PRODUCTION OF LIQUID BIOFERTIZER AND BIOCHEMICAL CHARACTERISATION OF COMPONENT NITROGEN-FIXING AND PHOSPHATE-SOLUBILISING BACTERIA SPECIES

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APPROVAL

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DEDICATION

…for my family.

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All glory be to God Almighty; Whose grace has been ever sufficient, always.

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ABSTRACT

Excessive use of chemical fertilizers have caused a large number of environmental pollutions in water, air and soil. Biofertilizers have, therefore, been developed as a safer and more effective alternative. Biofertilizers refer to any substance that contains living organisms whose activities can improve the plant growth by increasing availability of plant nutrients. This work is aimed at production of liquid biofertilizer using fruits, rice chaff, wheat chaff and soil containing growth-promoting microorganisms; as well as isolation and characterisation of the component nitrogen-fixing and phosphate-solubilising bacteria. *Azotobacter* and *Bacillus subtilis* were isolated using Mannitol Ashby and Pikovskaya agar media, respectively. They were characterised using biochemical tests. This can be used in further research to genetically engineer these organisms, in order to optimise their efficiency.

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CHAPTER ONE

INTRODUCTION

1.1. BACKGROUND

Geometric increase in world population, coupled with effects of global warming/climate change, have had deteriorative effects on agricultural productivity. With an estimated 815 million people undernourished in the world today (FAO, 2017), it is necessary to take fervent steps to improve agricultural productivity. Such steps could include improving seed quality, germination conditions, farming practices and soil quality.

Soil quality can be improved by the use of fertilizer, which can either be of chemical or biological sources. Chemical fertilizers have been in popular use since the 20^{th} century, especially since their contribution to the Third Agricultural (Green) Revolution.

However, excessive and extensive use of chemical fertilizers have resulted in a large number of environmental problems (Savci, 2012); which include water, soil and air pollution. Nitrate content from chemical fertilizers can get into water bodies by drainage, leaching, and flow. This cause eutrophication, leading to algal bloom and suffocation of aquatic life. Also, chemical fertilizers contain heavy metals, such as cadmium and chromium. As such, long-term use may result to accumulation of inorganic compounds in the soil, degrading its quality. Continuous use of chemical fertilizers effects soil degradation and deterioration of soil fertility. This is as it affects soil pH, usually with negative effects on soil organisms, such as worms, soil mite.

Chemical fertilizers also contribute to air pollutions during evaporation of ammonia (NH_3) from ammonia fertilizer; which may be oxidized to nitric acid, and cause acid rain. Also, emissions of nitrogen oxides (NO, N_2O, NO_2) contribute to global warming and climate change.

These detriments of chemicals propagated concerns on the best approach to increase agricultural productivity, while protecting the environment.

The advent of biofertilizer has served to counter the deleterious effects of chemical fertilizers, while being more advantageous. Biofertilizer is commonly referred to as the fertilizer that contains living micro-organisms and it is expected that their activities will influence the soil ecosystem and produce supplementary substance for the plants (Parr *et. al.,* 2002). They contain live and efficient formulates of bacteria, algae and fungi either separately or in combination that are capable of fixing atmospheric nitrogen, solubilising phosphorus, decomposing organic materials or oxidising sulphur and; on application will enhance the availability of nutrients for the benefits of the plants (Hanapi *et. al.,* 2012). They also accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants.

The first generation of commercial biofertilizer – 'Nitragin' – was developed in 1895 by Nobbe and Hiltner, from nitrogen-fixing rhizobacteria isolated from legumes; followed by the discovery of Azotobacter, then the blue green algae and a host of other microorganisms (Ghosh, 2003; Gavrilescu & Chisti, 2005).

Based on formulation, biofertilizers can be either solid or liquid (Chandra *et. al.,* 2005).

1.2. STATEMENT OF PROBLEM

Among the crop nutrients, nitrogen as well as phosphorus and potassium play important roles in increasing the crop productivity (Pindi & Satyanarayana, 2012). Biofertilizers provide an effective alternative to chemical fertilizers. However, with the short shelf-life and high risk of contamination encountered with solid biofertilizers, liquid biofertilizers serve as a better option.

Also, there is need to identify and confirm the component microorganisms of biofertilizer; to assist subsequent research into ways of optimising their performance.

1.3. AIM

This project is aimed at producing a liquid biofertilizer from readily available fruit and plant sources; and basically, identify and characterise the component nitrogen-fixing and phosphate-solubilising bacteria species.

1.4. OBJECTIVES

- Production of liquid biofertilizer
- Isolation of bacteria from the biofertilizer
- Biochemical identification and characterisation of component nitrogen-fixing and phosphate-solubilising bacteria species.

CHAPTER TWO

LITERATURE REVIEW

2.1. BIOFERTILIZER

According to Anubrata & Rajendra (2014), biofertilizers are preparations containing living or latent cells of efficient strains of microorganisms that help crop-plants' uptake of nutrients by their intentions in the rhizosphere, when applied through seed or soil.

Abdullahi *et. al.,* (2012) provide a simple definition, describing biofertilizers as preparations of living cells or efficient microorganisms that help in the uptake of nutrients for the growth of plants.

However, a more encompassing and, perhaps, standard definition comes from Vessey (2003). According to him, a biofertilizer is a substance that contains living organisms which, when applied to seeds, plant surface, or soil, colonise the rhizosphere or interior of the plant, and promotes growth by increasing the supply and/or availability of passing nutrients to the host plant.

The term 'Biofertilizer' does not refer to plant extracts, composted urban wastes, various microbial mixtures with unidentified constituents, or chemical fertilizer formulations supplemented with organic compounds. These can be referred to as 'organic fertilizers' or 'fertilizers containing organic matter'.

Biofertilizers accelerate, as well as augment, certain microbial processes in the soil which promote the availability of nutrients in forms easily assimilated by plants. Use of biofertilizer is one of the most important components of integrated nutrient management; as they are low-cost, effective and renewable. Excessive and extensive use of petrochemical-based fertilizers have caused detrimental effects to the soils, water and food supplies, animals, and even humans

(Laditi *et. al.*, 2012; Savci, 2012). Biofertilizers, on the hand, provide an excellent alternative to chemical fertilizers, as they are made up of, and produced from, biological components. Microorganisms composed in biofertilizer can be bacteria, fungi and/or blue-green algae.

The microorganisms in biofertilizers add nutrients through the natural processes of nitrogen fixation, solubilising and mobilising phosphorus through the synthesis of growth-promoting substances.

2.2. CLASSIFICATION OF BIOFERTILIZER

Biofertilizers can be classified into four major groups, based on their nature and functions:

1. Nitrogen-fixing biofertilizer
2. Phosphorus-solubilising biofertilizer
3. Phosphate-mobilising biofertilizer
4. Plant-growth-promoting biofertilizer

2.2.1. NITROGEN-FIXING BIOFERTILIZER

Nitrogen is considered the most important nutrient for plant growth. This is because it is a major component of both chlorophyll – the compound that facilitates photosynthesis, and amino acids – the building blocks of proteins (Day & Ludake, 1993).

While there is a relative abundance of molecular nitrogen in the atmosphere (about 78%), fixed nitrogen is a major limiting nutrient in plant growth (Ohyama, 2010; Bhat *et. al.*, 2014).

