertiliser products made of consortia of strains. However, the authors reported high microbial diversity in the free-living nitrogen-fixing products, contrary to that obtained in this study. The heterogeneity and competitive ability of indigenous strains over biofertiliser strains have caused the need to have consortium

inoculant formulation, which has a better competitive advantage to withstand local strains and ecological conditions (Faye et al., 2013; Kyei-Boahen et al., 2002). In the present study, 38% of products were made of consortia of strains.

The observed NGS sequences in the present study showed rhizobia products were generally of better quality with the lowest Shannon and Simpson Diversity Index. These are single strain carrier-based products, which suggests that carrier-based products in this study are of good quality compared to the liquid products. It may be that the single-strain products are selectively formulated, unlike the consortium-strain products that support the growth of various biofertiliser strains as well as other undesired microbes that could cause product-quality damage. This is in agreement with other studies that correlated the increase in microbial density of rhizobia biofertiliser to the carrier material properties, which selectively supported rhizobia optimal growth (Balume, 2013; Olsen et al., 1995). In general, the results showed a low level of product quality across the range of products analysed with free nitrogen-fixing products having the least quality.

5.3.2 Contamination of biofertiliser products

The Sanger and Illumina nucleotide sequences revealed that the products had more microbial communities than what manufacturers claimed, signifying a high level of microbial contamination. Aforementioned observations have been reported in previous studies where different levels of contamination and consequential effect on product quality had been reported in biofertilisers (Herridge et al., 2002; Herrmann et al., 2015; Lupwayi et al., 2000; Olsen et al., 1995). A substantial proportion of biofertiliser products, especially using non-sterile carriers, are of questionable quality and rarely support farmers' productivity due to low strain density and/or excessive levels of contamination (Bashan, 1998; Olsen et al., 1995). Ideally, carriers should selectively support optimal growth of biofertiliser strains in desired densities during production, transportation and storage (Herrmann et al., 2015). However, other non-biofertilisers strains are also supported, especially when sterilisation was not performed on the carrier (Malusá et al., 2012). El Fattah et al. (2013) observed that poor-quality carriers caused substantial growth of diverse undesirable microbes in biofertiliser products. All the biofertiliser products examined in the present study had different levels of contamination with major ones found in CB1L, CB6L, CB7L, CB8L, CB9L, CB10L CB11L, CB12L and CB13S (Table 4.12). It is worthwhile to note that these products, except CB1L and CB13S, were produced by the

same manufacturer and possibly, with similar materials and production processes, which suggests the reason for their analogous level of contaminants. These findings agree with that of Herrmann et al. (2015), who found similar levels of contamination in products from the same manufacturers.

Many of the contaminants have been reported as opportunistic pathogens, which are potentially dangerous to the health of humans, crops and the environment (Olsen et al., 1996). Similarly, Herrmann et al. (2015), observed that over 53% of the biofertiliser products tested had these harmful microbes. For example, *Acinetobacter junii, A. modestus, Arthrobacter oryzae* and *Alcaligenes faecalis* subsp. *faecalis* have been found to cause diseases in human beings and animals (Goodfellow et al., 2012; Saffarian et al., 2017; Tille, 2013), while *Bacillus paralicheniformis* and *B. siamensis* have also been found to be human pathogens (Siribaed, 1935). Similarly, *Brevibacillus laterosprus* has been reported as a pathogen of invertebrates (Ruiu, 2013). Other human and animal pathogenic strains included *Cellulomona denverensis, Cupriavidus metallidurans, Escherichia coli, Enterococcus ratti* and *Staphylococcus hominis* subsp. *novobiosepticus* (Brown et al., 2005; Chaves et al., 2005; Langevin et al., 2011; Rivas et al., 2015; Teixeira et al., 2001). Some of these pathogenic microbes have been found to inhibit the growth of beneficial microbes such as rhizobia in biofertiliser products (Gomez et al., 1997). Considering the dangers posed by these microbes, their occurrence in biofertiliser products are to be considered more seriously beyond being ordinary contaminants (Catroux et al., 2001).

5.3.3 The quality of imported and locally produced biofertiliser

The results showed that the majority of the poor-quality products were locally manufactured, signifying the need to improve locally manufactured products and quality-control systems. However, there is contradictory evidence relating to this in literature. Imported biofertiliser products have been reported to be of low quality due to quality challenges arising from ecological differences between the manufacturing country and the country where the products will be applied (Masso et al., 2015; Simiyu et al., 2013). Additionally, various studies have shown that native inoculants are more efficient than exotic commercial species (Kouadio et al., 2017; Oloke & Odeyemi, 1988). However, the conclusions in this study were based on quality parameter evaluation and not on-field experiments. Nevertheless, a poor-quality biofertiliser will not be effective when applied in the field (Faye et al., 2013).

5.3.4 Predictive metagenomics profiling of 16S rRNA gene nucleotide

The predictive metagenomics profiling (PMP) results revealed rhizobia, which is one of the most important nitrogen-fixing biofertilisers, to correlate more with nitrogenase genes, thereby confirming the long-established potential of the group in fixing nitrogen to usable forms of ammonia (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-) using these genes (De Bruijn, 2015; Dighe et al., 2010). Similarly, nitrogenase genes were predicted in some PGPR products (CB9L and CB10L), though in low abundance, suggesting the presence of microbial communities that can possibly participate in BNF. Several studies have reported the nitrogen-fixing ability of *Pseudomonas, Enterobacter* and *Citrobacter,* which were found in the PGPR products (Desnoues et al., 2003; Hatayama et al., 2005; Neilson & Sparell, 1976). Additionally, denitrification genes, which convert nitrate, nitrite or ammonia to nitrogen gas were also predicted to be present in all the products, but with high prevalence in six products. The occurrence of these genes was at a very low level in samples CB10L and CB12L, which were PGPR and rhizobia products respectively, suggesting the use of denitrification pathway amongst the bacterial communities in these products may not be prominent.

Furthermore, the presence of nitrate reductase genes was predicted in high abundance in the products, suggesting the use of nitrate as an alternative to oxygen in order to gain electrons for maintaining the proton-motive force in microbial cells (Tyx et al., 2016). In oxygen-deficient conditions, such as during product storage, respiratory nitrate reductase is often expressed where nitrate is present. However, the build-up of extracellular nitrite during the respiratory process is toxic to microbial cells. To overcome this situation, the nitrite-exporting enzymes are expressed. The nitrite genes were likewise predicted to be present in some of the bacterial communities. Most microorganisms with assimilation and denitrification pathways can further use the produced nitrite (Kraft et al., 2014; Lin & Stewart, 1997; Luque-Almagro et al., 2011).

