

**ANALYSES OF SOIL BACTERIA IN NGERE TEA  
CATCHMENT AREA OF MURANG'A COUNTY, KENYA**

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**Analyses of soil bacteria in Ngere tea catchment area of  
Murang'a County, Kenya**

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**A thesis submitted in partial fulfilment for the degree of  
Master of Science in Genetics in the Jomo Kenyatta University  
of Agriculture and Technology**

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## DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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## **DEDICATION**

This work is dedicated to my beloved parents and relatives, who encouraged and supported me all through to this level of education. Above all to God, the creator of all beings, who provided strength, health and favour to enable me see this output.

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**MAY GOD BLESS YOU ALL!**

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## LIST OF ABBREVIATIONS

|              |  |
|--------------|--|
| <b>CFB</b>   | Cytophaga Flavobacteria and Bacteroides      |
| <b>BCG</b>   | Biogeochemical Cycles                        |
| <b>DNA</b>   | Deoxyribonucleic Acid                        |
| <b>MRNA</b>  | messenger Ribonucleic Acid                   |
| <b>PCR</b>   | Polymerase Chain Reaction                    |
| <b>rDNA</b>  | Ribosomal Deoxyribonucleic Acid              |
| <b>KTDA</b>  | Kenya Tea Development Agency                 |
| <b>MR-VP</b> | Methyl Red-Voges-Proskauer                   |
| <b>PGPR</b>  | Plant Growth Promoting Rhizobium             |
| <b>NCBI</b>  | National Centre of Biotechnology Information |
| <b>BLAST</b> | Basic Local Alignment Search Tool            |
| <b>HCN</b>   | Hydrogen Cyanide                             |
| <b>UV</b>    | Ultra Violet                                 |
| <b>ARDRA</b> | Amplified Ribosomal DNA Restriction Analysis |

|             |                                    |
|-------------|------------------------------------|
| <b>SIM</b>  | Sulphur Indole Motility            |
| <b>KOH</b>  | Potassium Hydroxide                |
| <b>CFU</b>  | Colony forming Unit                |
| <b>DNBA</b> | Dilute Nutrient Broth Agar         |
| <b>TSA</b>  | Tryptone Soy Agar                  |
| <b>PSB</b>  | Phosphate Solubilizing Bacteria    |
| <b>GOK</b>  | Government of Kenya                |
| <b>C</b>    | Carbon                             |
| <b>N</b>    | Nitrogen                           |
| <b>pH</b>   | Hydrogen Potential                 |
| <b>Bp</b>   | Base pairs                         |
| <b>EDTA</b> | Ethylene diamine tetra-acetic Acid |
| <b>SDS</b>  | Sodium Dodecyl Sulphate            |
| <b>TRFK</b> | Tea Research Foundation of Kenya   |
| <b>GDP</b>  | Gross Domestic Product             |



## ABSTRACT

Bacteria are a very diverse group of organisms in soil, with major taxonomic groups being represented in most soils. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health, since a wide range of bacteria are involved in the important soil functions. The objectives of this study were to isolate, characterise and identify groups of bacteria that are associated with soil fertility in tea growing areas of Ngere. Thirty eight isolates were obtained using two categories of media, namely dilute nutrient broth agar and Tryptone soy agar. Ngere tea soils had a pH range of 3.9 to 5.0 and organic carbon content that ranged from 3% to 19%. The isolates were characterized using cultural and biochemical techniques. The Gram stain reaction showed that 53% of the isolates were Gram positive while 47% were Gram negative, and they grew well at pH ranging from 5 - 6.5 and temperature range of 25°C to 35°C. Identification was done by Polymerase Chain Reaction (PCR) amplification of the 16S rDNA region, sequencing and phylogenetic analysis. BLAST analysis of the partial sequences showed that 45 % were from the genus *Bacillus* within the Firmicutes in the domain bacteria with similarities between 83 % and 99 %, 23% and belonged to the genus *Pseudomonas* with similarities between 94 % and 99 %. The genus *Enterobacter* represented 13% with similarities between 83 % and 90 %. Other genera such *Burkholderia*, *Chryseobacterium*, *Acinetobacter* and *Serratia* constituted 9 % with similarity of 88% to 99 %. D66 showed identity of 95 - 97 % similarity with the previously known sequences in the GenBank database. This represents novel species of organisms within Ngere tea soils. Isolates D63, S49, D16, D5, S48, D51, D80, D1, D79

and S31 showed identity of 80 -93 % similarity, representing novel genera of organisms within the tea soils. This study demonstrated that Ngere tea soils harbours diverse bacteria with specific biochemical properties like the ability to reduce nitrate to nitrite, nitrogen fixation, ability to produce urease enzyme that splits urea into carbon dioxide and ammonia, ability to hydrolyse starch and to solubilisation phosphate suggests their involvement in the nutrient recycling within the tea soils hence improving soil fertility. Thus these can be used as indicators of soil health or as biofertilizers.

## CHAPTER ONE

### 1.0 INTRODUCTION AND LITERATURE REVIEW

#### 1.1 Background information

Tea (*Camellia sinensis*) is a major cash crop in many countries, including China, India, Sri Lanka and Kenya (International Tea Committee, 2004). There were about 2.72 million hectares of land under tea cultivation globally in 2004 (International Tea Committee, 2004). Tea is grown in many countries ranging from as far north as 49° N, outer Carpathians to as far as 30° S, Natal, South Africa (Shoubo, 1989). Tea grows in various latitudes from the sea level in Japan to 2700 M above mean sea level (amsl) in Olenguruone, Kenya and Gisovu, Rwanda (Owuor *et al.*, 2008).

The plant is widely adaptable to geographical areas with large variations in climate and physical features which affect rates of growth, yields and quality (Ng'etich *et al.*, 2001). Several studies have demonstrated wide response ranges in yield (Ng'etich *et al.*, 2001); yield partitioning (Ng'etich *et al.*, 2001), growth (Ng'etich and Stephens, 2001a, b), shoot population density and dry matter partitioning (Ng'etich and Stephens, 2001b) of tea genotypes to different environments (Wachira *et al.*, 2002) including water stress (Carr, 1997), temperature (Tanton, 1982) and altitude (Obaga, *et al.*, 1989).

Tea is an unusual crop because the soil becomes strongly acidified following the planting of tea (Song and Liu, 1990; Ding and Huang, 1991). The soil is acidified by the tea plant itself and also by use of fertilizers, especially ammonium sulphate and urea (Shi *et al.*, 1999). Therefore, the fields with high production normally have a pH of about 4 (Han *et al.*, 2002). Soil pH is a vital characteristic in determining the chemical environment of higher plants and soil microbes (Marschner, 1986). There are few reactions involving soil or its biology that are not affected by soil pH. This sensitivity must be recognized in any soil management system (Brady and Weil, 1999) and continues to decrease with the increase of stand age (Song and Liu, 1990; Ding and Huang, 1991).

## **1.2 Soil microbial biomass**

Soil microorganisms are important components of ecosystem functioning as they determine the mineralization of soil organic matter and energy flow (Robertson and Groffman, 2007). Microbial recycling of crop residues provides an important component to improve the soil organic matter pool and soil productivity in agricultural management systems, particularly in the tropics, where microbial soil organic matter turnover time is usually shorter as opposed to temperate agro-ecosystems (Oelbermann *et al.*, 2004).

The turnover and mineralization of crop residues is largely dependent on soil microbial processes warranting the recycling of nutrients (e.g. nitrogen) supplied to both plants and microbes (Crecchio *et al.*, 2004). Differences in residue decomposition have mainly

been explained by differences in biochemical quality of residues (e.g. C-to-N ratio) (Kaewpradit *et al.*, 2008). Residue quality is known to alter microbial decomposition processes by containing compounds of varying recalcitrance (Palm *et al.*, 2001), requiring specific microbial communities for their degradation. Nitrogen availability controls the decomposition rate of crop residues (Hadas *et al.*, 2004), particularly those with a high C-to-N ratio (e.g. in maize, soil bacteria play pivotal roles in various biogeochemical cycles (BGC) (Wall and Virginia, 1999) and are responsible for the cycling of organic compounds.

Our knowledge of soil microbial diversity is limited in part by our inability to culture soil microorganisms (Torsvik *et al.*, 1990a, b). It is estimated that in 1 g of soil there are 4000 different bacterial “genomic units” based on DNA – DNA association. It has also been estimated that about 5000 bacterial species have been described (Pace, 1997). Approximately 1% of the soil bacterial population can be cultured by standard laboratory practices. It is not known if this 1% is representative of the bacterial population (Torsvik *et al.*, 1998). Another problem with this approach is that soil is heterogeneous, containing many microhabitats that are suitable for microbial growth. As a result, bacteria are highly aggregated in soil existing in clumps or “hot spots”. Plants also influence the spatial distribution of soil bacteria (Wall and Virginia, 1999) and fungi, as shown by an approximately two-fold increase in bacterial numbers in the rhizosphere over bulk soil (Curl and Truelove, 1986).