Atmospheric nitrogen can only become available to plants as ammonia (NH_3), through a biological process known as nitrogen fixation. Nitrogen fixation is a process in which nitrogen in the atmosphere is converted into ammonia (NH_3) or other molecules available to living

organisms (Postgate, 1998). Nitrogen fixation occurs naturally in the air by the means of nitrogen oxides (NO_X) production during lightning (Hill *et. al.,* 1979), of which the NO_X may react with water to make nitrous acid or nitric acid; this seeps into the soil, where it becomes nitrate.

Nitrogen fixation can also be done biologically by nitrogen-fixing bacteria; this accounts for about 90% of nitrogen fixation (Encyclopaedia Britannica, 2018). These bacteria may be free-living, symbiotic or associative symbiotic.

- **Free-living nitrogen-fixing bacteria**: These include cyanobacteria (blue-green algae), Azotobacter, Clostridium. The reduction of atmospheric nitrogen to ammonia (nitrogen fixation) is catalysed by the enzyme, nitrogenase (Burk, 1934; Burk *et. al.,* 1934). Nitrogenase requires a large amount of energy (230-420kJ^{-1}). Free-living bacteria obtain the necessary nutrients for supplying this energy. Even as they exist in relatively small concentrations, they are especially important in fixing nitrogen for crops that do not favour symbiotic bacteria, such as corn and wheat.

- **Symbiotic nitrogen-fixing bacteria**: Also called mutualistic, these bacteria form a beneficial relationship with the plants (roots). The plant provides the bacteria with nutrients in form of exudates, which are sugars that act both as carbon source and energy source. The bacteria, in turn, invade the root hair, where they multiply and stimulate formation of root nodules; within which they convert free nitrogen to ammonia, which the host plant utilises for its development. This relationship is most common in leguminous species (e.g. beans, peas), ensuring their optimum growth.

- **Associative symbiotic nitrogen-fixing bacteria**: This group of bacteria does not form symbiotic structures in the host plant. They, however, invade cortical and vascular tissues of the host, and enhance growth of more lateral root hairs. This results in an increase of mineral uptake,

which is due to phytochrome production. This class of bacteria are more common among grasses.

2.2.2. PHOSPHATE-SOLUBILISING BIOFERTILIZER

Phosphorus is the second major plant nutrient-limiting factor for crop productivity (Rathi & Gaur, 2016). It plays a vital role in virtually every plant process that requires energy transfer; as well as in photosynthesis and genetic transfer. Deficiency of Phosphorus reduces plant size and growth.

Although abundant in soils in both organic and inorganic forms, its availability is restricted, as it occurs mostly in insoluble forms (Sharma *et. al.*, 2013); only about 0.1% of total soil phosphorus exists in soluble form, available for plant uptake (Zhou *et. al.*, 1992).

Phosphate-solubilising biofertilizers consist of several strains of bacterial and fungal species that have been observed to solubilise phosphate. These organisms are ubiquitous, but vary in density and mineral-phosphate-solubilising (m.p.s) ability from soil to soil or from one production system to another.

2.2.3. PHOSPHATE-MOBILISING BIOFERTILIZER

Phosphorus is an immobile nutrient (Ludwick, 1998). In most plants, the transportation of phosphorus to the root, rather than the root uptake of phosphorus from the soil, is the main limiting factor for phosphorus uptake (Barber, 1995).

Orthophosphates (Pi), a derivative of Phosphorus, is supplied to the roots by diffusion rather than mass flow; and the rate of diffusion of orthophosphates is slow in the soil. This challenge is

overcome by the symbiotic relationship between plant roots and mycorrhizae. This association is based on bi-directional nutrient transfer between soil fungi and the roots of vascular plants. The plant supplies the fungi with sugars produced by photosynthesis, while the hyphae network improves the plant's capacity to absorb water and nutrients, especially phosphorus (Smith *et. al.*, 2003; Plenchette *et. al.*, 2005).

- **Vesicular Arbuscular Mycorrhizae:** Also referred to as endomycorrhizae; they are soil fungi of the phylum Glomeromycota, forming symbiotic relations with circa 80% of all known plant species (SchuBler *et. al.*, 2001). Arbuscular mycorrhizae are unique in that they penetrate the roots, as well as form arbuscules and vesicles in the cortical cells of the roots Vesicles are thick walled swollen structures and arbuscules are branched haustorial branches of mycelium .They serve as food storage organs of the fungus in the cortical cells of the roots of a vascular plant. Paleobiological and molecular evidence indicate that arbuscular mycorrhizae symbiosis is an association that has lasted at least 460 million years ago, which may have facilitated the development of land plants (Simon *et. al.*, 1993).
- **Ectomycorrhizae:** These fungi form symbiotic relationships with the roots of about 2% of plant species, which tend to be composed of woody plants, including species from the birch, willow, pine and rose families (Smith & Read, 2010). etc.). In general root hairs are absent on roots of some higher plants; therefore roots are infected by mycorrhizal fungi and form a mantle. The hyphae grow intercellularly and develops a Hartig net in the cortex, thus establishing a bridge between the soil and root through the mycelia. They absorb nitrogen, phosphorus, potassium and calcium and produce growth promoting substances i.e. cytokinins.
- **Erocoid Mycorrhizae:** These forms symbiotic relationships between members of the plant family Ericaceae and several lineages of fungi. It represents an important adaptation to the

acidic and nutrient-poor soils in areas typically inhabited by Ericaceae species such as boral forests, bogs and heartlands (Cairney & Meharg, 2003). Ericoid mycorrhizae are characterised by fungal coils that form in the epidermal cells of the fine hair roots of ericaceous species.

- **Orchid mycorrhizae:** These are fungi that form symbiotic relationships with the roots of plants of the family Orchidaceae and a variety of fungi. They are critically important during orchid germination, as an orchid seed has virtually no energy reserve, and obtains its carbon from the fungal symbiont (McCornick *et. al.,* 2012; Sathiyadash *et. al.,* 2012).

2.2.4. BIOFERTILIZER FOR MICRO-NUTRIENTS

Micro-nutrients in plants are essential elements required by plants in small quantities, to ensure and promote pant growth (Tucker, 2016). Also called Trace Elements, they include Boron (B), Copper (Cu), Iron (Fe), Chloride (Cl), Manganese (Mn), Molybdenum (Mo) and Zinc (Zn).

Though a trace element, zinc is very crucial to plant development. Besides being a key constituent of many enzymes and proteins, it plays an important role in a wide range of processes, such as growth hormone production and internode elongation.

Plants can take up zinc as a divalent cation (Kabata-Pendias & Pendias, 2001), but only a very minute portion of total zinc is present in soil in soluble form. Most of the zinc on soil is in the form of insoluble complexes and minerals (Alloway, 2008).

Zinc-solubilising microorganisms, such as *Bacillus aryabhattai*, provide a solution to this challenge, as they solubilise zinc compounds into simpler ones, thus making zinc available to the plants (Kamran *et. al.,* 2017).

Even though not listed among plant macro- and micro-nutrients, silicon is an important element for plant development and increases plant resistance to biotic and abiotic stresses (Ng *et. al.,* 2016), such as salinity, drought, heavy metal toxicities and diseases.

Despite the relative abundance of Si in the soil, most of its sources are not available for plant uptake due to the low solubility of the Si compounds in the soil. To improve plant-availability of Si, silicate-solubilising bacteria, such as *Burkholderia eburnea, Burkholderia vietnamiensis* are important in solubilising insoluble forms of silicate (Santi & Goenadi, 2017).