The PMP showed the dominance of alkaline phosphatase, an important enzyme in phosphate mineralisation, signifying the ability of the community to mineralise phosphate in alkaline pH environments (Behera et al., 2017; Fitriatin et al., 2011). Furthermore, the presence of sulphur-degrading enzymes, especially arylsulphatase, may indicate the role of biofertiliser communities in sulphur cycling and mineralisation in the soil. The ferric-chelate reductase involved in plant iron uptake was also predicted and this could improve crop growth and development through direct or indirect mechanisms of the microbial community (Sayyed et al.,

2010). Similarly, genes encoding for galactosidase and glucosidase predicted in high abundance across the community suggest the ability of the microbial community to use different sources of carbohydrates, such as galactose and glucose. Other important genes predicted were the Shiga, leucocidin and haemolysin toxin genes that have great potential in causing diseases in human beings. These toxins are frequently cytotoxic, destroying host cells by creating unregulated pores in the membranes of the host plants (Gouaux et al., 1997; Laohachai et al., 2003). *Escherichia-Shigella*, a major genus responsible for the production of Shiga toxin, was also a factor in some of these products (Laohachai et al., 2003). The microbial community producing the toxin genes may suffer from loss of viable beneficial strains due to the effect of the toxins, thereby causing poor-quality biofertiliser products (Gomez et al., 1997).

5.3.5 Microbial viable cell density

The microbial cell density results showed that most of the products were of low quality. When compared with the acceptable standards in India (> 5×10^7 CFU/g, solid and >10^8 CFU/ml, liquid) (Malusá & Vassilev, 2014) and South Africa (legume inoculants standard 5×10^8 CFU/g) (Strijdom, 1998), only products CB4L and CB3L had acceptable viable cell density. This accentuated the need to maintain quality viable cells that are metabolically and physiologically competent to unleash the desired benefits of biofertiliser products when used on the field (Xavier et al., 2004). Several biofertiliser quality assessments showed that over 50% of products evaluated had less than the acceptable viable rhizobia per gram of carrier (Rodríguez-Navarro et al., 2010; Singleton et al., 1997). In other instances, some biofertiliser products sold in developed and developing countries have been reported not to contain any rhizobia inoculant (Lupwayi et al., 2000).

Arbuscular Mycorrhizal Fungal biofertiliser quality depends on spore viability, as well as the infectivity potential of the spores on host plants (Habte & Osorio, 2001). The results show that more than 100 viable spores per gram of the product were obtained. This is in agreement with AMF products' acceptable standard (Yadav & Chandra, 2014). It is essential to maintain a high quantity of viable spores in the product to increase the infectivity potential with the host plant. Microbial enumeration is a widely recognised practice in biofertiliser quality control (Elhassan et al., 2010; Lesueur et al., 2016). This is because the number of viable cells transferred to the field is essential in improving the efficiency of the inoculum. Evidence has shown that increasing rhizobial cell numbers applied per seed results in increased nodulation and nitrogen-

fixation (Olsen et al., 1995). According to Hume and Blair (1992), increasing rhizobia population from 10^5 to 10^6 for seed inoculation improved soybean yield by 24%. In addition, increasing the rate of inoculation has been suggested to reduce the dominance of indigenous soil microbes. Therefore, it is important that biofertilisers supply the adequate amount of inoculum to the field for improved competitive advantage over the indigenous strains as well as for inoculum efficiency. Consequently, quality assessment by enumerating the viable microbial density is a seamless index of the potential efficiency of inoculant (Lupwayi et al., 2000).

5.3.6 Limitations of Sanger and next-generation sequencing technologies

Advances in NGS technologies have revolutionised biological sciences through the analysis of environmental DNA using specific gene markers such as species-specific DNA barcodes. A major advantage of NGS is that it bypasses the need for laboratory cultivation and isolation of microbial specimens, a major process necessary in conventional Sanger DNA sequencing (Shokralla et al., 2012). With the challenges of culturing microbes and considering the fact that approximately 99% of all microbes are still unculturable (Vartoukian et al., 2010), Sanger sequencing is highly limited and inadequate for analysing and processing complex environmental samples, especially for large-scale studies (Christine, 2004; Sanger et al., 1977). In addition, most environmental samples contain mixtures of DNA from several hundred or thousands of individuals. Although Sanger sequencing has provided the most efficient technique for building large DNA-barcode reference libraries, the number of individuals in environmental samples is far beyond the scope of its ability (Hajibabaei et al., 2011). However, the isolation of DNA sequences from thousands of species in the complex environmental samples requires the ability to read DNA from multiple templates simultaneously, a process that NGS technologies do effectively and at a lower cost. This was evident in the present study where high numbers of microbial isolates were observed with Illumina MiSeq compared to Sanger sequences (Shokralla et al., 2012).

Generally, obtained nucleotide sequences are compared to a growing standard reference library of known organisms and the correctness of the taxa depends on the completeness of the reference library. For example, the Silver 123 QIIME release and NCBI were used in this study for Illumina and Sanger sequencing respectively, suggesting the taxa results are as good as the database at the time of analysis. Hence, sequences cannot align with recent DNA barcodes not

yet updated in these reference libraries (Quast et al., 2012). This is a limitation that impacts on sequencing-output taxa accuracy. Furthermore, Sanger sequencing is able to recover up to 1 kb of sequence data from a single specimen at a time and with the most advanced version of an automated Sanger sequencer, up to 1 kb for 96 individual specimens at a time can be recovered. However, NGS technologies can potentially generate several hundred thousand to ten millions of sequencing reads in parallel, e.g. from a pool of PCR-amplified molecules (amplicon sequencing) (Shokralla et al., 2012). This may infer a better efficiency in taxonomy. Over 2.88 million reads with 5,791 OTUs were generated in this study.

Despite the huge benefits of NGS technologies, several challenges have been encountered. The relatively short-read length of Illumina MiSeq sequencing outputs due to signal decay and dephasing is a challenge. This limits the application where no reference sequence is available. There is also a high accumulation of error rates with longer sequencing reads (Zhou et al., 2010). In addition, similar to Sanger sequencing, there are problems relating to amplification steps before sequencing. These may include PCR-bias, the formation of chimeric sequences and other secondary related strictures (Shendure & Ji, 2008). Summarily, the use of both techniques in this study compensates for the limitations of each method and therefore increases the robustness of the results, which can give efficient and convincing conclusions.