In the final analysis, the plants growing on the soil subsist on the products of microbial activity, for the microorganisms are continually oxidizing the dead plant remains and

leaving behind, in a form available to the plant, the nitrogenous and mineral compounds needed by the plants for their growth (Van Elsas *et al.*, 1997). On this concept, a fertile soil is one, which therefore contains either an adequate supply of plant nutrients in an available form, or a microbial population, which is releasing nutrients fast enough to maintain rapid plant growth (Filion *et al.*, 1999). Infertile soil is one in which this does not happen, as for example, if the microorganisms are removing and locking up available plant nutrients from the soil (Silvia *et al.*, 1998).

Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition (Timonen *et al.*, 1996), plant health (Filion *et al.*, 1999; Smith and Goodman, 1999), soil structure (Dodd *et al.*, 2000) and soil fertility (O'Donnell *et al.*, 2001). Much of the recent studies have focused on tea production in relation to different ecological environment (Carr and Stephens, 1992) with little knowledge on soil microbial composition and their genetic diversity. This research focused on the diversity of soil bacteria, their importance as well as their possible contribution to soil fertility in small scale tea growing areas of Ngere tea catchment, Murang'a County, Kenya.

### **1.3 Economic importance of tea in Kenya**

Tea is one of the leading cash crops in Kenya and makes a significant contribution to the economy. In the year 2010, the country produced 399,000 metric tons of black tea (TRFK, 2011). Over 95% of the tea was exported mainly in bulk earning over Kshs. 97 billion in foreign exchange (TRFK, 2011). This represents about 26% of the total

export earnings, and about 4% of Kenya's GDP. Tea is a rural based enterprise, and contributes directly to the objectives of the Agricultural Sector Development Strategy, 2009-2020 (ASDS), Kenya's Vision 2030, National Development Plan and the Medium Term Plan 2008-2012 (TRFK, 2011). An estimated 4 million Kenyans (about 10% of the total population) derive their livelihoods from the tea industry. About 50% of the workforce in the enterprise is women and thus tea contributes to gender empowerment. The crop also contributes significantly to the development of rural infrastructure and contributes to stemming rural-urban migration (GOK, 2010b). It directly contributes to environmental conservation through enhanced water infiltration, reduced surface erosion, and mitigation of global warming through carbon sequestration (TRFK, 2011).

#### **1.4 Statement of the problem**

Soil supports an extraordinary diversity of microorganisms; however, surveys of soil indicate that a substantial number of bacteria have not been identified and characterized (Curtis *et al.*, 2002). There has been challenge to link new information on the composition and function of soil bacteria back to the soil processes that have long been the focus of soil fertility. Tea is a globally important crop and is unique because it both requires an acid soil and acidifies soil. Tea stands tend to be extremely heavily fertilized in order to improve yield and quality, resulting in a great potential for diffuse pollution. With the growing interest in sustainable agriculture, and in reducing synthetic fertilizer inputs in tea growing soils, new research was necessary to

understand the role of bacteria in the soil. The microbial ecology of tea soils remains poorly understood; an improved understanding is necessary as processes affecting nutrient availability and loss pathways are microbially mediated.

### **1.5 Justification**

Agriculture, the mainstay of Kenya's economy, currently contributes 26 per cent of the GDP directly and another 25 per cent indirectly. The sector also accounts for 65 per cent of Kenya's total exports and provides more than 18 per cent of formal employment. Like other cash crops, tea is the leading export crop and is the second largest exporter after horticultural production (GOK, 2010a). In Africa, Kenya is the largest tea producer and globally Kenya is ranked third in annual tea production after China and India (TRFK, 2011). The tea produced in Kenya accounts for about 10% of the world production and about 26% of the export share and about 4% of the GDP. Tea farming plays a leading role in earning the country foreign exchange. Thus it's with this reason that intensive studies are needed to expand the productivity and quality of the tea being produced. In addition, every year farmers have to apply fertilizers to ensure good produce however, the price of fertilizers is too high for the poor farmers to afford. Therefore, the aim of this study was to establish exactly what groups of bacteria are involved in nutrient recycling and their role in promoting soil fertility.



## **1.6 Hypotheses**

- i. There is no dominant soil bacteria in Ngere tea catchment area of Murang'a County, Kenya
- ii. Bacteria found in soils of tea growing farms do not influence soil fertility

## **1.7 Objectives**

### **1.7.1 General objective**

To determine the diversity of soil bacteria and their possible contribution to soil fertility from Ngere tea Catchment area of Murang'a County, Kenya

### **1.7.2 Specific objectives**

- 1) To carry out morphological and biochemical characterization of dominant soil bacteria in Ngere tea catchment area
- 2) To characterise and identify the bacterial isolates using molecular techniques
- 3) To determine the possible contribution of the characterised bacteria to soil fertility

## 1.8 Literature review

### 1.8.1 General characteristics of soil bacteria

Bacteria are prokaryotic, single celled organisms that lack the cytoskeleton and contain double stranded circular DNA. They are classified according to the morphological appearance into bacillus (rod), coccus (round), Spirilla (spiral) or filamentous. In terms of numbers, *Bacilli* are most numerous followed by Cocci and Spirilla in soil (Tate, 2000). Bacterial cells are about one tenth the sizes of eukaryotic cells and are typically 0.5–5.0 micrometres in length (Schulz, 2001). They move either by cilia or by flagella. They nourish themselves heterotrophically either as saprophytes, parasites or autotrophs depending on the species (Paul *et al.*, 1996). Others can extract nitrogen directly from the air or break down some toxic substances (Bardgett *et al.*, 1998). Populations of microbes can boom or bust in the space of a few days in response to changes in soil moisture, soil temperature or carbon substrate. To gain advantage in this process, many microbes release antibiotic substances to suppress particular competitors. In this way some species can suppress other disease-causing microorganisms (Greg, *et al.*, 2005).

As many as 13,000 bacterial species are estimated to be in soils based on analysis of DNA (Wackett, 2001). Bacteria are also classified on the basis of physiological activity or mode of nutrition, especially the manner in which they obtain their carbon, nitrogen, energy and other nutrient requirements (Joseph *et al.*, 2007). They are broadly divided into two groups namely Autotrophs and Heterotrophs. Autotrophic

bacteria are capable of synthesizing their food from simple inorganic nutrients, while heterotrophic bacteria depend on pre-formed food for nutrition (Bardgett *et al.*, 1998). All autotrophic bacteria utilize carbon dioxide from atmosphere as carbon source and derive energy either from sunlight photoautotrophs such as, *Rhodospseudomonas* or from the oxidation of simple inorganic substances present in soil. Majority of soil bacteria are heterotrophic in nature and derive their carbon and energy from complex organic substances/organic matter, decaying roots and plant residues (Glick, 1995). They obtain their nitrogen from nitrates and ammonium compounds like proteins from the decomposing soil organic matter. Certain bacteria also require amino acids, B-Vitamins and other growth promoting substances (Kloepper *et al.*, 2004). They are relatively resistant to desiccation and are abundant in desert soils. Previously, specific groups of bacteria that were thought to be most abundant in soil corresponded only to those organisms that could be cultured on laboratory media such as Gram-negative bacteria, spore-forming bacteria and actinomycetes (Coyne, 1999). Microbial characteristics of soils are being evaluated increasingly as sensitive indicators of soil health because of the clear relationships between microbial diversity, soil and plant quality, and ecosystem sustainability (Doran *et al.*, 1996).

### **1.8.2 16s ribosomal RNA**

16S ribosomal RNA (16S rRNA) is a component of the 30S subunit of prokaryotic ribosomes. It is 1,542 bp in length and is used for phylogenetic studies (Weisburg *et al.*, 1991). It is highly conserved between different species of bacteria and archaea (Coyne

*et al.*, 2003). In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions which can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification (Clarridge, 2004).

### **1.8.3 Methods for characterization of bacteria**

Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species (Ovreas, 2000). Methods to measure microbial diversity in soil can be categorized into two groups: morphological based techniques and molecular-based techniques. Typically, diversity studies include the relative diversities of communities across a gradient of stress, disturbance or other biotic or abiotic difference (Hughes *et al.*, 2001). It is difficult with current techniques to study true diversity since we do not know what is present and we have no way of determining the accuracy of our extraction or detection methods (Priest *et al.*, 1994). Often researchers attempt to reduce the information gathered by diversity studies into discrete, numerical measurements such as diversity indices (Atlas and Bartha, 1993).

## **1.9 Major groups of soil bacteria**

### **1.9.1 Decomposers**

Bacteria play an important role in decomposition of organic materials, especially in the early stages of decomposition when moisture levels are high. In the later stages of

decomposition, fungi tend to dominate. *Bacillus subtilis* and *Pseudomonas fluorescens* are examples of decomposer bacteria (Soil biology, 2011).