2.3. MICROORGANISMS THAT ACT AS BIOFERTILIZERS

2.3.1. NITROGEN-FIXING

2.3.1.1. *Rhizobia*

Legume plants have root nodules, where atmospheric nitrogen fixation is done by bacteria belonging to genera, *Rhizobium*, *Bradyrhizodium*, *Azorhizobium*; collectively called rhizobia. When rhizobial culture is inoculated in field, pulse crops yield can be increased due to rhizobial symbiosis (Dubey, 2006). Rhizobium can increase crop yield up to 20%.

- ***Azorhizobium***

It is a stem nodule forming bacteria and fixes nitrogen symbionts of the stem nodule. Also, it produces a large amount of indole acetic acid (IAA) that promotes plant growth.

- ***Bradyrhizobium***

Bradyrhizobium strain inoculation with mucuna seeds enhances total organic carbon, N_2, phosphorus and potassium in the soil, increases plant growth and consequently plant biomass, reduces the weed population and increases soil microbial population.

2.3.1.2. *Diazotrophs*

These are aerobic chemolithotrophs and anaerobic photoautotrophs. These are non-nodule forming bacteria and include numbers of the families:

- ***Azotobacteracae: e.g. Azotobacter***

They are the free living aerobic, photoautotrophic, non-symbiotic bacteria. They secrete vitamin-B complex, gibberellins, napthalene, acetic acid and other substances that inhibit certain root pathogens and improve root growth and uptake of plant nutrients. *Azotobacter indicum* occurs in acidic soil in sugarcane plant roots. It can be applied in cereals, millets, vegetables and flowers through seed, seedling and soil treatments.

- ***Spirillaceae: e.g. Azospirillum and Herbaspirillum***

These are gram- negative, free living, associative symbiotic and non-nodule forming, aerobic bacteria, that occur in the roots of dicots and monocot plants i.e. corn, sorghum, wheat etc. They are easy to culture and identify.

Azospirillum is found to be very effective in increasing 10-15% yield of cereal crops and fixing N_2. Inoculation of different *A. brasiliense* strains in the wheat seed causes increase in seed germination, plant growth, plumule and radicle length. *Herbaspirillum* species occurs in roots, stems and leaves of sugarcane and rice. They produce growth promoters (IAA, gibberillins, cytokinins) and enhance root development and uptake of plant nutrients (N, P & K).

- ***Acetobacter diazotrophicus***

Acetobacter diazotrophicus is another diazotroph that occurs in roots, stem and leaves of sugarcane and sugar beet crops as nitrogen fixer and applied through soil treatment.

It also produces growth promoters e.g. IAA and helps in nutrients uptake, seed germination, and root growth. This bacterium enhances crop yield by up to 0.5 – 1% (Gahukar, 2005-06).

2.3.1.3. *Cyanobacteria (Blue green algae)*

Nostoc, *Anabaena*, *Oscillatoria*, *Aulosira*, *Lyngbya* etc. are prokaryotic organisms and are phototropic in nature. They play an important role in enriching paddy field soils by fixing atmospheric nitrogen and supply vitamin B complex and growth promoting substances which make the plant to grow vigorously. Cyanobacteria increase crop yield by 10-15% when applied. Youssef and Ali (1998) reported that three blue-green algae, *Anabena oryzae*, *Nostoc calcicola* and *Spirulina* sp. reduced number of galls and eggmasses caused by the root knot nematode *Meloidogyne incognita*, which infects cowpea; thus improving plant growth.

2.3.1.4. *Azolla – Anabaena symbiosis*

Azolla is a free-floating, aquatic fern found on water surface, which has a cyanobacterial symbiont, *Anabaena azollae* in its leaves. It fixes atmospheric nitrogen in paddy fields and excretes organic nitrogen in water during its growth and also immediately upon trampling. *Azolla* contributes nitrogen, phosphorus, potassium and organic carbon; as well as increases 10-20% yield of paddy crops and also suppresses weed growth. *Azolla* also absorbs traces of potassium from irrigation water and can be used as green manure before rice planting. *Azolla spp.* are metal-tolerant, hence can be applied near heavy metal polluted areas.

2.3.2. PHOSPHATE-SOLUBILISING:

Pseudomonas fluorescens, *Bacillus megatherium* var. *phosphaticum*, *Acrobacter acrogens*, *nitrobacter spp*., *Escherichia freundii*, *Serratia* spp., *Pseudomonas striata*, *Bacillus polymyxa* are the bacteria which have phosphate solubilising ability. Phosphobacterin are the bacterial fertilizers containing cells of *Bacillus megatherium* var. *phosphaticum* (first prepared by USSR

scientists). They increased crop yield by about 10-20% (Cooper, 1959) and also produced plant growth promoting hormones which helped in phosphate solubilizing activity of soil.

Al-Rehiayani *et. al*. (1999) found that *B. megaterium* reduced penetration of *M. chitwoodi* and *Pratylenchus penetrans* into potato roots by 50%. Padgham & Sikora (2007) stated that treatment with *Bacillus megatherium* resulted in 40% reduction in nematode penetration and gall formation compared with non-treated rice plants. Khan *et. al*. (2007) stated that biofertilizers, based on plant-growth microorganisms, particularly phosphate-solubilising microorganisms in place of inorganic fertilizers, could also be used in nematode disease management.

Some fungi also have phosphate-dissolving ability e.g. *Aspergillus niger, Aspergillus awamori, Penicillium digitatum* etc.

2.3.3. PHOSPHATE-MOBILISING:

2.3.3.1. MYCORRHIZAE

Mycorrhizae are developed due to the symbiosis between some specific root-inhabiting fungi and plant roots, and are used as biofertilizers. They absorb nutrients such as manganese, phosphorus, iron, sulphur, zinc etc. from the soil and pass them to the plant. Mycorrhizal fungi increase the yield of crops by 30-40% and also produces plant growth promoting substances

- **Vesicular Arbuscular Mycorrhizae (VAM) or Endo-mycorrhizae**

They occur commonly in the roots of crop plants. VAM fungal hyphae enhance the uptake of phosphorus and other nutrients that are responsible for plant growth stimulation including roots and shoot length. VAM also enhances the growth of black pepper and protects from

Phytophthora capsici, *Radopholus similis* and *Meloidogyne incognita* (Anandraj *et. al.,* 2001). VAM fungi enhance water uptake in plants and also provide heavy metal-tolerance to plants. Bagyaraj *et. al.* (1979) reported that inoculation of tomato roots with root knot nematodes enhanced infection and spore production by vesicular arbuscular mycorrhizal fungus, *Glomus fasciculatus.* Inoculation of tomato plants with this fungus significantly reduced the number and size of the root knot galls produced by root knot nematode, *Meloidogyne incognita* and improved plant growth criteria.

Suresh *et. al.* (1885) showed that the number of giant cells caused by *M. incognita* and formed in mycorrhizal plants were significantly low, Root extract from the mycorrhizal plants brought about 50% mortality of the nematode larvae in four days. Hajra *et. al.* (2013) reported that leaf area and plant height were increased in mycorrhizal plants than non-mycorrhizal, while they showed a sharp decrease in nematode-infected plants. The same plants showed less water content due to xylem vessel damage. In mycorrhizal plants, roots had large amount of carbohydrates indicating transfer of photosynthates to the fungal partner. Nematode-infected roots have least amount of carbohydrates showing a great sink of carbon to rhizosphere.

2.3.4. PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

They are also called as microbial pesticides e.g. *Bacillus spp.* and *Pseudomonas fluorescens*. Species such as *Serratia spp.* and *Ochrobactrum spp.* are able to promote growth of plants; while application of *P. fluorescens* to black pepper enhances uptake of nutrients, which increase plant biomass.