5.4 Biochemical characterisation of isolates

The biochemical test results showed a high number of isolates can ferment various carbohydrates such as glucose, lactose and sucrose, with the production of acid as a by-product (Park et al., 2005). Hydrogen sulphide is an inorganic acid used by some PGPR in phosphorous solubilisation. However, hydrogen sulphide production from sugar fermentation was not common amongst the test isolates, implying the use hydrogen sulphide mechanism for phosphorous solubilisation was not dominant (Sharma et al., 2013). The majority of the isolates that tested positive for citrate metabolism have the ability to use sodium citrates as their sole carbon source, producing pyruvic acid and carbon dioxide (Faidy & Ali-Shtayeh, 2000). In addition, isolates with ammonia-production abilities have the potential to supply nitrogen in deficient soils and to be used as biofertiliser for improving crop productivity. The ability of the isolates to produce enzymes such as hydrogen cyanide synthase, cytochrome oxidase, lipase and catalase could contribute to the survival of these strains in extreme environments. The production of extracellular lipase and hydrogen cyanide contribute immensely to the biocontrol

ability of biofertiliser strains in suppressing fungal and bacterial pathogens (Ghodsalavi et al., 2013; Khan et al., 2012).

The majority of the isolates could decompose hydrogen peroxide using the catalase while the hydrolysis of urea to ammonia was by urease production. Isolates producing urease are useful in the agricultural soil where urea fertilisers have been applied by increasing the availability of ammonia for crop uptake and as well in maintaining soil health through their functions in the nitrogen cycle (Das & Varma, 2010). Isolates positive for Methyl red and Voges Proskauer were able to metabolise glucose to pyruvic acids or lactic, formic and acetic acids (Goldman & Green, 2015). Several studies have reported beneficial rhizobacteria with different biochemical characteristics similar to the observations in this study (Ahmad et al., 2008; Ghodsalavi et al., 2013; Majeed et al., 2015; Mohan et al., 2008).

5.5 Functional attributes of isolates

5.5.1 Nitrogen fixation ability

In the present study, approximately 40% of the tested isolates could grow on nitrogen-free medium, implying that the isolates have the potential to fix nitrogen when used on nitrogen-deficient soils (Rodrigues et al., 2016). This capability is aided by the nitrogenase gene, which is responsible for the reduction of nitrogen to ammonia (De Bruijn, 2015). Different isolates such as *Bacillus, Alcaligenes* and *Pseudomonas* were found to fix nitrogen with complementary ability to solubilise phosphorous and as well synthesise IAA, making them a good PGPR for biofertiliser formulation (Beneduzi et al., 2008). The results showed similar observations with previous studies. For example, the genera of *Alcaligenes* (You & Zhou, 1989), *Bacillus* (Seldin & Dubnau, 1985) and *Pseudomonas* (Hatayama et al., 2005) have been reported to fix nitrogen. Nitrogen remains one of the essential macronutrients required by plants for metabolism and growth. Despite being in abundance in the atmosphere, it is not accessible by plants, except when it is converted to usable forms of ammonia and nitrate (Galloway et al., 2004). Therefore, the nitrogen-fixation ability of microorganisms is the most desirable traits in BNF.

5.5.2 Phosphate solubilisation ability and acid phosphatase production

The dominant genera associated with high phosphate solubilisation ability were *Pseudomonas, Bacillus, Citrobacter, Alcaligenes* and *Enterococcus*. In phosphorous-deficient or immobilised soils, phosphate solubilising biofertiliser (PSB) is a good alternative to improve plant phosphorous uptake (Jain & Khichi, 2014; Parani & Saha, 2012). The major mechanism of inorganic phosphorous solubilisation has always involved the production of low molecular weight organic acids such as gluconic, oxalic and citric acids to solubilise phosphorous. This ability is ascertained by the halo zone formation on phosphate media (Goldstein & Krishnaraj, 2007; Richardson & Simpson, 2011). Therefore, the *in vitro* assessment of phosphorous solubilisation as measured by the level of halo zone formed could define the solubilisation potential of the isolates and their use as biofertilisers (Sharma et al., 2013). The high phosphate solubilisation index (PSI) (> 2) exhibited by some of the isolates suggest they are good PSB (Behera et al., 2017; Bello-Akinosho et al., 2016; Majeed et al., 2015). Phosphate solubilisation by microbes may be hindered by available soluble phosphorous, toxic metabolites and changes in pH of the medium (Yasmin & Bano, 2011). Similar to this, after seven days of incubation, the rate of phosphate solubilisation amongst the isolates reduced.

Some of the tested isolates had high acid phosphatase production in the range of 7.33–36.96 µg/ml, indicating their organic phosphate-mineralisation potential. Rhizosphere bacteria produce a range of phosphatases and are able to utilise phosphate from different organic sources when cultured in laboratory media. The para-nitrophenyl phosphate used as organic phosphate in this study was hydrolysed to inorganic phosphate, a process catalysed by the phosphatase (Richardson & Simpson, 2011). Phosphatases are essential enzymes with huge potential in increasing sustainability in the rhizosphere and as indicators of soil health. The enzyme plays a crucial role in the phosphorus cycle and has been correlated with soil phosphorous stress and plant growth (Das & Varma, 2010). Different studies have reported similar phosphatase activities in some of the test isolates. For instance, Fitriatin et al. (2011), reported a range of 2.0-4.96 µg/ml of phosphatase in *Bacillus, Pseudomonas, Micrococcus* and *Flavobacterium,* while Behera et al. (2017) reported a maximum enzyme activity of 92.7 U/ml in 2.5 mg/ml of substrate concentration in *Serratia* sp. Most phosphatases found in the soil are produced by rhizosphere microorganisms; therefore, the search for microorganisms producing the enzymes is imperative for formulating an efficient phosphate biofertiliser products (Ribeiro & Cardoso, 2012).

5.5.3 Indole acetic acid production

A varying amount of indole acetic acid (IAA), also known as auxin, was produced by the test isolates. Microbial IAA has been established to stimulate in plants long-term responses such as cell division and tissue differentiation, as well as short-term responses such as increased cell elongation and root architecture (Ji et al., 2014). In addition, IAA influences the activity of ACC (1-aminocyclopropane-1-carboxylic acid) deaminase (Khan et al., 2016). Therefore, isolates producing IAA have huge potential in biofertiliser technology for increased crop growth and development. Several studies have reported varying levels of IAA production in different microorganisms. For example, *Trichoderma* sp. (15.7-39.60 µg/ml) (Dixit et al., 2015), *Pseudomonas* sp. (23.4-53.2 µg/ml) (Ahmad et al., 2005) and *Bacillus* (29.3-51.3 µg/ml) (Islam et al., 2016) have been reported with varying IAA production under different substrate concentrations. The results in this study showed a range of 1.6-115.3 µg/ml amongst the test isolates, with *Hafnia paralvei* producing the lowest concentration at 1.6 µg/ml and *Bacillus velensis* producing the highest concentration at 115.3 µg/ml. Essentially, strains with a high level of IAA production such as *Pseudomonas, Bacillus* and *Enterococcus* could be good biofertiliser with multiple functional abilities; producing growth promoting substances as well as making nutrients available to crops.