### **1.9.2 Nitrogen fixers**

Bacteria can be inoculated onto legume seeds to fix nitrogen in the soil such as Rhizobium. These nitrogen-fixing bacteria live in special root nodules on legumes such as clover, beans, medic and wattles. They extract nitrogen gas from the air and convert it into forms that plants can use (Sprent, 2001). Non-symbiotic nitrogen fixation has a great agronomic significance. Some important non-symbiotic nitrogen-fixing bacteria include *Gluconacetobacter* and *Azotobacter* sp. (Vessey, 2003, Bariuso *et al.*, 2008); *Bacillus*, *Corynebacterium*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*, (Saxena and Tilak, 1998). This form of nitrogen fixation can add the equivalent of more than 100kg of nitrogen per hectare per year. To date, inoculating the soil with these organisms has not proved an effective means of increasing nitrogen fixation for non-legume crops (Boland, *et al.*, 1998).

### **1.9.3 Disease suppressors**

*Bacillus megaterium* is an example of a bacterium that has been used on some crops to suppress the disease-causing fungus (Banasco *et al.*, 1998). *Pseudomonas fluorescens* may also be useful against this disease. *Bacillus subtilis* has been used to suppress seedling blight of sunflowers, caused by *Alternaria helianthi* (Shang *et al.*, 1999). A number of bacteria have been commercialized worldwide for disease suppression.

However, suppression is often specific to particular diseases of particular crops and may only be effective in certain circumstances (Van Dijk and Nelson, 2000).

#### **1.9.4 Aerobes and anaerobes**

Aerobic bacteria are those that need oxygen, so where soil is well drained aerobes tend to dominate. Anaerobes are bacteria that do not need oxygen and may find it toxic. This group includes very ancient types of bacteria that live inside soil aggregates. Anaerobic bacteria favour wet, poorly drained soils and can produce toxic compounds that can limit root growth and predispose plants to root diseases. Example of these groups of bacteria belongs to *Pseudomonas*, *Bacillus*, *Micrococcus* and *Burkholderia* genera (Adebusoye *et al.*, 2008).

#### **1.9.5 Sulphur oxidizers**

The sulphur oxidizing bacteria are primarily the Gram negative currently classified as species of *Thiobacillus*, *Thiomicrospira* and *Thiosphaera* (Lane *et al.*, 1992), but heterotrophs, such as some species of *paracoccus*, *Xanthobacter*, *Alcaligenes* and *pseudomonas* can also exhibit chemolithotrophic growth on inorganic sulphur compounds (Kuenen *et al.*, 1982). Studies on the distribution of *Thiobacillus thiooxidans* and *T. thioparus* showed that these bacteria are found in an active state mainly in soils fertilized with sulphur (Brock *et al.*, 2006).

### **1.9.6 Phosphate solubilizing bacteria**

Phosphorus is one of the essential nutrients for plant growth and the absence of this element in the soil could limit plant development (Igual *et al.*, 2001). It is well known that a large proportion of the inorganic phosphorus added to the soil as fertilizer is not available for plants because of its rapid immobilization (Mehta and Nautiyal, 2001). The existence of soil microorganisms bacteria, actinomycetes and some fungi that solubilize soil-precipitated or soil-attached phosphate has been reported previously (Haas *et al.*, 2005) (Reyes *et al.*, 2001). Most of the reported phosphate solubilizing bacteria (PSB) belongs to *Pseudomonas*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Mesorhizobium*, *Burkholderia*, *Azotobacter*, *Serratia*, *Azospirillum* and *Erwinia* genera (Haas *et al.*, 2005).

## **1.10.0 Major microbial processes in the soil**

### **1.10.1 Gaseous exchange**

Soil microorganisms are important regulators of the Earth's atmosphere through the gases they emit and consume. Soil microorganisms are involved in the cycling of all major elements, and many of these elements have gaseous forms (Tate, 2000). Low molecular weight, volatile compounds produced by microorganisms include carbon dioxide, from respiration; methane, from methanogenic processes in anaerobic environments; and N<sub>2</sub>, NO, and N<sub>2</sub>O from denitrification and hydrogen sulphide from sulphate reduction in anoxic environments (Paul *et al.*, 1996). Gases emitted by microorganisms are, in turn, consumed by other types of microorganisms. Thus carbon

dioxide is consumed by autotrophic bacteria, methane by methanotrophic bacteria, nitrogen gas by nitrogen-fixing bacteria, and hydrogen sulphide by sulphur oxidizing bacteria (Silvia *et al.*, 1998).

### **1.10.2 Elemental cycling of the nutrients**

Bacteria bring about a number of changes and biochemical transformations in the soil and thereby directly or indirectly help in the nutrition of higher plants growing in the soil (Killham, 1994). The important transformations and processes in which soil bacteria play vital roles are: decomposition of cellulose and other carbohydrates, ammonification (proteins ammonia), nitrification (ammonia-nitrites-nitrates), denitrification (release of free elemental nitrogen), biological fixation of atmospheric nitrogen (symbiotic and non-symbiotic) oxidation and reduction of sulphur and iron compounds (Sprent, 2001). All these processes play a significant role in plant nutrition and organic matter recycling.

### **1.10.3 Decomposition, mineralization, and immobilization**

Organic compounds produced by plants via photosynthesis continually enter the soil as root exudates when the plant is living and as debris when the plant dies. These complex organic polymers are broken down into smaller organic and mineral components by the combined forces of soil fauna and microorganisms in a process called decomposition (Coyne, 1999). The nutrients released by this process are thus made available for uptake and incorporation into plants and other organisms. Nutrients are released from organic residues by the process of mineralization (Killham, 1994).



Mineralization is defined as the conversion of organic chemicals to their inorganic constituents, primarily carbon dioxide, water, and/or ammonia. During anabolism, soil microorganisms assimilate inorganic forms of elements into their cells in a process referred to as immobilization (Coyne, 1999). Although both mineralization and immobilization are always occurring simultaneously, the carbon to nitrogen ratio of available organic substrates determines which process will dominate. Thus net immobilization of inorganic nitrogen is expected during decomposition of compounds with high carbon to nitrogen ratios (Paul *et al.*, 1996). A large portion of immobilized elements eventually becomes available for plant uptake when microorganisms are preyed on by protozoa or lysed by environmental conditions (Silvia *et al.*, 1998). An important product of decomposition is soil humus. Humus is defined as dark-coloured organic by-products consisting of microbial cell walls and other resistant molecules formed from free-radical reactions of sugars, amino acids, and products of lignin decomposition. Thus, microorganisms play an important role in humus formation (Paul *et al.*, 1996).

#### **1.10.4 Biodegradation and transformation of pollutants**

Many organic and inorganic pollutants end up in soil intentionally, through their use as pesticides, and unintentionally when they migrate from contaminated waste sites or are deposited on soil from the atmosphere (Okoh, 2006). Biodegradation of organic pollutants can be considered under the general category of decomposition (Coyne, 1999). In its most general form, biodegradation is defined as an alteration in the

chemical composition of a molecule mediated by a biological process (Adams and Ribbons, 1988). Microorganisms are able to utilize many organic pollutants as sources of energy and carbon and use some of the highly chlorinated pollutants as electron acceptors under anoxic conditions (Obire, 1997). Bioremediation is defined as the decontamination of polluted environments via biological activity.

### **10.11 Tea growing in Kenya**

Tea growing regions in Kenya are found in the Great Rift Valley - a spectacular natural geographical wonder that divides the country almost asymmetrically. In the East of the Rift are the cool Aberdare highlands, the home to the snow-capped Mt. Kenya and the panoramic Nyabene hills. In the West of the Rift defined by the Mau escarpment are the Nandi Hills, highlands around Kericho, Mt. Elgon and the Kisii highlands (KTDA, 2011). Tea is grown on tropical red loam mixed with volcanic soils found in high altitude areas of Kenya. It is on the slopes of these highlands within the altitudes of between 1500 M to 2700 M above sea level that tea is grown (Owuor *et al.*, 2006). Tea does well in an environment with well-distributed rainfall ranging from 1200mm to 2500mm annually with long sunny intervals (Owuor *et al.*, 2008). Suitable temperature for tea growth ranges from minimum 12°C to a maximum 28°C. Soil type and condition is very important factor to tea growth; therefore the soils must be well drained with 2m depth. Tea grows well on highland well drained soils having a good depth, acidic pH in the range 4.5 to 6.5 beyond which the tea is restarted and more than 2% organic matter (KTDA, 2011). Fertilizer application is mandatory in tea production

and use of NPK fertilizers is recommended in Kenya. The figure 1.1 shows the tea growing regions of Kenya (Owuor *et al.*, 2006).

#### **10.12 Ngere tea catchment area**

It is located in the eastern site of the rift valley in Kariara Location of Gatanga District, Murang'a County Kenya; it falls within the KTDA Administrative regions (KTDA, 2011). It is 80 Kilometers from Nairobi and 42 Kilometers from Thika town. The Ngere tea factory is built on a 10 hectares Piece of land and the first bush was planted in 1958. The factory was commissioned in 1993. In Ngere tea catchment area there are seventy five registered tea collection centres and sixty three tea buying centres. It lies at an altitude of 1633 meters above sea level and receives an annual rainfall of 1225mm. Temperature range from 13° C and 26° C (KTDA, 2011).