Fluorescent rhizobacteria improve the growth of *Hevea brasiliensis* plants. Bevivino *et. al.* (1998) found that rhizobacteria could stimulate plant growth; directly by producing growth

hormones and improving nutrient uptake, and indirectly by changing microbial balance in the rhizosphere in favour of beneficial microorganisms.

2.4. STATES OF BIOFERTILIZER

At present, biofertilizers can be found in solid and liquid forms (Ahmad *et. al.,* 2011).

2.4.1. SOLID-CARRIER BIOFERTILIZER

This involves a carrier material, which is used as a vehicle for the microorganisms to be used as biofertilizer (Brar *et. al.,* 2012), for subsequent seed or soil inoculation.

Such materials may have a role in maintaining the viability (shelf-life) of the microorganisms prior to its release into the field as well as also providing a suitable microenvironment for rapid growth of the organisms upon their release. A carrier could be a material, such as peat, vermiculite, lignite powder, clay, talc, rice bran, seed, rock phosphate pellet, charcoal, soil, paddy straw compost, wheat bran or a mixture of such materials. In common practice, for better shelf-life of biofertilizer formulation, a carrier or a mixture of such carrier materials are selected based on the viability of the microorganisms mixed with them. For preparation of seed inoculant, the carrier material is fine powder with particle size of 10-40μm (Ma & Kalaiyarasi, 2015).

According to the "Handbook for Rhizobia" (Somasegaran & Hoben, 1994), the properties of a good carrier material for seed inoculation are:

(i) Non–toxic to inoculant bacteria strain.

(ii) Good moisture absorption capacity.

(iii) Easy to process and free of lump-forming material.

(iv) Easy to sterilize by autoclaving or gamma-irradiation.

(v) Available in adequate amount.

(vi) Inexpensive.

(vii) Good adhesion to seed.

(viii) Good pH buffering capacity.

(ix) Non–toxic to plant.

Other essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered:

(i) Survival of the inoculant bacteria on seed. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying condition until placed into soil.

(ii) Survival of the inoculant bacteria during the storage period.

(iii) Survival of the inoculant bacteria in soil. After being introduced into the soil, the inoculant bacteria have to compete with native soil microorganisms for the nutrient and habitable niche, and have to survive against grazing protozoa.

Such carrier materials that offer the available nutrient and/or habitable micro-pore to the inoculant bacteria will be desirable. In this sense, materials with micro-porous structure, such as soil aggregate and charcoal, will be good carriers for soil inoculant.

2.4.1.1. STERILIZATION

Sterilization of carrier material is essential to keep high number of inoculant bacteria on carrier for a long storage period.

Gamma-irradiation is the most suitable way of carrier sterilization, because the sterilization process makes almost no change in physical and chemical properties of the material. The carrier material is packed in thin-walled polyethylene bag, and then gamma-irradiated at 50 kGy (5 Mrads).

Another way of carrier sterilization is autoclaving. Carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121ºC. It should be noted that during autoclaving, some materials change their properties and produce substances toxic to some bacterial strains.

2.4.2. LIQUID BIOFERTILIZER

According to Pindi & Satyanarayana (2012), liquid biofertilizer is a consortium of microorganisms provided with suitable liquid medium to keep up their viability for certain period which aids in enhancing the biological activity of the target site. Hegde (2008) defined liquid bio-fertilizers as special liquid formulation containing not only the desired microorganisms and their nutrients but also special cell protectants or chemicals that promote formation of resting spores or cysts for longer shelf life and tolerance to adverse conditions.

Liquid biofertilizers contain living species of microorganisms which have the ability to mobilize nutritionally important elements from non-usable to usable forms through biological processes such as nitrogen fixation, phosphate solubilization or mobilization, excretion of plant growth promoting substances or cellulose or lignin degradation, to increase the crop productivity.

Challenges encountered in solid-carrier biofertilizer include low shelf-life of the microorganisms – six months, with an initial population density of 10^8 c.f.u/ml (Bhattacharyya and Kumar,

2000), non-tolerance to UV rays and temperatures more than 30°C. However, these challenges are overcome by liquid biofertilizers.

Microorganisms in liquid biofertilizers have an average shelf-life of two years, a population count as high as 10^9 c.f.u/ml, which is maintained up to two years; they are also tolerant to high temperatures (up to 55°C) and ultraviolet radiations (Mahdi *et. al.,* 2010).

2.5. BIOFERTILIZER APPLICATION METHODOLOGY

2.5.1. SOLID BIOFERTILIZER

There are three ways of applying solid biofertilizers.

- Seed treatment
- Root dipping
- Soil application (Singh & Kumar, 2015).

2.5.1.1. SEED TREATMENT

Seed treatment is a most common method adopted for all types of inoculants (TNAU Agritech Portal, 2014). The seed treatment is effective and economic. Seed Treatment can be done with *Rhizobium*, *Azotobacter*, *Azospirillum*, along with Phosphate Solubilising Microorganisms (PSM).

There is no antagonistic effect when seed treatment is done with more than two bacteria. The important things that has to be kept in mind are that the seeds must be coated first with *Rhizobium, Azotobacter* or *Azospirillum*. When each seed gets a layer of above bacteria then

PSM inoculant can be coated as outer layer. This method will provide maximum number of each bacteria required for better results. Treatments of seed with any two bacteria will not provide maximum number of bacteria on individual seed.

2.5.1.2. ROOT DIPPING

This method is used for transplanted crops (Motghare & Gauraha, 2012). The seedling roots of crops are treated for half an hour in a biofertilizer solution before transplantation in the field. For this, a bucket having adequate quantity of water is taken and the biofertilizer is mixed properly. The roots of the seedlings are then dipped in this mixture so as to enable the roots to get inoculum. These seedlings are then transplanted. This method has been found very much suitable for crops like tomato, rice, onion, cole crops and flowers.

2.5.1.3. SOIL APPLICATION

This method is mostly used for fruit crops, sugarcane and other crops where localized application is needed. Before use, the inoculants are incubated with the desired amount of well-decomposed granulated farmyard manure (FYM) for 24 hours. The FYM acts as nutrition medium and adjuvant (carrier) for the biofertilizer. Sometimes, biofertilizers are also introduced in the soil but this may require four to ten times more amount of biofertilizer.

2.5.2. LIQUID BIOFERTILIZER

Liquid biofertilizers can be applied by spraying and fertigation (Bhawsar, 2011). Liquid biofertilizers are usually concentrated; and thus need to be diluted with water before application

to the intended plants, in order to prevent chances of fertilizer burn. Spraying is recommended for standing citrus plants, vines, mango, guava, apple and peach orchards. In fertigation, the biofertilizer is mixed with water and other micronutrients in a tank. It is distributed to individual plants by irrigation sprinklers/sprayers/piping. Fertigation is usually employed in shade nets or greenhouses.

2.6. CONSTRAINTS OF BIOFERTILIZER

Despite the aforementioned benefits and attributes of biofertilizers, there are constraints that hinder its widespread adoption and acceptance. These can be grouped under the following categories:

- Technical
- Infrastructural
- Human Resources
- Environmental
- Social
- Marketing (Bodake *et. al.*, 2009; Purohit & Dodiya, 2014; Singh & Kumar, 2015)

2.6.1. TECHNICAL CONSTRAINTS

- Unavailability of good quality carrier material.
- Use of improper, less efficient strains for production.
- Short shelf life of inoculants.