5.5.4 Siderophore production

One-third of the tested isolates predominantly belonging to the *Pseudomonas, Alcaligenes, Enterococcus* and *Bacillus* genera produced siderophore. Siderophores are low molecular mass iron-transport agents, which significantly enhance the uptake of metals such as zinc, iron and copper by crops (Beneduzi et al., 2012; Pal & Gokarn, 2010). The highest siderophore production was observed in the genus, *Pseudomonas*, similar to the observation by Ribeiro and Cardoso (2012). The microbes producing extracellular siderophore may indirectly inhibit the growth of harmful fungi and bacteria when it chelates available iron in the soil, thereby depriving native microflora of iron. On the other hand, siderophores may directly suppress the growth of pathogenic organisms stimulating the biosynthesis of antimicrobial compounds (Mathew et al., 2014; Solanki et al., 2014). Therefore, isolates producing siderophore have great potential to confer disease resistance to plants and thus can be used in biocontrol products. For example, siderophores produced by *Pseudomona*s and *Bacillus* have been reported to attack the common fusarium wilt of potato and maize (Beneduzi et al., 2012). However, the antagonistic property of siderophore against plant pathogens was not established in this study.

Chapter Six

Conclusion and Recommendations

6. Conclusion and recommendations

6.1 Conclusion

The underlying objectives of this study were to understand the level of awareness of biofertiliser amongst SHFs and whether good-quality products are available in the South African agro-market. Data obtained revealed that biofertiliser awareness amongst SHFs in the study area was very low and most of the farmers were not aware of the various commercial products available in the agromarket. The low awareness was attributed to inaccessibility of the products as well as low farming experience and low level of education amongst SHFs. Education is an important factor in the adoption of biofertilisers. Soil nutrient management is complex and farmers need some level of knowledge through extension services to comprehend this. Invariably, the aforementioned reasons may have contributed to the non-adoption of biofertiliser among SHFs. This situation suggests the need for efficient biofertiliser policy and promotional strategy that will provide economic incentives and opportunities for improving the awareness and application of biofertilisers amongst SHFs.

Furthermore, the laboratory experiments showed that microbial densities of the majority of the investigated products were lower than the acceptable quality standards. Similarly, some of the products did not contain the claimed microbial strains, implying that the products cannot be effective if used on the field. Findings of this study also revealed that the products had different levels of contamination, which could be a potential risk to the environment as well as animal and human health. Therefore, it is imperative that biofertilisers be made with sterile carriers that are free of contaminants and that supports only the biofertiliser strains.

The general ability of the isolated strains in nitrogen-fixation, phosphate-solubilisation, and indole-3-acetic acid and siderophore production is an indication that some of the isolates may be efficient in biofertiliser production. It is important that strains with dual-functional capabilities be employed in the biofertiliser formulation. In this study, most species of Pseudomonas and *Bacillus* could effectively perform at least two of the major functions of biofertiliser, viz., nitrogen-fixation, phosphate-solubilisation and IAA production. Therefore, consortium products with these species have been suggested in product formulation, for improved field efficiency.

It is also important to state that the majority of the locally manufactured products investigated in this study were of poor quality, emphasising the need to improve national quality-control systems in South Africa. Given that the quality assessment undertaken in this study is only preliminary, other studies investigating the field efficiency of biofertiliser products, especially on various crops and ecological regions are required. These studies will help to demonstrate the benefits of biofertiliser in increasing crop productivity and invariably, increasing SHFs confidence in biofertiliser products.

6.2 Recommendations

Urgent attention should be given to biofertiliser policy and standard formulation in order to ensure that official regulations of biofertilisers quality are enforced. This will increase quality-control awareness on the part of the manufacturer as well as guide biofertiliser manufacturers in maintaining acceptable quality parameters in terms of particle sizes, water-holding capacity, pH, cell density, and moisture content. Such quality-controlled biofertiliser products can enhance optimal crop yield when applied on the field. In addition, government financial support in the form of subsidies should be extended to biofertiliser production to promote biofertiliser commercialisation and market expansion.

Furthermore, the development of biofertilisers with an increased shelf life at room temperature and for durations exceeding 6 months, will help maintain biofertiliser quality between the time of production and field application. Similarly, formulation of biofertilisers using consortia of strains with multiple functional capabilities as well as the use of sterilised carrier materials are essential to maintaining product quality. In addition, the implication of cell-cell communication in the microbial community of biofertiliser products should be considered for future studies.

There is a need to increase biofertiliser awareness and knowledge through capacity building, creating farmer group and efficient extension services. This will increase easy access to training, product samples and information on biofertiliser. Building technical capacity of extension service officers and agro-dealers through “train the trainers” learning technique is also important. Such an approach will aid the dissemination of information on biofertilisers and their applications. These objectives can be achieved through public-private partnership. Moreso, regular collaboration between South Africa and developed countries for the exchange of new technologies and ideas on biofertiliser development may also be considered.

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Appendices

Appendix 1: Questionnaire

Department of Environmental Sciences
College of Agriculture and Environmental Sciences,
University of South Africa

My name is Adekunle Raimi, a student in the Department of Environmental Sciences at the University of South Africa. I am currently carrying out a study to assess the awareness and quality of commercial biofertilisers used by smallholder farmers in South Africa. I would be grateful if you would complete the following questions. This survey is completely anonymous and purely an academic exercise. Any information supplied shall be treated with strict confidence.

Instruction

Please indicate your answers by ticking or writing in the columns provided as you deem appropriate.

Section A.

1. Age: (a) 20-29 years [ ] (b) 30-39 years [ ] (c) 40-49years [ ] (d) 50 and above [ ]
2. Please indicate your educational level:
   (a) diploma Cert./ Degree [ ] (c) Grade 7 Certificate [ ]
   (b) Grade 12 Cert. [ ] (d) None [ ]
3. How long have you been a farmer? …….
   What types of crop do you cultivate? a) …… b) …… c)……..... d)……
4. Do you use fertiliser for your crops? Yes [ ] No [ ]
5. What type do you use? a) Inorganic fertiliser [ ] b) Organic fertiliser [ ]
   C) Biofertiliser [ ] d) Others [ ]
6. Do you know how to apply biofertiliser? Yes [ ] No [ ]
7. How often do you use biofertiliser?
   a) Every season [ ] b) Every other Seasonal [ ]
   c) When necessary [ ] d) Not at all [ ]
8. Please tick the biofertiliser product(s)/ brand you use for your crops?