## CHAPTER TWO

### 2.0 MATERIALS AND METHODS

#### 2.1 Study site

The soil samples were collected from Ngere tea catchment area in Gatanga District, Murang'a County Kenya. It is located at latitude  $0^{\circ} 56' 0''$  S and longitude  $36^{\circ} 58' 0''$  E. It lies at an altitude of 1633 meters above sea level and receives an annual rainfall of 1225mm. Temperature range from  $13^{\circ}$  C and  $26^{\circ}$  C. Laboratory work was carried out at Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Thika District.



**Figure 2.2:** Map showing Gatanga district

## 2.2 Study design

Cross-sectional study involving stratified random sampling was used. The study area was divided into strata based on the tea collection centres. In Ngere tea catchment area there are seventy five registered tea collection centres and sixty three tea buying centres were selected using the formula below.

## 2.3 The sample size

The sample size was adjusted using the following formula (Israel, 2009)

$$n = \frac{N}{1 + N(e^2)} = \frac{75}{1 + 75(0.05^2)} = \frac{75}{1.1875} \quad n=63$$

Where:  $n$  = sample size,  $N$  = Population size of tea collection centres,  $e$  = precision at 95% confidence level and  $P= 0.05$ .

A total of 63 soil samples were used for the study.

## 2.4 Sampling and collection of samples

Soil cores were collected from the rhizosphere of tea bushes using a soil auger along the four cardinal directions at the base of each tea plant in order to cover much of the rhizosphere. The soil samples were taken from a depth of 0-20 cm and 20-40 cm. In the sampling farm 3-15 points were randomly selected using a zigzag format. Soil samples from different points within a farm were thoroughly mixed to constitute a composite sample. Collection dates of the soil samples were recorded and then transported intact in cool box at ambient temperature of 4° C in sealed polyethylene bags to the laboratory for processing. The soil cores were sieved to remove stones and

leaves in readiness for cultivation experiments. Control experiment was done using soils obtained from Napier grass (Adegbite *et al.*, 2006).

### **2.5 Soil pH determination**

Soil pH was determined by drying the soil then sieving to remove stones, sticks and leaves. Twenty five grams of the soil was measured and put in a conical flask then added 50 ml of distilled water then followed by shaking with a reciprocator machine for 20 minutes. The pH meter was calibrated according to manufacturer's instructions over the appropriate range (Missouri Agricultural Station, 1998).

### **2.6 Determination of soil organic carbon content**

The total organic carbon content of the tea soil was determined by Walkley and Black method (Missouri Agricultural Station, 1998) whereby 0.1 g of soil dried and sieve was measured and transferred to the conical flask then 10 ml of potassium dichromate solution was added and swirled gently. In the fume hood 15 ml of concentrated sulphuric acid was added and gently swirled for 1 minute then it was allowed to stand for 30 minutes, followed by addition of 150 ml of distilled water using the measuring cylinder and allowed to cool. Five millimetre of orthophosphoric acid and 10 drops of diphenylamine indicator were added and stirred using a magnetic stirrer. Finally it was titrated with ferrous ammonium solution, the colour changes a bit, then to a dirty green (due to the masking effects of excess dichromate) but shortly before the end point, which is extremely sharp to a ferrous sulphate solution clear green (Missouri

Agricultural Station, 1998). The blank titre included all ingredients minus the sample.

The calculation was done according to the formula:

$$\%C = \frac{B - T \times 0.3 \times V \times 100}{W \times B \times 75}$$

Where B=Blank titre, T=Sample titre, W=Weight of soil and V=Volume of potassium dichromate

## **2.7 Enrichment of soil bacteria**

Dilute Nutrient Broth Agar (DNBA) was used for the cultivation of non-fastidious soil bacteria. Difco nutrient broth (DNB) consisted of Difco nutrient broth (BD Diagnostic Systems, Sparks, MD), at a concentration of 8 g per litre of distilled water. For solid media, 15 g of washed Difco technical agar (BD Diagnostic's systems) was added and 10-fold-diluted Tryptone soy agar 0.1 X TSA was prepared as described by Joseph *et al.*, 2003. In order To adjust the medium pH to 6.0, 0.1M hydrochloric acid solution was added. The media were then autoclaved at 121° C for 15 minutes after which it was then dispensed into 90-mm-diameter polystyrene sterile plastic petri dishes. The freshly sieved soil was carefully mixed and pulverized with spatula on the larger piece of paper. One gram of soil transferred immediately to the conical flask containing 150 ml of normal saline (Janssen *et al.*, 2002). The soil was stirring with Teflon-coated magnetic bars for 15 minutes at approximately 200 g. The soil suspension was then serially diluted with 1ml of the soil suspension added to 9 ml test tube of normal saline. Dilution ratios included:  $10^0$ ,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ . These preparations were vortexed at approximately 150 g for 1 minute and 1ml of aliquots was rapidly transferred to other 9



ml tubes. For plate count experiments, 200 µl aliquots from different dilutions were transferred to petri dishes containing both Tryptone soy agar and dilute nutrient broth agar (DNBA) and spread over the surface with a sterile glass spreading rod. Each dilution series was used to inoculate a series of plates with two plates at each dilution level. This was followed by incubation at 25 ° C for 24 to 72 hours in the dark. Sub culturing was done on dilute DNBA to isolate pure cultures.

To measure the survival efficiency, colonies from 10<sup>-1</sup> dilutions were counted following the formula adapted by James (1978):

$$\text{Viable cell count(CFU/g soil)} = \frac{\text{Number of colonies}}{\text{Volume of inoculum}} \times \text{Dilution factor}$$

## **2.8 Identification and characterization of bacteria isolates using morphological techniques**

### **2.8.1 Morphological characterization**

Preliminary characterization of the isolates involved the examination of colony morphology and culture features such as colour, pigmentation, elevation, shape, size and growth form, of safranin-stained bacterial isolates under the dissecting and compound microscope using the slide procedure.

### **2.8.2 Gram Staining of the isolated bacteria**

Smears of bacteria cultures were prepared and heat-fixed. Slides were placed on the staining rack and flooded with crystal violet. This was allowed to stand for 30 seconds.

The slide was then rinsed with water for 5 seconds then covered with Gram's iodine mordant. The slide was allowed to stand for 1 minute and rinsed with water for 5 seconds. Decolonization was done with 95% ethanol for 15 to 30 seconds. This was followed by rinsing with water for 5 seconds. Counterstaining was done using Safranin for about 60 to 80 seconds and the slides were rinsed again with water for 5 seconds. Blot drying was done with bibulous paper and examined using microscope at x100 under oil immersion (Keast *et al.*, 1984). Gram-positive organisms stained blue to purple; Gram-negative organisms stained pink to red. Gram staining results were confirmed by using the 3% KOH test (Halebian *et al.*, 1981). This test was performed by adding a drop of 3% KOH on a slide. A loop full of the bacteria was introduced and mixed thoroughly. Positive results were observed if the culture pulled along with the wire loop when raised up and negative results were recorded if nothing was pulled along the wire loop.

## **2.9 Biochemical characterization of isolated bacteria**

### **2.9.1 Catalase test**

Catalase test detects the activity of enzyme catalase, present in most cytochrome-containing aerobic bacteria. These microbes produce hydrogen peroxide during the aerobic breakdown of sugars. Catalase decomposes hydrogen peroxide to water and oxygen.

Catalase test was done by scooping a colony of a 24-hour culture, placing it on a glass slide and adding a drop of 3% hydrogen peroxide solution. A positive reaction was

indicated by the formation of bubbles, while the absence of air bubbles indicated a negative catalase test (Cappuccino and Sherman, 2002).

### **2.9.2 Nitrate reduction**

This tests the production of enzyme nitrate reductase which reduces nitrates that the cell uses as a final hydrogen acceptor during anaerobic respiration, to nitrites or free nitrogen gas and water. The nitrite combines with the test reagents sulphanic acid and alpha-naphthylamine to form a diazo red dye.

Nitrate reduction was tested by inoculating substrates into nitrate broth medium supplemented with 1% potassium nitrate and incubating them for 72 hours at 37 °C (Mwatha *et al.*, 1993). After incubation, drops of sulphanic acid and alpha-naphthylamine were added. Nitrate reduction was detected by withdrawing 0.5 ml samples and adding 0.2 ml naphthylamine and 0.2 ml sulphanic acid reagent to each tube, as described by Smibert and Krieg, 1981. Positive reactions produced an immediate cherry red colour while negative reactions remained yellow. The results were confirmed by the addition of zinc powder, in which two uninoculated tubes were used as controls (Korn-Wendisch and Kutzner, 1992; Cappuccino and Sherman, 2002). Formation of a red colour indicated negative results while no colour change indicated that reduction was beyond nitrite (to ammonia or molecular nitrogen) (Harold, 2002; Cappuccino and Sherman, 2002).