2.6.2. INFRASTRUCTURAL CONSTRAINTS

- Non-availability of suitable facilities for production.
- Lack of essential equipment, power supply etc. for adequate incubation and storage of inoculants.

2.6.3. HUMAN RESOURCES CONSTRAINTS

- Lack of technically qualified staff in the production units.
- Lack of suitable training on the production techniques.

2.6.4. ENVIRONMENTAL CONSTRAINTS

- Soil characteristics like salinity, acidity, drought, water logging, toxicity etc.
- Simultaneous cropping operations and planting practices.

2.6.5. SOCIAL CONSTRAINTS

- Unawareness on the benefits of the technology.
- Lack of confidence towards different biofertilizer practices
- Unawareness on the damages caused on the ecosystem by continuous application of inorganic fertilizers.

2.6.6. MARKETING CONSTRAINTS

- Lack of retail outlets or the market network for the producers.
- Unavailability of proper transportation and storage facilities.
- Limited demand.

CHAPTER THREE

MATERIALS AND METHODS

The objectives of this work are:

- Production of liquid biofertilizer
- Isolation of bacteria from the biofertilizer
- Biochemical identification and characterisation of component nitrogen-fixing and phosphate-solubilising bacteria.

This work was carried in the Biotechnology Laboratory of Godfrey Okoye University, Thinkers' Corner, Enugu, Enugu State, Nigeria.

The soil sample used was gotten from the rhizosphere of a fully-developed mango tree, about 5cm beneath the soil surface. The fruits used were gotten from a local market.

3.1. LIQUID BIOFERTILIZER PRODUCTION

3.1.1. MATERIALS

Fresh soil sample, wheat chaff, rice chaff, orange, cucumber, banana, grapefruit, moringa leaves, brown sugar, water.

3.1.2. MICROORGANISM CULTURE

The following procedure was used to culture the microorganisms:

1. 250g of the fresh soil sample was mixed with 250g of the wheat chaff and 250g of rice chaff until a homogenous mixture was formed. This was done in a low depth bowl.

2. 250ml of water was mixed into the mixture.

3. A depression was made at the centre of the mixture to enable proper air circulation.

4. The bowl was covered and kept to stand under a shady area for 7 days.

White hyphae were observed to form on top of the mixture from the second day of incubation.

3.1.3. PRODUCTION OF THE LIQUID BIOFERTILIZER

The following materials were used in the production of the liquid biofertilizer: orange, cucumber, banana, grapefruit, moringa leaves, brown sugar, microorganism culture.

The following procedure was used:

1. 1kg orange, 1kg cucumber, 1kg banana, 1kg grapefruit, 500g moringa leaves were cut into pieces and put in a container.

2. 500g of brown sugar and 500g of the microorganism culture was added to the container.

3. The materials were mixed properly, until the mixture became consistent.

4. 15L of water was added to the mixture; this was stirred in one direction for some time.

5. The container was tightly sealed and kept to stand in a dark place for two weeks, without disturbance.

White hyphae were observed to form on top of the mixture after two weeks. The mixture was sieved; and the resulting liquid, which had a golden-yellow colour, was transferred into a gallon. The liquid is the biofertilizer, and was stored for further analysis.

3.2. ISOLATION OF NITROGEN-FIXING BACTERIA AND PHOSPHATE-SOLUBILISING BACTERIA

The sample (liquid biofertilizer) was cultured, using two selective media: Mannitol Ashby agar media for Azotobacter and Pikovskaya agar media for Phosphorus-solubilising bacteria.

3.2.1. MATERIALS

Petri dishes, test tubes, measuring cylinder, Bunsen burner, test tube rack, distilled water, glass spreader, ethanol, cotton wool, aluminium foil, autoclave, weighing balance, spatula, flat bottom flasks, large beaker, Pikovskaya agar media, Mannitol Ashby agar media.

3.2.2. ISOLATION OF NITROGEN-FIXING BACTERIA

3.2.2.1. PROCEDURE

1. 100ml of Mannitol Ashby agar media was formulated with the following components:

Mannitol	2g
Potassium Hydrogen Phosphate (K_2HPO_4)	0.02g
Sodium Chloride (NaCl)	0.02g
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	0.02g
Potassium Sulphate (K_2SO_4)	0.01g
Calcium Carbonate ($CaCO_3$)	0.5g
Agar-agar	2g
Distilled water	100ml

2. The agar media was sterilised in an autoclave at 121°C for 15 minutes.

3. The media was poured into a petri dish, and was allowed to solidify at room temperature.

4. Serial dilution of the sample was made, and the 10^{-3} diluent was spread on the solidified media with a glass spreader.

5. The sample was incubated at 37°C.

3.2.3. ISOLATION OF PHOSPHATE-SOLUBILISING BACTERIA

3.2.3.1. PROCEDURE

1. 100ml of Pikovskaya agar media was formulated with the following components:

Glucose	1g
Calcium Phosphate ($Ca_3(PO_4)$	0.5g
Sodium Chloride (NaCl)	0.02g
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	0.01g
Manganese (II) Sulphate Dihydrate ($MnSO_4.2H_2O$)	0.00025g
Iron (II) Sulphate Heptahydrate ($FeSO_4.7H_2O$)	0.00025g
Yeast	0.05g
Agar-agar	2g
Distilled water	100ml

2. The agar media was sterilised in an autoclave at 121°C for 15 minutes.

3. The media was poured into a petri dish, and was allowed to solidify at room temperature.

4. Serial dilution of the sample was made, and the 10^{-3} diluent was spread on the solidified media with a glass spreader.

5. The sample was incubated at 37°C.

3.3. GRAM STAINING

3.3.1. MATERIALS

Wire loop, normal saline, Bunsen burner, glass slides, oil immersion, distilled water, Crystal violet, Lugol's iodine, safranin, iodine, light microscope.

3.3.2. PROCEDURE

1. A smear of the bacteria sample was made. This was done by placing a drop of sterile saline on a clean glass slide. The wire loop was then flamed to red hot and allowed to cool, and then a small amount of cells from an isolated colony on a culture plate was collected and mixed in the drop of saline; and then spread to make a thin, uniform smear.

2. The smear was allowed to air dry before heat-fixing by carefully passing the slide over a Bunsen burner flame six to eight times.

3. The smear was flooded with crystal violet staining reagent for a minute. The slide was washed with distilled water and drained.

4. The slide was flooded with Lugol's iodine (the mordant) for a minute. It was washed off gently with distilled water and drained.

5. The slide was flooded with acetone (the decolourising agent) for 3 seconds, and it was washed off quickly with distilled water and drained.

6. The slide was flooded with safranin (the counter stain) for one minute. The slide was washed with distilled water and air-dried.

7. The smear was viewed x100 oil-immersion objective of a light microscope.

3.4. BIOCHEMICAL TESTS FOR ANALYSIS OF ISOLATED MICROORGANISMS

3.4.1. INDOLE TEST

3.4.1.1. MATERIALS

Kovac's reagent, peptone water, wire loop incubator.

3.4.1.2. PROCEDURE

1. The test organism was inoculated in sterilised tube containing peptone water broth.

2. The solution was incubated at 37ºC for 24 hours.

3. 0.5ml of Kovac's reagent was added to the broth.

4. The result was noted.

3.4.2. METHYL-RED TEST

3.4.2.1. MATERIALS

MR-VP medium (glucose broth), methyl-red indicator, wire loop.