Waterbac		Nitrasec		Bio-Potash		Eco-Rhizo	
Prosoil		Bio-zinc		Histick		Mycoroot Super grow	
Bio-Azo		Bio-Azospirillum		Lifeforce		Rhizovital	
Bio-Phosphate		Seedbac		Activate N		Mycoroot Booster	
Biostart		Vital soil QCM 360		Firstbase		Vital soil microbe max	
Landbac		Organo		Rhizo flow		Likuiq Semia	

9. Please state any other brand not listed above I) …… II)…… III) …….....
10. What is your major reason for using biofertilisers
    a) Increase nitrogen supply b) Increase phosphorous supply c) Increase potassium supply
    d) Plant growth promoting substances e) Increase crop yield f) All of the above

Section B. Choose the most appropriate that best answers the question.

S/No	Question	**Poor**	**Fair**	**Average**	**Good**	**Excellent**	**Don't know**
1	How would you describe your knowledge about biofertilisers?	○ Poor	○ Fair	○ Average	○ Good	○ Excellent	○ Don't know
2	Overall, what is your perception of the quality of biofertilisers you know of?	○ Poor	○ Fair	○ Average	○ Good	○ Excellent	○ Don't know
3	How would you rate the performance of biofertilisers with respect to other fertilisers	○ Poor	○ Fair	○ Average	○ Good	○ Excellent	○ Don't know
4	How would you rate extension services on biofertilisers	○ Poor	○ Fair	○ Average	○ Good	○ Excellent	○ Don't know
5	Describe the accessibility to commercial biofertilisers	○ Poor	○ Fair	○ Average	○ Good	○ Excellent	○ Don't know
6	How is the support/policy of the government on biofertilisers	○ Poor	○ Fair	○ Average	○ Good	○ Excellent	○ Don't know

Kindly use the space below for other comments you would like to make on commercial biofertilisers.

……
……

Signature: …… Date: ……

Appendix 2: Approval to engage smallholder farmers.

GAUTENG PROVINCE
AGRICULTURE AND RURAL DEVELOPMENT
REPUBLIC OF SOUTH AFRICA

The Coordinator
Department of Environmental Sciences
College of Agriculture and Environmental Science
University of South Africa
PRETORIA
0028

Email: RaimiA@arc.agric.za

To whom it may concern

PERMISSION TO ENGAGE SMALLHOLDER FARMERS IN GAUTENG PROVINCE IN A STUDY THROUGH THE UNIVERSITY OF UNISA

The department has reviewed the request submitted by Mr Raimi for the engagement of smallholder farmers in the Gauteng Province in his study on different types of bio-fertilizers used by such farmers.

The importance of the study for the department has been considered. Therefore, the department grants permission of the ethical clearance for Mr Raimi's independent MSc research project to be conducted through the University of South Africa.

The department wishes Mr Raimi all the best in his studies.

Yours sincerely

Mr A Ismail
ACTING HEAD OF DEPARTMENT
DATE: 24.3.16

11 Diagonal Street, Diamond Building, Newtown, Johannesburg, 2000. Tel: 011 240 25000, Fax: 011 240 2700
Website: www.gdard.gpg.gov.za

Appendix 3: UNISA CAES Ethical approval

UNISA university of south africa

CAES RESEARCH ETHICS REVIEW COMMITTEE

Date: 07/04/2016

Ref #: **2016/CAES/056**
Name of applicant: **Mr A Raimi**
Student #: **57670617**

Dear Mr Raimi,

Decision: Ethics Approval

Proposal: Assessment of the quality of commercial bio-fertilizers used by smallholder farmers in South Africa

Supervisor: Dr R Adeleke

Qualification: Postgraduate degree

Thank you for the application for research ethics clearance by the CAES Research Ethics Review Committee for the above mentioned research. Final approval is granted for the duration of the project.

Please note point 4 below for further action.

The application was reviewed in compliance with the Unisa Policy on Research Ethics by the CAES Research Ethics Review Committee on 06 April 2016.

The proposed research may now commence with the proviso that:

1) *The researcher/s will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.*
2) *Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study, as well as changes in the methodology, should be communicated in writing to the CAES Research Ethics Review Committee. An amended application could be requested if there are substantial changes from the existing proposal, especially if those changes affect any of the study-related risks for the research participants.*
3) *The researcher will ensure that the research project adheres to any applicable*

University of South Africa
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national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study.
4) *The researcher is reminded to adhere to the stipulations in the confidentiality agreement signed with the Gauteng Department of Agriculture.*

Note:
The reference number [top right corner of this communiqué] should be clearly indicated on all forms of communication [e.g. Webmail, E-mail messages, letters] with the intended research participants, as well as with the CAES RERC.

Kind regards,

Signature
CAES RERC Chair: Prof EL Kempen

Signature
CAES Executive Dean: Prof MJ Linington

NB Point 4.

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Appendix 4: Triple Sugar Iron agar (TSI)

Reagent	Composition (g/l)
Yeast Extract	3.0
Beef Extract	3.0
Peptone	15.0
Sodium chloride	5.0
Sucrose	10.0
Dextrose	1.0
Lactose	10.0
Ferrous sulphate	0.2
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	15
pH @ 25 °C	7.0

Appendix 5: Simmon Citrate Agar

Reagent	Composition (g/l)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0
pH @ 25 °C	6.8 ± 0.2

Appendix 6: Ammonia Nessler's reagent

Reagent	Composition (g/l)
Mercuric chloride	22.0
Potassium iodide	50.0
Sodium hydroxide	200 ml
pH at 25 °C	13.2 ± 0.05

With continuous stirring, 50 g of potassium iodide is dissolved in 50 ml of cold distilled water. A saturated solution of mercury chloride (about 22 g in 350 ml of distilled water) is added until precipitates appear indicating excess mercury chloride. Add 200 ml of 5 N of NaOH and ammonia free distilled water to a final volume of 1 litre

Appendix 7: Tween 20 agar medium

Reagent	Composition (g/l)
Peptone	10.0
Calcium chloride	0.1
Sodium chloride	5.0
Tween 20	10 ml
Agar	15.0
pH @ 25 °C	6 ± 0.2

Appendix 8: Urea agar base

Reagent	Composition (g/l)
Peptone	1.0
Glucose	1.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	5.0
Urea	20.0
Phenol Red	0.012

Appendix 9: MR-VP Broth

Reagent	Composition (g/l)
Peptone	7.0
Dextrose	5.0
Potassium phosphate	5.0

Appendix 10: Methyl red indicator and Barritt's reagent

Methyl Red indicator

Dissolve 0.1 g of Methyl Red in 300 ml of ethanol (95%). Add 200 ml of deionised water to make 500 ml of a 0.05% (w/v) solution in 60% (v/v) ethanol. Store solution at 4ºC.