### **2.9.3 Gelatine liquefaction/hydrolysis**

Gelatine liquefaction detects the breakdown of gelatine to polypeptides and amino acids by enzyme gelatinase (Harold, 2002). Gelatine protein is produced by hydrolysis of a component of the connective tissues and tendons of animals known as collagen. Gelatine is solid at room temperature but above 25 °C it turns into liquid. When gelatinase hydrolyses this protein into amino acids, it remains liquefied even at the low temperatures of an ice bath (Cappuccino and Sherman, 2002).

The bacterial isolates were inoculated onto nutrient broth supplemented with 12% gelatine and 1.5 % agar, to demonstrate hydrolytic activity of gelatinase. One uninoculated tube was used as control for each isolate (Cappuccino and Sherman, 2002). After incubation, cultures that remained liquefied when placed in refrigerator at 4 °C for 30 minutes were considered positive for gelatine hydrolysis.

#### **2.9.4 Methyl Red-Voges- Proskauer (MR-VP) test**

MR-VP is used to identify mixed acid fermenting bacteria. The test detects the ability of the isolates to oxidize glucose by detecting the production of sufficient acid end products (Harold, 2002). MR test detects mixed acids, which are the characteristic end products of a particular fermentation pathway that make the medium more acidic ( $\text{pH} \leq 4.5$ ). This is detected when an indicator is added (Cappuccino and Sherman, 2002). The VP test is used to identify bacteria that produce non-acidic or neutral end products from the organic acid products of the glucose fermentation. It specifically detects an intermediate product of the fermentation pathway that yields 2, 3-butandiol known as acetoin, by the addition of Barrit's reagent (4% KOH and 5% alpha naphthol

in 95% ethanol). MR-VP broth was inoculated with each of the isolates, in duplicates, shaken and then incubated at 37 °C for 72 hours after which, drops of Methyl red indicator methyl red test or Barrit's reagent for VP test respectively, was added to aliquots of each culture. Positive reactions were those that turned red while negative reactions turned pale yellow for MR test, while for the VP test, positive tests produced a deep red coloration (Cappuccino and Sherman, 2002).

### **2.9.5 Indole, Motility and Hydrogen sulphide production tests**

The test identifies isolates with the ability to produce the enzymes tryptophanase that removes the amino group from tryptophan to form Indole, pyruvic acid and ammonia, and cysteine desulfurase, that produces pyruvate, ammonia and hydrogen sulphide from sulphur containing amino acids. Indole reacts with Kovacs reagent (*p*-dimethylamino-benzaldehyde) (Harold, 2002), to form a deep red colour while the Iron in the medium reacts with hydrogen sulphide to produce a black precipitate (Cappuccino and Sherman, 2002). The isolates were inoculated in Sulphur-Indole Motility (SIM) agar media by stabbing method in duplicate for replication, and then incubated at 37 °C for 48 hours. Two uninoculated tubes were used as controls. Kovac's reagent was the added to each of the 48 hour culture according to the protocol of (Harold, 2002). The presence of a cherry red layer in the media indicated positive result for Indole production while negative results were indicated by colour remaining brown (Cappuccino and Sherman, 2002). The presence of a black coloration in the media after incubation indicated lack of hydrogen sulphide in the media (Cappuccino

and Sherman, 2002). Lack of motility was detected by the confinement of the bacteria along the line of inoculation.

#### **2.9.6 Citrate utilization**

This test determines the ability of a microbe to use citrate as the sole source of carbon (Harold, 2002). Citrate utilization is indicated by growth accompanied by an alkaline pH (Cappuccino and Sherman, 2002). Simmon's citrate agar slants containing the pH indicator bromothymol blue were inoculated by streaking with the isolates in duplicates and incubated at 37 °C for 72 hours. One uninoculated tube with the same media served as the control. Positive test were indicated by growth of the bacteria accompanied by colour change in the medium from olive green to Prussian or deep blue. Green colour medium a negative test (Cappuccino and Sherman, 2002).

#### **2.9.7 Urease test**

This test is used to determine the ability of a microorganism to produce the enzyme urease that splits urea, into carbon dioxide and ammonia (Harold, 2002). Ammonia makes the medium alkaline. The isolates were inoculated in urea broth media containing phenol red indicator followed by Incubation at 37 °C colour change was monitored for 4 days. Positive reaction was indicated by presence of a deep pink colour, while negative tests remained yellow (Cappuccino and Sherman, 2002).

### **2.9.8 Starch Hydrolysis**

Using the wax pencil, starch agar plate was divided into three straight sections. Labelling was done each with the bacterium to be inoculated. Using aseptic technique streaking was done with the respective bacteria onto the plate in a straight line within the section. Incubation of the plate for 24 to 48 hours at 35 ° C was done. Drops of Gram's iodine were placed on each of the line streaked on the starch agar plate. If the area around the line of growth was clear, starch had been hydrolysed, and the test was positive; if it was not clear or the entire medium turned blue, starch has not been hydrolysed, and the test was negative.

### **2.9.9 Oxidase test**

This tests the ability of a microbe to oxidize certain aromatic amines like p-aminodimethylaniline to form coloured end products. Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein), which are components of the electron transport chain of specific organisms (Kovac, 1956). These enzymes catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The oxidase reagent, (N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride) acts as an artificial electron acceptor for the enzyme oxidase. When its oxidised in the presence of alpha naphthol, the reagent forms the coloured compound indophenol blue.

To test for the activity of this enzyme, some 24- hour old bacterial cultures were scrapped onto plastic N,N-dimethyl-1,4-phenylene diamine and alpha naphthol

impregnated oxidase strips. The strips were left on the clean bench and observed after 1 minute. The development of a deep blue color at the position of the wiped colony after the 1 minute indicated a positive result for oxidase test and lack of this blue colour indicated a negative test (Cappuccino and Sherman, 2002).

### **2.10 Nitrogen fixation test**

Nitrogen fixation was tested using nitrogen free medium. Five ml of the media was placed in 10 ml vials this was followed by inoculating the organisms. Incubation was done at 35° C for 48 hours in an incubator shaker. The growth was determined using a Shimadzu UV spectrophotometer (UV- Mini- 1240, Japan) by taking the optical density at wave length 570 nm. Uninoculated nitrogen free medium was used to set the reference.

### **2.11 Phosphate solubilisation test**

Phosphate solubilization medium was used for screening phosphate solubilizing microorganisms using plate assay method. The phosphate solubilization media was prepared and dispensed on sterile petri dishes. A sterile wire loop was then used to place inoculums onto the media. The plates were then placed in a clean incubator at 30°C for two weeks, after which observations were done (Cappuccino and Sherman, 2002). Formation of a halo around the bacterial colonies indicates a positive test for phosphate solubilization.



## **2.12 Molecular characterization**

### **2.12.1 DNA extraction**

Genomic DNA was extracted from bacterial cells at exponential growth phase grown aerobically in nutrient broth. Prior to extraction, bacterial cells were harvested from broth by centrifuging 1 ml of culture in a 1.5 ml Eppendorf tube at 13,000 g for ten minutes. The pellet was washed by re-suspending the cells in equal volume of TE buffer, centrifuged (Hettich, Micro 200, Germany) for 5 minutes at 13,000 g and the supernatant discarded. The cells were then re-suspended in 200 µl of solution 1[50 mM Tris (pH 8.5), 50 mM EDTA (pH 8.0) and 25% sucrose solution] 5 µl of lysozyme (20 mg/ml) (Sigma Aldrich, Steinheim, Germany) and 5 µl of RNase A (20 mg/ml) (Sigma Aldrich, Steinheim, Germany) then mixed gently (Sambrook *et al.*, 1989). The mixture was then incubated at 37 °C for 1 hour 600 µl of solution 2[10 mM Tris (pH 8.7), 5mM EDTA (pH 8.0) and 1% sodium dodecyl sulphate] and 10 µl of proteinase K (20 mg/ml) (Sigma Aldrich, Steinheim, Germany) were added and mixed gently. The mixture was then incubated at 55 °C for 30 minutes (Sambrook *et al.*, 1989). 400 µl of phenol-chloroform (ratio 1:1) was added and spun for 10 minutes at 13,000 g; carefully the upper aqueous layer was transferred into a separate 1.5 ml Eppendorf tube. This step was repeated before adding 500 µl of chloroform: Isoamylalcohol (24:1) to wash off the phenol. This mixture was then spun at 13,000 g for 10 minutes and the supernatant carefully discarded (Sambrook *et al.*, 1989). The procedure was repeated twice. The DNA was then precipitated by adding 250 µl of ice cold absolute ethanol and 50 µl of sodium chloride (Sigma Aldrich, Steinheim, Germany) and left overnight at -20 °C. The

pellet was precipitated by centrifugation at 13,000 g for 10 minutes and the supernatant discarded. 500 µl of 70% ethanol (Scharlab S.L., Spain) was added and centrifuged at 13,000 g for 10 minutes. Supernatant was discarded carefully not to discard the pellet. This procedure was repeated twice before completely air-drying the pellet at room temperature (this step eliminates residual ethanol) (Sambrook *et al.*, 1989). The dry pellet was then re-suspended in 100 µl of TE buffer (the advantage of the TE is that EDTA chelates magnesium ions which makes it more difficult for residual DNases to degrade the DNA) and then kept at -20 °C for future use (Magarvey *et al.*, 2004). The DNA was visualised on a 1% agarose gel in 1XTBE buffer under UV after staining with ethidium bromide (Sambrook *et al.*, 1989). The DNA quantification was done using spectrophotometer with the absorbance at 260 nm and 280 nm used to determine the purity of the DNA.