3.4.2.2. PROCEDURE

1. A sterile inoculating loop was used to inoculate the bacterial sample into the fresh, sterile medium.

2. The inoculated tube was incubated at 35-37°C for three days.

3. After incubation, add 5 drops of Methyl Red reagent was added to the broth.

4. The colour was observed.

3.4.3. VOGES-PROSKAUER TEST

3.4.3.1. MATERIALS

MR-VP broth, alpha naphthol, 40% KOH, deionized water.

3.4.3.2. PROCEDURE

1. A tube containing the MR/VP broth was inoculated with the pure culture of the bacteria sample and was incubated at 35^{o}C at 24 hours.

2. 1.5ml of 5% alpha naphthol and 0.5ml of 40% KOH was added and mixed properly.

3. The tubes were left to stand for 5 minutes for aeration.

4. Colour changes were observed.

3.4.4. CITRATE TEST

3.4.4.1. MATERIALS

Simmon's citrate agar, test tubes, wire loop.

3.4.4.2. PROCEDURE

1. Simmon's citrate agar was prepared and autoclaved at 121°C for 15 minutes. 5ml was used to prepare slant medium in test tubes.

2. The bacteria sample was then inoculated on the Simmon's citrate agar using sterile wire loop.

3. The tubes were incubated at 37^{o}C for 36 hours.

4. Colour change was observed.

3.4.5. OXIDASE TEST

3.4.5.1. MATERIALS

Oxidase reagent, petri dishes, Whatman's No 2 filter paper, wire loop.

3.4.5.2. PROCEDURE

1. A piece of filter paper was placed in a clean Petri-dish and 3 drops of freshly prepared oxidase reagents were added.

2. Using a sterile loop, the bacteria sample was smeared over a small area of the filter paper.

3. The colour change was examined after 10 seconds.

3.4.6. CATALASE TEST

3.4.6.1. MATERIALS

3% H_2O_2, glass slide, test tubes, wire loop, normal saline.

3.4.6.2. PROCEDURE

1. A drop of normal saline was placed on the glass slide.

2. A smear of the bacteria sample was made using a sterile wire loop.

3. A drop of 3% H_2O_2 was placed on the smear.

4. The sample was observed for immediate bubbling.

CHAPTER FOUR

RESULTS

This chapter contains the results of the production of the biofertilizer, as well as of isolation and characterisation of the nitrogen-fixing and phosphate-solubilising bacteria species.