Voges Proskauer reagent:

Barritt's reagent A: 5% (w/v) a-naphthol in absolute ethanol (add 0.6 ml to the test isolates)

Barritt's reagent B: 40% (w/v) KOH in deionized water (add 0.2 ml to the test isolates).

Appendix 11: National Botanical Research Institute Phosphate medium (NBRIP)

Reagent	Composition (g/l)
Glucose	10.0
Tricalcium phosphate	5.0
Agar	15.0
Magnesium chloride hexahydrate	5.0
Magnesium sulphate heptahydrate	0.25
Potassium chloride	0.20
Diammonium sulphate	0.10

Appendix 12: Burk's media

Reagent	Composition (g/l)
Sucrose	20.0
Magnesium sulphate	0.20
Calcium sulphate	0.13
Dipotassium phosphate	0.80
Monopotassium phosphate	0.20
Ferric chloride	0.00145
Sodium molybdate	0.000253

Appendix 13: Nitrogen free bromothymol blue medium

Reagent	Composition (g/l)
DL-Malic acid	5.0
Dipotassium phosphate	0.4
Monopotassium phosphate	0.1
Iron III chloride hexahydrate	0.01
Magnesium sulphate	0.20
Sodium chloride	0.10
Bromothymol blue	0.002
Sodium molybdate	0.002
Calcium chloride	0.02
Agar	1.75
Biotin	0.001
Potassium hydroxide	4.50
pH	7±2 °C

Appendix 14: Congo Red Yeast Extract Mannitol Agar medium (CRYEMA)

Reagent	Composition (g/l)
Agar	20.0
Yeast extract	1.0
Mannitol	10.0
Potassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Congo red	0.025
pH	6.8±2 °C

Appendix 15: Potato Dextrose Rose Bengal Agar

Reagent	Composition (g/l)
Potato infusion	200.0
Dextrose	20.0
Rose Bengal	0.008
Agar	15.0

Appendix 16: CAS agar preparation

Solution A:

1 Dissolve 0.06 g of Chrome Azurol S in 50 ml of distilled water
2 Dissolve 0.0027 g of iron (III) chloride hexahydrate in 10 ml of 10 mM HCl
3 Dissolve 0.073 g of hexadecyltrimethylammonium bromide in 40 ml of distilled water
4 Add solution (1) with 9 ml of solution (2), then mix with solution (3)

Note: The resulting solution should be a blue colour. Autoclave and cool to 50 °C.

Solution B

a. A minimal media 9 (MM9), salt solution stock was made by dissolving 15 g KH_2PO_4, 25 g NaCl and 50 g NH_4Cl in 500 ml of distilled water
b. 20% Glucose stock: dissolve 20 g glucose in 100 ml of distilled water
c. NaOH stock: dissolve 25 g of NaOH in 150 ml of distilled water, pH ~ 12
d. Casamino acid solution: dissolve 3 g of casamino acid in 27 ml of distilled water

CAS agar preparation

i. Add 100 ml of MM 9 solution to 750 ml of distilled water
ii. Dissolve 32.24 g piperazine-N, N-bis (2-ethane sulfonic acid) PIPE.

Note PIPE will not dissolve at pH below 5, bring pH to 6.8 and slowly add PIPE while stirring

iii. Add 15 g Bacto sugar
iv. Autoclave and cool to 50 °C
v. Add 30 ml of sterile casamino acid solution and 10 ml of sterile 20% glucose solution
vi. Slowly add 100 ml of blue dye solution along the glass wall with enough shaking
vii. Aseptically pour plates.

Appendix 17: Table showing chemical rea*g*ents and their preparation for phosphatase assay

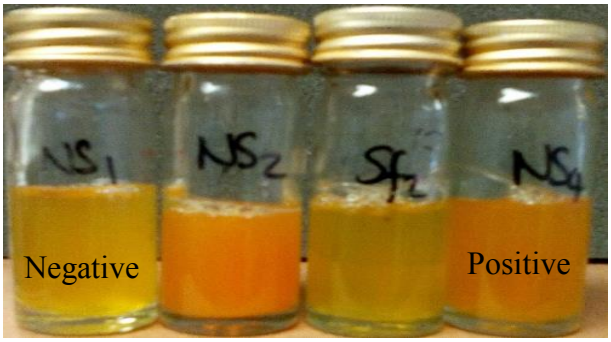
SN	Reagent stock	Preparation
1	Modified universal buffer (MUB) stock	Dissolve all the reagents in 1 L of deionized water *12.1 g of Tris (hydroxymethyl) aminomethane (THAM) *11.6 g of maleic acid, *14.0 g of citric acid, *6.3 g of boric acid
2	MUB working solution pH 6.5 and pH 11.0	Place 200 ml MUB stock solution in a beaker and titrate to pH 6.5 with 0.1 M HCl and while stirring with a magnetic stirrer. Adjust the volume to 1 L
3	ρ-Nitrophenyl phosphate solution (PNP) 0.05 M	dissolve 0.840 g disodium PNP tetrahydrate in 40 ml MUB (pH 6.5) and dilute to 50 ml with the same buffer
4	Calcium Chloride 0.5 M	Dissolve 73.5 g of $CaCl_2$. $2H_2O$ in 1 L of deionized water
5	Sodium hydroxide 0.5 M	dissolve 20 g NaOH in 1 L of deionized water
6	ρ-Nitrophenol (PNP) standard solution	dissolve 1 g of PNP in 1 L of deionized water

Appendix 18: (a) Carbohydrate utilization, (b) Citrate utilization, (c) Ammonia production and (d) Catalase test

(a)

(b)

(c)



(d)

Appendix 19**:** Consent form

CONSENT FORM

TITLE OF RESEARCH PROJECT

QUALITY ASSESSMENT OF COMMERCIAL BIOFERTILISERS AND THE AWARENESS OF SMALLHOLDER FARMERS IN SOUTH AFRICA.

Dear Mr/Mrs/Miss/Ms _____ Date.... /...../.....

NATURE AND PURPOSE OF THE STUDY

Biofertilisers are substances that have been formulated with living beneficial microorganisms which stimulate plant growth by increasing the availability of essential nutrients to the crops. They have been found to fix nitrogen and solubilise phosphorous and potassium. They are cheap, environmentally friendly and have a lower cost of production and application. Hence, the application of biofertiliser can improve the economic value of smallholder farmers through increased crop productivity. However, for optimal efficiency and increased productivity, the microbial and carrier material composition of biofertiisers must conform to the acceptable quality standards. Quality is a factor that can affect the efficiency of biofertilisers. Therefore, the quality of biofertiiser products used among smallholder farmers in South Africa needs to be investigated. This is the purpose of this study.