### **2.12.2 DNA amplification**

Total genomic DNA was used as a template for amplification of the 16S rDNA gene. The gene encoding the 16S rRNA was amplified by PCR using bacterial universal primer pair combination of forward primer Bac27-F (5'TAGAGTTTGATCCTGGCTCAG3') and the reverse primer Bac1392-R (5'GACGGGCGGTGTGTACA3'), (Bioneer, USA) in relation to *Escherichia coli* gene sequence (Lane, 1991; Embley and Stackebrandt, 1994). Amplification was performed using an advanced Eppendorf 96 AG, model 22331 thermal cycler (Hamburg). Amplification was carried out in a 50 µl mixture containing 0.20 µl of gene script Taq,

1.0 µl (10-pmol) of 27F, 1.0 µl (10-pmol) of 1392R reverse primer, 1 µl of template DNA, and 4.0 µl of dNTP's mix (1.25 mM), 4.0 µl PCR 10 X buffer (genescrypt) 38.8 µl of PCR water. The control contained all the above except the DNA template (Sambrook *et al.*, 1989). Reaction mixtures were subjected to the following temperature cycling profiles repeated for 32 cycles: Initial denaturation 94 °C for 5 minutes, Denaturation at 94 °C for 1 minute, primer annealing at 49 °C for 1 minute, extension at 72 °C for 2 minutes for 32 cycles and a final extension at 72 °C for 10 minutes (Roux, 1995). Amplification products (7 µl) were separated on a 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining (Sambrook *et al.*, 1989).

### **2.12.3 Restriction of the PCR products**

The preliminary genetic diversity was determined by amplified ribosomal DNA restriction analysis (ARDRA) of 16S rDNA using a modified procedure (Desaint *et al.*, 2000). Aliquot of 8 µl of the PCR product was digested in a final volume of 30µl for 12 hours at 37° C with 0.5 µl of a restriction endonuclease (*RsaI*) according to the manufacturer's specifications (Sigma Aldrich, Steinheim, Germany). Digested DNA fragments were separated by electrophoresis in 1.5 (w/v) agarose gels (Sigma Aldrich, Steinheim, Germany) for 2 hours at 80 V. The gel was stained with ethidium bromide and DNA fragments visualised under UV illumination (BTS-20.M, EEC, Taiwan). Similarity among strains was estimated from the proposition of shared restriction fragments bands generated by *RsaI* digestion.

#### **2.12.4 Agarose gel electrophoresis**

1.0% Agarose gel (w/v) was prepared by dissolving 1.0 g of agarose powder into 100 ml of 1XTBE buffer. The gel solution was stirred, brought to boil in a microwave for 3 minutes to completely dissolve the powder, the cooled gel solution was poured in a casting tray having combs and left for sometimes to gel (polymerise). Ethidium bromide (3  $\mu$ l) was incorporated in the gel to facilitate visualisation of DNA under UV light. The PCR products (7  $\mu$ l) was mixed with 3  $\mu$ l of loading dye (Bromophenol blue) and loaded into the well and subjected to electrophoresis at 80 V for 45 minutes (Sambrook *et al.*, 1989).

#### **2.12.5 Purification of PCR products**

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (binding buffer) (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed. The mixture was then transferred to QIAquick spin column and then centrifuged for three minutes at 8000 g. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 740 $\mu$ l buffer PE (washing buffer) was added to the QIAquick column twice and centrifuged for 1 minute. The flow-through was discarded and the column centrifuged again for an additional 1 minute at 8000 g to remove residual ethanol from buffer PE. The QIAquick column was placed in a 1.5 ml microcentrifuge and 30  $\mu$ l of buffer EB (elution buffer) (10 mM Tris-Chloride, pH 8.5) added to elute DNA. The tubes were then centrifuged for 1-minute,

the spin column removed and DNA. The tubes were then centrifuged for 1 minute at 8000 g, the spin column removed and DNA stored at -20° C (Sambrook *et al.*, 1989).

#### **2.12.6 Sequencing and phylogenetic analysis**

Sequencing of purified PCR products was done by a commercial service provider (Macrogen South Korea). In this case, dye-terminator sequencing technique was used.

The sequences were compared to the sequences in the public databases using Basic Local Alignment Search Tool (BLAST) (NCBI, 2012). Alignment was done using CLUSTAL W 1.6 software (EBI, 2012). The 16S rDNA gene sequences with high similarity to those determined in the study were retrieved and added to database and aligned with Mega 5 (Tamura *et al.*, 2011). To show the evolutionary relationships of these taxa, the evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987; Tamura *et al.*, 2007). Bootstrap analysis using Mega 5 software, was performed to attach confidence estimates for the tree topologies (Felsenstein, 1983). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and were in the units of the number of base substitutions per site.

#### **2.12.7 Data Analysis**

Data entry management and preliminary summaries such as averages, percentages were done in Microsoft Excel Spreadsheet. Colonies counts from repeated experiments were subjected to student t-test for the comparison of the mean between different

layers of the soil using Portable SPSS version 18. The Biochemical characterization procedures were done in replicates of three. Observations were made on these replicates to define the nature of each of the qualitative tests as either positive or negative. This information was used during the generation of phylogenetic trees to identify the bacterial isolates. 16S rRNA gene sequences of the bacteria isolates were viewed and edited using Chromas Pro 5 software package. They were then aligned using CLUSTAL W 1.6 to provide full sequences of about 1500 nucleotide bases. The sequences were compared to sequences in the public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website to find closely related bacterial 16S rRNA gene sequences and parameters such as percentage similarities and E-values were used. The 16S rRNA gene sequences of the isolates and those of the closely related bacteria were then aligned and processed to produce phylogenetic trees using MEGA 5 software package.

## **CHAPTER THREE**

### **3.0 RESULTS**

#### **3.1 Isolation and characterization of Bacteria**

The inoculated plates were incubated at 25° C and observations were made as from day two of growth (Plate 3.1a-c). Using enrichment culture methods, a total of thirty eight pure isolates were obtained from Ngere tea catchment area. Among them thirteen were from Tryptone soy agar and twenty five were from dilute nutrient broth agar

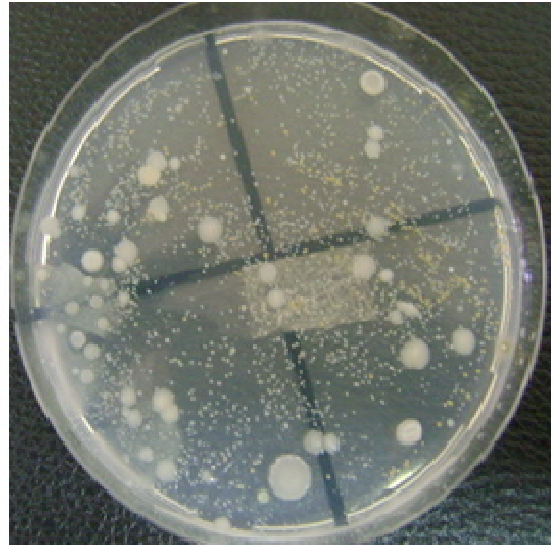
#### **3.2 Morphological characterization of bacterial isolates**

##### **3.2.1 Colony and Cell morphology**

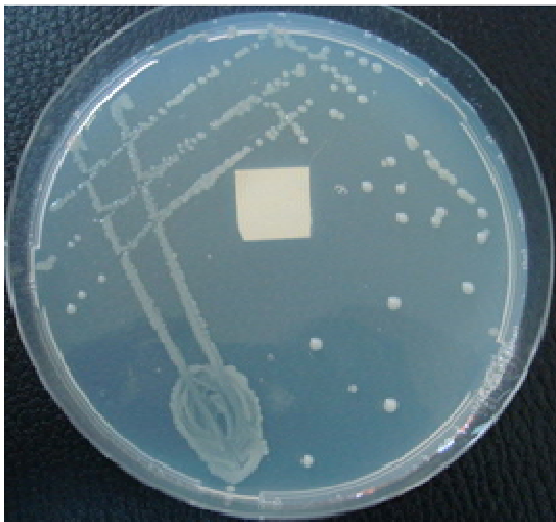
Morphological characterization was based on classical macroscopic attributes of colour, form, shape, and elevation of pure colonies. Most colonies were able to grow within 2-3 days of incubation at 25 °C. The colony morphology of the isolates obtained from Ngere tea catchment area ranged from circular, entire, flat and filamentous or branching (Plate 3.2). They were smooth or rough and the colour ranged from white to cream and brown (Table 3.1). 53 % of the isolates were Gram positive while 47 % were Gram negative (Plate 3.3). The cells ranged from long rods, short rods to coccus (Table 3.1). Most (96%) isolates were rods and only 4% were cocci in shape.