Fig. 1. Liquid Biofertilizer

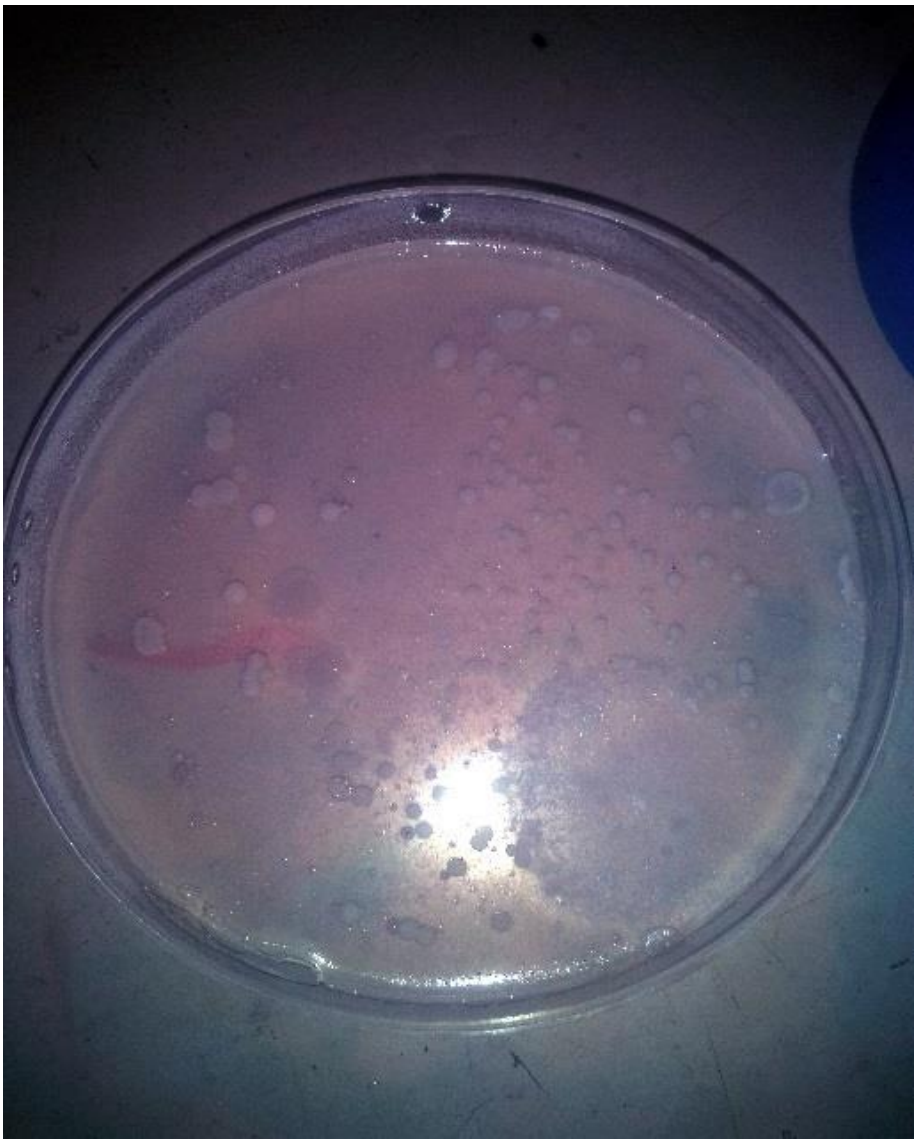

Fig. 2. Nitrogen-fixing Bacteria, with visible colonies

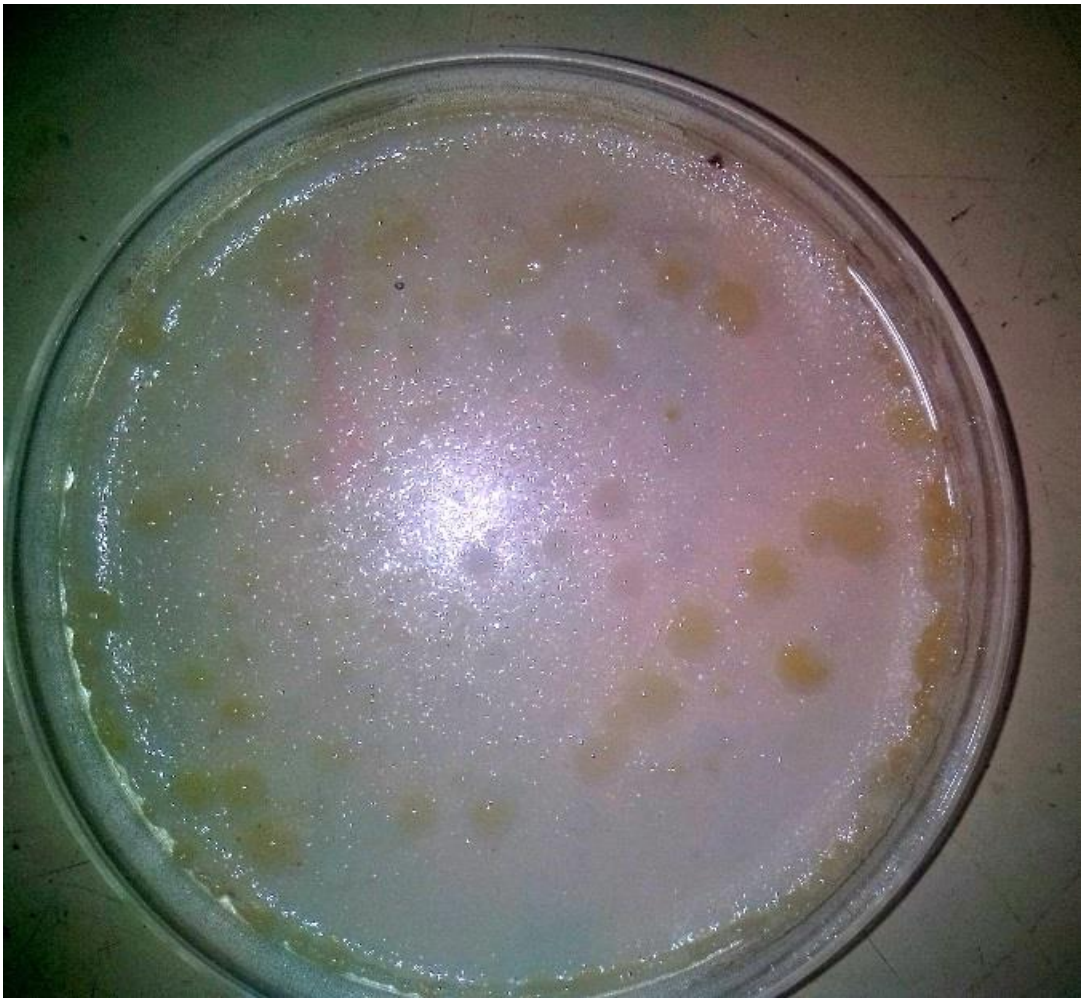

Fig. 3. Phosphate-solubilising Bacteria, with visible colonies

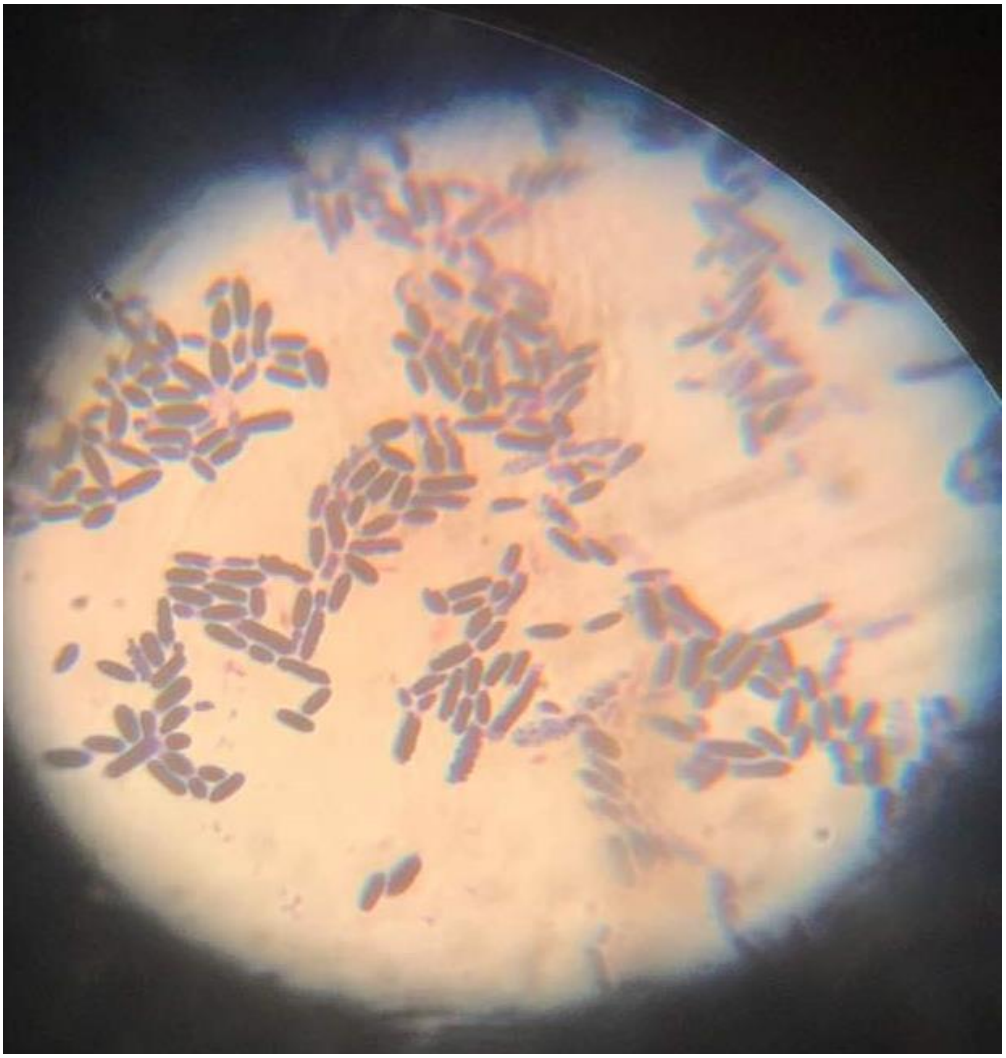

Fig. 4. Gram-stained Phosphate-solubilising Bacteria

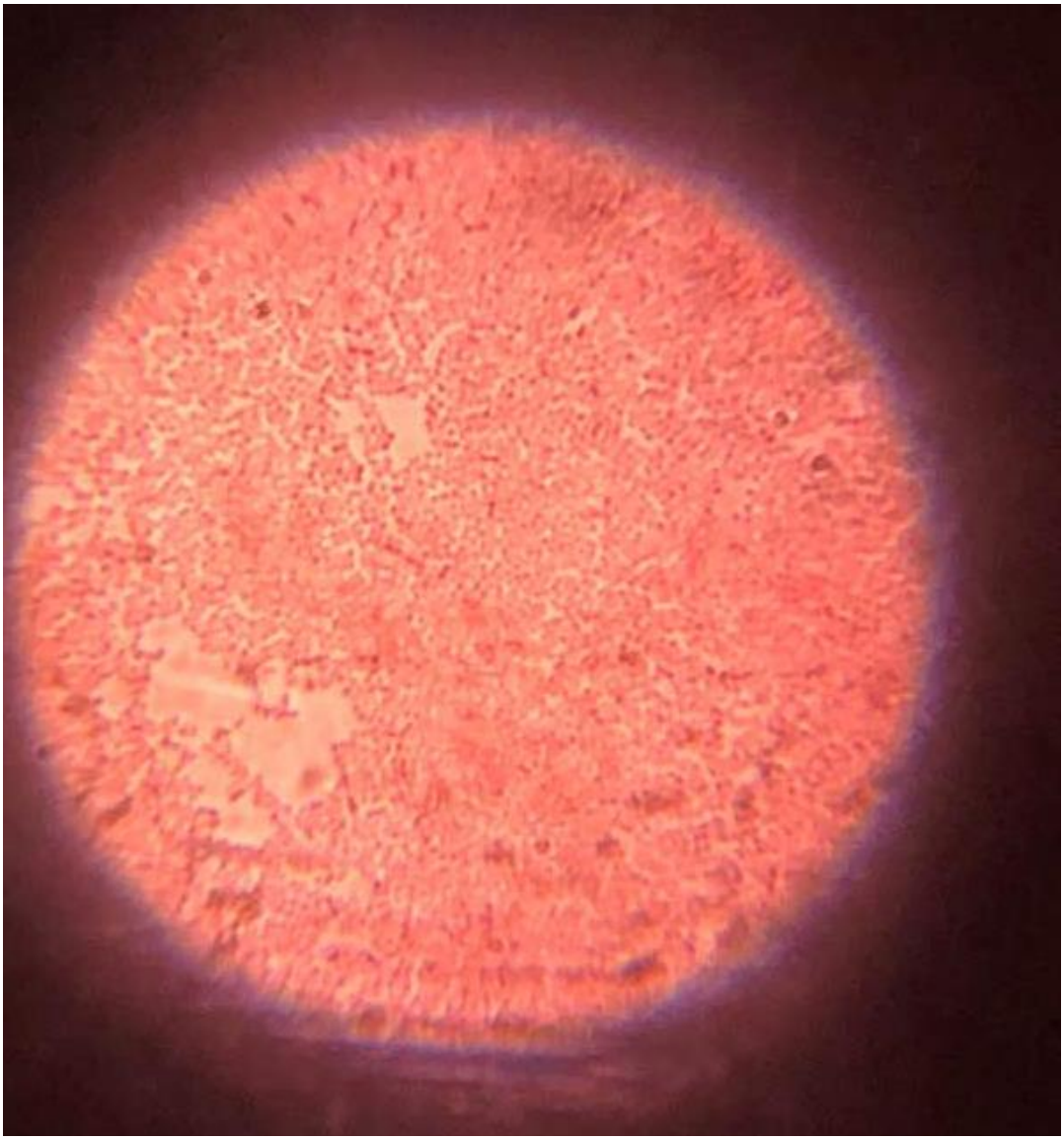

Fig. 5. Gram-stained Nitrogen-fixing bacteria.

4.1. LIQUID BIOFERTILIZER

This is the liquid biofertilizer produced from the anaerobic fermentation of the fruits, and from which the microorganisms were isolated. A strong, pungent odour was released upon opening the mixture, which quickly diffused. The sieving of the mixture gave a golden-yellow liquid, as shown in the figure. Refer to Figure 1.

4.2. NITROGEN-FIXING BACTERIA

These bacteria were isolated from the liquid biofertilizer using Mannitol Ashby agar media for nitrogen-fixing bacteria. Mannitol Ashby agar media is a formulated selective media used to isolate *Azotobacter* species, a nitrogen-fixing bacterium that can use mannitol and atmospheric nitrogen as carbon and nitrogen sources respectively. The medium contains various essential ions required to promote the growth of *Azotobacter*, while the dipotassium phosphate acts as a buffer in the medium. Refer to Figure 2.

4.3. PHOSPHATE-SOLUBILISING BACTERIA

These bacteria were isolated from the liquid biofertilizer using Pikoskaya agar media for phosphate solubilising bacteria. Pikovskaya agar medium is a selective medium used to isolate phosphate-solubilising bacteria. The yeast extract in the medium provides nitrogen and other nutrients necessary to support bacterial growth. Glucose acts as an energy source, while other salts support the growth of the microorganisms. Refer to Figure 3.

4.4. GRAM-STAINED PHOSPHATE-SOLUBILISING BACTERIA

This is the image gotten from viewing the gram-stained phosphate-solubilising bacteria under the x100 oil immersion objective lens of a light microscope. As shown in the figure, the gram-staining identified the isolated bacteria as a gram +ve bacilli. Refer to Figure 4.

4.5. GRAM-STAINED NITROGEN-FIXING BACTERIA

This is the image gotten from viewing the gram-stained nitrogen-fixing bacteria under the x100 oil immersion objective lens of a light microscope. As shown in the figure, the gram-staining identified the isolated microorganism as a gram –ve cocci. Refer to Figure 5.

TABLE 1: RESULTS OF GRAM'S STAINING AND BIOCHEMICAL TESTS

	Nitrogen-fixing bacteria	Phosphate-solubilising bacteria
Gram's staining	-ve cocci	+ve bacilli
Indole Test	+ve	-ve
Methyl-Red Test	-ve	-ve
Voges- Proskauer Test	+ve	+ve
Citrate Test	-ve	+ve
Oxidase Test	+ve	+ve
Catalase Test	+ve	+ve

TABLE 2: COLONY MORPHOLOGIES OF THE ISOLATES

S/N	COLONY TYPE	COLOUR	TEXTURE	SHAPE	ELEVATION
1	Nitrogen-fixing bacteria	Milky	Smooth	Irregular	Raised
2	Phosphate-solubilising bacteria	Yellow	Smooth	Irregular	Raised

4.6. ISOLATED NITROGEN-FIXING ORGANISM

The isolated nitrogen-fixing organism is *Azotobacter*, as the Mannitol Ashby agar medium used is a selective medium for *Azotobacter*.