RESEARCH PROCESS

The study requires your participation in the following manner:

1. 100 voluntary respondents who are smallholders will be required as the targeted research group
2. Respondents must be the owner or manager of a smallholder farm
3. Maybe representative of any age (above 18 years), ethnicity, economic class or gender.
4. Basic demographic information will be required from you such as age and academic qualifications.
5. The questionnaire is mainly about the information on the awareness and types of biofertiiser products used for your crops.
6. You are not required to prepare anything in advance.
7. The questionnaires contain few questions that can be completed in less than 20 minutes

CONFIDENTIALITY

The information given will be treated as strictly confidential and only people working on this study will have access to the information. This is why any form of identity will not be made compulsory in completing this research instrument. No data published in dissertations and journals will contain any information through which you may be identified. Your anonymity is therefore ensured.

WITHDRAWAL CLAUSE

This is a voluntary obligation and you may withdraw from the study at any time without any liability.

POTENTIAL BENEFITS OF THE STUDY

The use of biofertiisers has been considered one of the practices that can be adopted by the smallholders not only to maintain cost-effective operations and increased productivity but also in maintaining ecological balance. Therefore, factors that affect its success such as quality should be researched into in South Africa. At the end of this study, it is expected that the various types and quality of biofertiisers currently available to smallholder farmers in South Africa would have been ascertained. With this information, It is an opportunity for smallholder farmers to know the different types of biofertiisers available in South Africa as well as the quality of biofertiiser products that can increase their productivity. In addition, the manufacturer or importer of these products will use this information to appraise their quality control performance and make amend where necessary.

INFORMATION

If there is any inquiry concerning this study, kindly contact Prof Rasheed Adeleke at Agricultural Research Council, Institute of Soil, Climate and Water. Telephone number: 0123102519 or 0728843243

CONSENT

I, the undersigned…… (full name) have read the above information relating to the project and have also heard the verbal version, and declare that I understand it. I have been afforded the opportunity to discuss relevant aspects of the project with the project leader, and hereby declare that I agree voluntarily to participate in the project.

I indemnify the university and any employee or student of the university against any liability that I may incur during the course of the project.

I further undertake to make no claim against the university in respect of damages to my person or reputation that may be incurred as a result of the project/trial or through the fault of other participants, unless resulting from negligence on the part of the university, its employees or students.

I have received a signed copy of this consent form.

Signature of participant: .....

Signed at …… on ……

WITNESSES

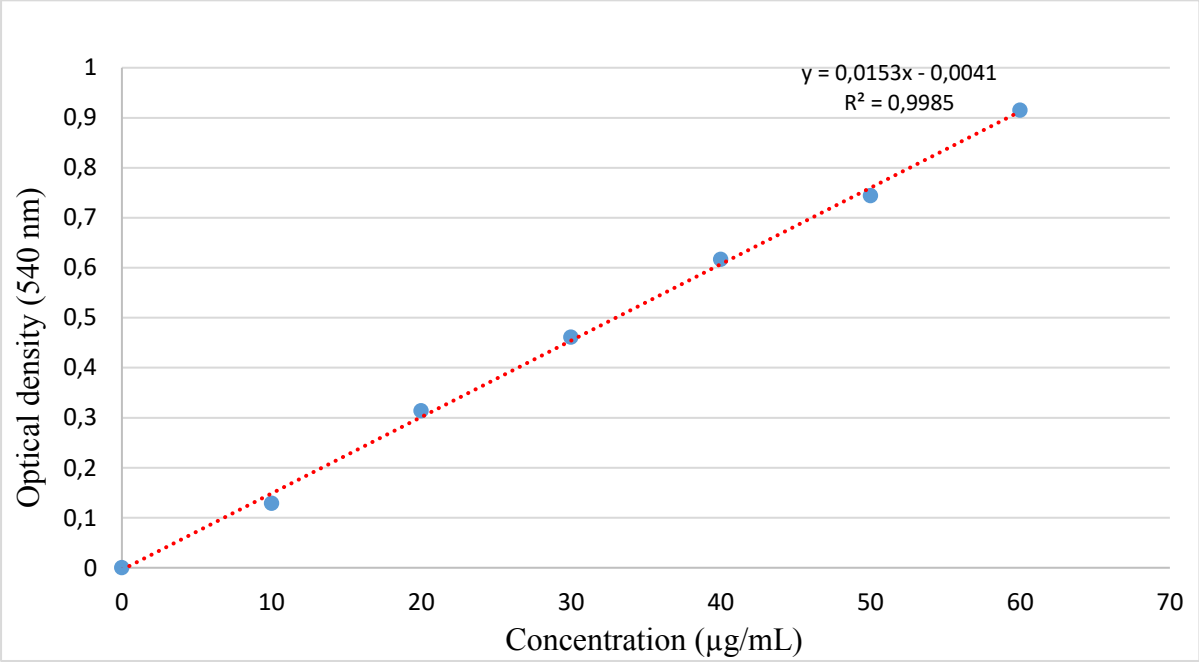
1 .....

2 .....

Appendix 20: Standard curve table for indole-3-acetic acid concentrations at absorbance 540 nm.

Tube No.	Concentration µg/ml	Absorbance (540 nm)
B = blank	0	0
1	10	0,129
2	20	0,314
3	30	0,461
4	40	0,617
5	50	0,744
6	60	0,915

Appendix 21: Indole-3-acetic acid standard curve using Salkowski reagent.