**Plate 1 a**



**Plate 1 b**



**Plate 1 c**

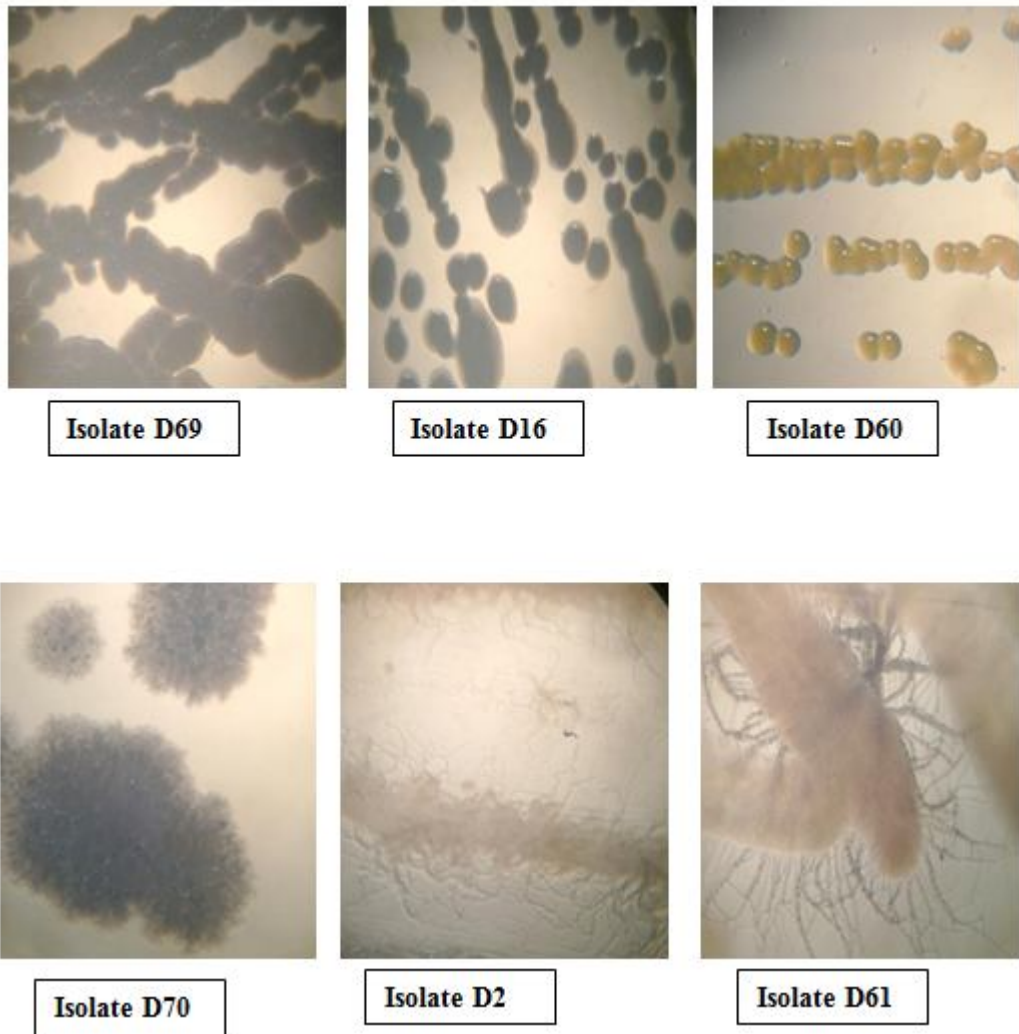
**Plate 3.1:** (1a) Tryptone soy agar medium plate with different colonies before isolation of individual colonies (low diversity with a few colonies). (1 b) Dilute nutrient broth agar medium culture plate with different colonies before isolation of individual colonies (high diversity). (1 c) Culture plate with individual colonies



**Table 3.1:** Morphological characteristics of bacterial isolates obtained from Ngere tea soils

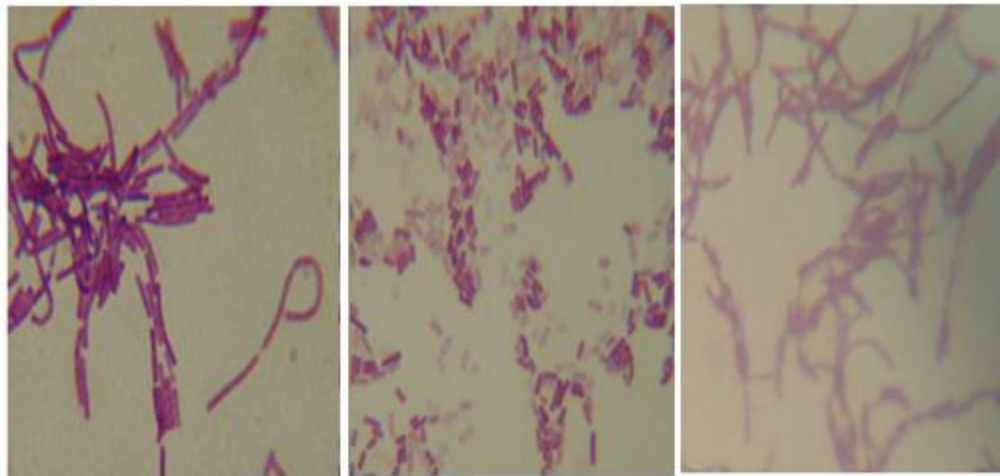
| Isolate | Colony Characterization |           |           |            | Cell characterization |               |
|---------|-------------------------|-----------|-----------|------------|-----------------------|---------------|
|         | colour                  | form      | elevation | margin     | Cell arrangement      | Gram reaction |
| D16an   | Cream                   | Irregular | Flat      | Ciliate    | Large rods            | Positive      |
| D19     | White                   | Irregular | Flat      | Undulate   | Small rods            | Positive      |
| D3      | Cream                   | Irregular | Flat      | Undulate   | Short rods            | Positive      |
| D5      | White                   | Irregular | Flat      | Whip- like | Large rods            | Positive      |
| D72     | Cream                   | Irregular | Flat      | Undulate   | Large rods            | Positive      |
| D62     | Cream                   | Irregular | Flat      | Branching  | Large rods            | Positive      |
| S6      | Cream                   | Irregular | Flat      | Undulate   | Large rods            | Positive      |
| D61     | Cream                   | Irregular | Flat      | Branching  | Small rods            | Positive      |
| D70     | Brown                   | Irregular | Flat      | Undulate   | Large rods            | Positive      |
| D2      | Brown                   | Irregular | Flat      | Ciliate    | Large rods            | Positive      |
| D29     | Cream                   | Irregular | Flat      | Undulate   | Large rods            | Positive      |
| D51     | Cream                   | Irregular | Flat      | Ciliate    | Short rods            | Positive      |
| C5      | Cream                   | Irregular | Flat      | Undulate   | Short rods            | Positive      |
| S42     | Brown                   | Irregular | Flat      | Ciliate    | Large rods            | Positive      |
| S31     | Cream                   | Irregular | Flat      | Ciliate    | Large rods            | Positive      |
| S12     | White                   | Irregular | Flat      | Ciliate    | Short rods            | Positive      |
| D68an   | Brown                   | Irregular | Flat      | Undulate   | Large Rods            | Positive      |
| S30     | White                   | Irregular | Flat      | Entire     | Large rods            | Positive      |
| D49an   | Brown                   | Circular  | Raised    | Entire     | Short rods            | Negative      |
| S48     | Cream                   | Circular  | Flat      | Smooth     | Large rods            | Negative      |
| S49     | Cream                   | Irregular | Raised    | Smooth     | Large rods            | Negative      |
| D79     | Brown                   | Circular  | Flat      | Entire     | Short rods            | Negative      |
| D60     | Yellow                  | Circular  | Flat      | Entire     | Large rods            | Negative      |
| D28     | Brown                   | Circular  | Raised    | Smooth     | Short rods            | Negative      |
| D63an   | Cream                   | Circular  | Flat      | Smooth     | Short rods            | Negative      |
| S20     | Brown                   | Circular  | Raised    | Smooth     | Short rods            | Negative      |
| D66an   | Brown                   | Irregular | Raised    | Entire     | Short rods            | Negative      |
| C53     | Cream                   | Circular  | Flat      | Entire     | Short rods            | Negative      |
| D69     | Cream                   | Irregular | Raised    | Smooth     | Short rods            | Negative      |
| D13an   | Cream                   | Irregular | Flat      | Entire     | Coccus                | Positive      |
| D78     | Cream                   | Circular  | Raised    | Smooth     | Coccus                | Positive      |
| D64an   | Cream                   | Circular  | Flat      | Entire     | Large rods            | Negative      |
| D80     | Cream                   | Irregular | Flat      | Raised     | Short rods            | Negative      |
| S55     | Cream                   | Circular  | Flat      | Smooth     | Large rods            | Negative      |
| D1      | Brown                   | Irregular | Flat      | Undulate   | Short Rods            | Negative      |
| S50     | Cream                   | Round     | Flat      | Smooth     | Short rods            | Negative      |
| S23     | White                   | Round     | Raised    | Smooth     | Short rods            | Negative      |
| D77     | Cream                   | Round     | Raised    | Smooth     | Large rods            | Negative      |