4.7. ISOLATED PHOSPHATE-SOLUBILISING ORGANISM

Based on the results of the biochemical tests, and reference from *Bergey's Manual of Determinative Bacteriology*, the isolated organism is identified to be *Bacillus subtilis*.

CHAPTER FIVE

DISCUSSION

According to the results, *Azotobacter* was isolated as the nitrogen-fixing bacteria. This agrees with results from Onyeze *et al.* (2013), who isolated *Azospirillum*, *Azotobacter* and *Clostridium* soil samples from bean, maize and legume farmlands and Jimenez *et al.* (2011), who also reported the isolation of *Azotobacter* from soil samples from vegetable farmlands. The isolation of *Azotobacter* from the liquid biofertilizer produced, and in the reports above, proves *Azotobacter* as a common nitrogen-fixing microorganism; irrespective of the type of plant and soil condition.

The phosphate-solubilising bacteria isolated, according to the results, was *Bacillus subtilis*. Gupta & Tewari (2012) reported the isolation of *Pseudomonas synxantha*, *Burkholderia gladioli*, *Enterobacter hormaechei* and *Serratia marcescens* as the phosphate-solubilising bacteria responsible for the enhanced growth of *Aloe barbadensis*. Zhu *et al.* (2011) isolated *Kushneria* sp. as a halophilic phosphate-solubilising bacterium from the eastern coast of China. Kidiri *et al.* (2013) reported the isolation of *Bacillus* sp., *Klebsiella* sp., *Neisseria* sp., *Enterobacter* sp., *Pseudomonas* sp. and *Proteus* sp. as phosphate-solubilising microorganisms. The result, and reports above, show a wider range of phosphate-solubilising bacteria, which may be relative to plant, soil conditions, and/or environment.

Microorganisms are ubiquitous. As such, there may always be a presence of nitrogen-fixing and/or phosphate-solubilising bacteria. The essence of biofertilizer production and application is to augment the native rhizobacteria, and enhance their efficiency; thus resulting in improved plant growth.

Also, it is expected that biofertilizers prepared using soil samples from the rhizosphere of a young, developing plant would yield a larger variety of plant-growth-promoting microorganisms; as such plant requires more nutrients for proper growth and development.

CONCLUSION

This project work serves to isolate and characterise soil bacteria that promote plant growth. The selective media and biochemical tests were used to isolate and identify the bacteria, promoting further research on possible optimisation of their efficiency.

This research is a progressive one, as it can be developed in biotechnological sub-fields, such as genetic and/or metabolic engineering of the identified species, as well as direct inoculation of the microorganisms to promote plant growth.

Molecular characterisation of the isolated microorganisms may also be carried out, to determine their specific strains as well as for bioinformatics purposes, and may submitted as a supplementary research work.

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