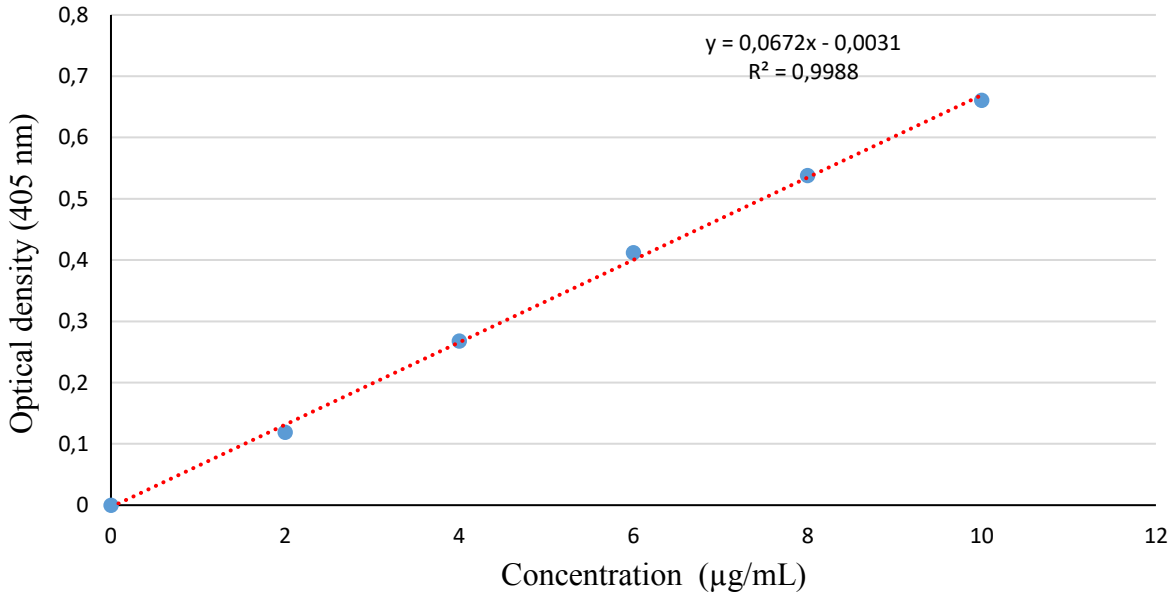
A standard curve was generated from the above table (Appendix 20) and the obtained linear equation ($y = 0.0153x - 0.0041$) from the line of best fit was used to calculate the various concentrations of IAA, having obtained their respective absorbance values (Appendix 21).

Appendix 22: Different concentrations of ρNP and the corresponding absorbance

Tube No.	Stock ρNP (ml)	Acid buffer (ml)	[ρNP] (µg/ml)	Absorbance (405 nm)
B = blank	0	5	0	0
1	1	4	2	0.119
2	2	3	4	0.268
3	3	2	6	0.412
4	4	1	8	0.538
5	5	0	10	0.661

The concentration of the stock solution was 0.001 g/ml made by adding 1 g ρNP in 1000 ml of the buffer. One millilitre of this solution was diluted to 100 ml with distilled water. Aliquots 0, 1, 2, 3, 4, and 5 ml of this solution (conc. 10 µg/ml) were pipetted into sterile McCartney bottles and diluted with distilled water to 5 ml.

Appendix 23: Standard curve of ρ-Nitrophenol concentrations at different optical densities.



A standard curve was generated from the above table (Appendix 22) and the linear equation ($y = 0.0673x - 0.0048$) obtained from the line of best fit was used to calculate the different concentrations of ρ nitrophenol, having obtained their absorbance values.

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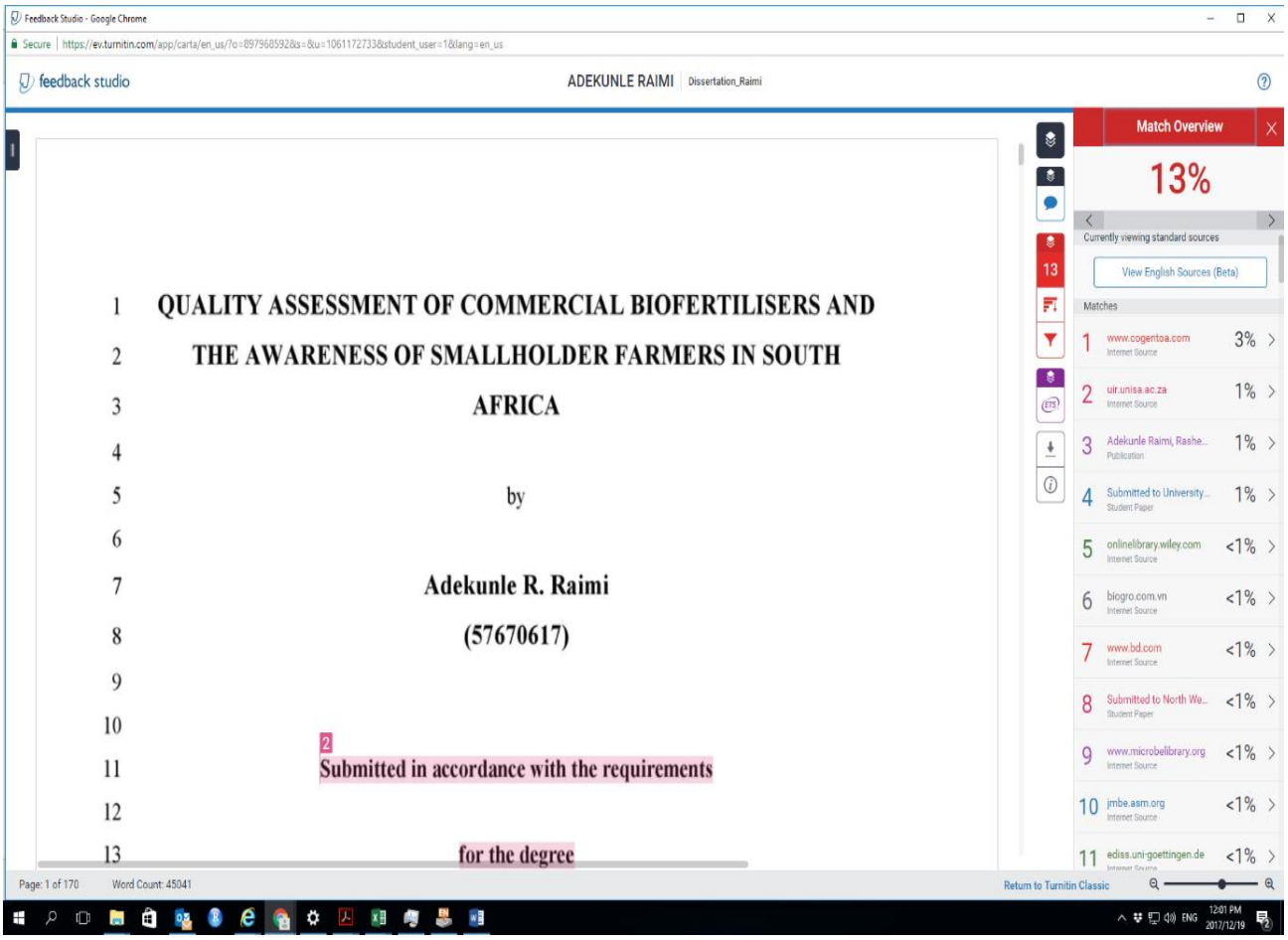

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