**KEY:** Isolates C5 and C53 highlighted in yellow are the controls



**Plate 3.2:** Some of the bacterial colonies, as seen under dissection microscope

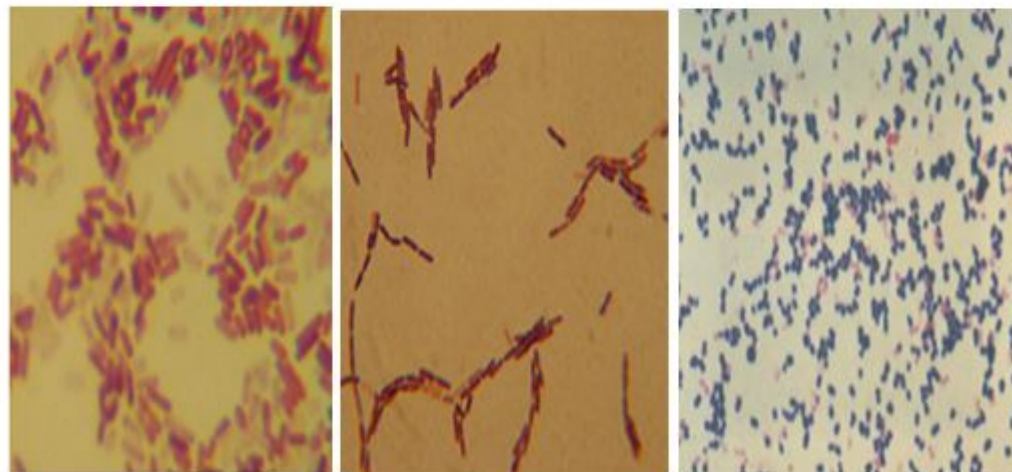
**Legend:** Irregular, raised and smooth (**D69**), irregular, flat and ciliate (**D16**), circular, flat and entire (**D60**), irregular, flat and undulate (**D70**), irregular, flat and ciliate (**D2**), irregular, flat and branching (**D61**)



Isolate D16

Isolate D2

Isolate D61



Isolate S23

Isolate D69

Isolate D78

**Plate 3.3:** Gram reaction of selected bacterial isolates.

**Legend:** Gram positive rods [ (D16), (D2), (D61)], gram negative rods [ (S23), (D69)], gram positive coccus [ (D78)]

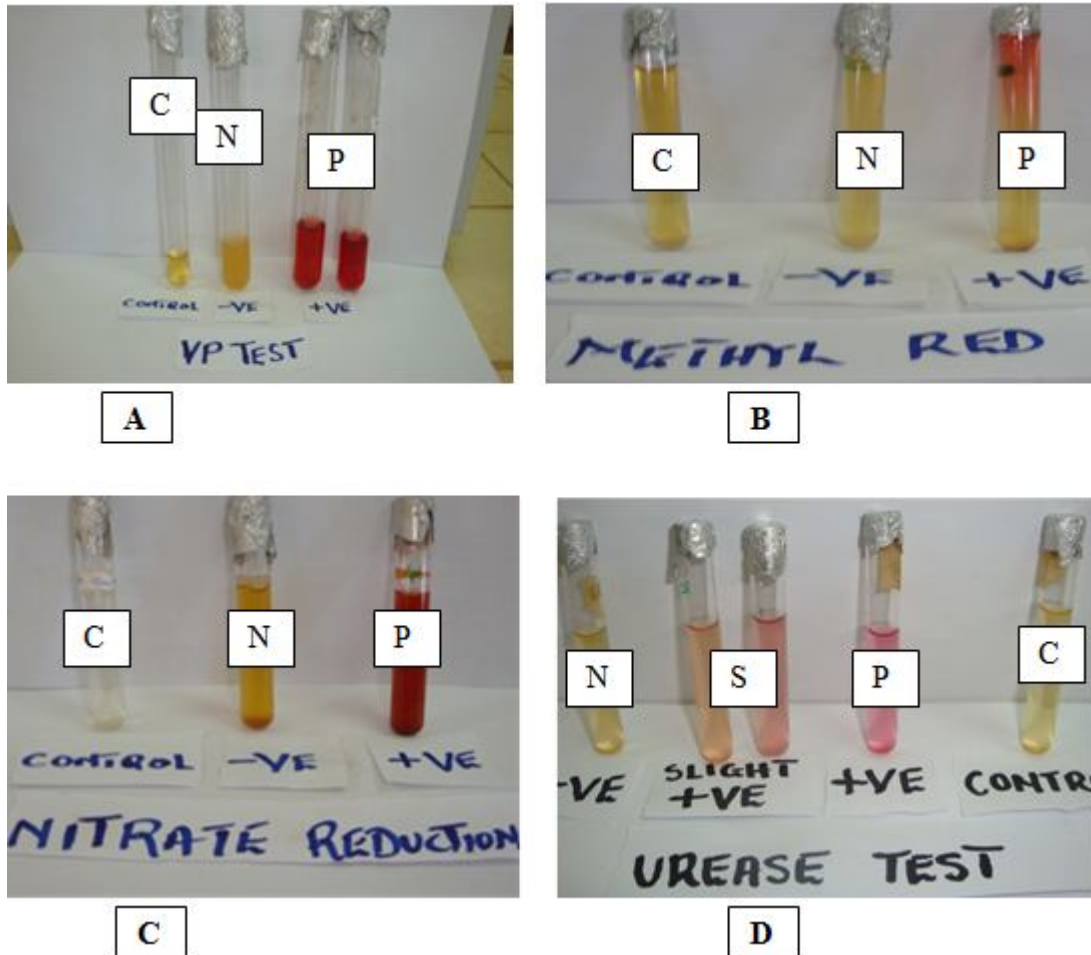
### **3.3 Biochemical tests of the isolates**

Results for the various Biochemical assays: (Catalase test, Oxidase, Nitrate Reduction Test, Methyl Red test, Voges-Proskauer Test, Indole Test, Starch Hydrolysis, Motility test, Urease Test, Citrate Utilization test, Gelatine Hydrolysis and Hydrogen Sulphide Gas production) are shown on Table 3.2. Motility test done on SIM medium showed that some of the isolates were motile with the presence of flagella D1, S55, D13, D78, D60, D29 and S6 were negative for motility (Table 3.2). The ability of the isolates to excrete intracellular enzymes was determined through tests on catalase reaction, urease, Voges-Proskauer. Catalase test revealed that all isolates were positive for hydrogen peroxide production an end product of oxidation of sugars among the soil bacteria. Some isolates were positive for nitrate reduction test since the addition of sulphanilic acid and alpha-naphthylamine reacted with nitrite released from nitrate and turned red in colour (Plate 3.4 C) these isolates included; S49, D79, D66, D69, S20, S23, S50, D70, D51, S30, S31, S42, D16, D5, D61 and D19. Most isolates were negative for methyl red test indicating the inability of the isolates to ferment glucose and produce a lot of mixed acids as end products (Table 3.2) except isolates D1, D49, D79, D63, D77, S5 and D72 were positive for the test. Urease a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product ammonia that causes the phenol red in the urea broth medium to turn a deep pink (Plate 3.4 D). Isolates D1, D78, D69, D77, D70, D51, S30, S31, S12, S5, D16, D5, D61, D19 and D72 were positive for the test (Plate 3.2)

**Table 3.2:** Biochemical characteristics of bacterial isolates obtained from Ngera tea soils

| Isolates | Biochemical Tests |        |          |         |          |      |         |    |    |          |         |     |                  |           |
|----------|-------------------|--------|----------|---------|----------|------|---------|----|----|----------|---------|-----|------------------|-----------|
|          | Starch            | Indole | Catalase | Nitrate | Motility | Urea | Citrate | MR | VP | Gelatine | Oxidase | NFT | H <sub>2</sub> S | Phosphate |
| D1       | +                 | -      | +        | -       | -        | +    | +       | +  | -  | -        | -       | +   | -                | +         |
| S55      | +                 | -      | +        | -       | -        | -    | +       | -  | -  | -        | -       | -   | -                | +         |
| D13      | -                 | -      | +        | -       | -        | -    | +       | -  | -  | -        | +       | +   | -                | +         |
| D78      | -                 | -      | +        | -       | -        | +    | +       | -  | -  | -        | +       | +   | -                | +         |
| S48      | +                 | -      | +        | -       | +        | -    | +       | -  | +  | -        | +       | +   | -                | +         |
| D49      | +                 | -      | +        | -       | +        | -    | +       | +  | -  | -        | +       | +   |                  | +         |
| S49      | +                 | -      | +        | +       | +        | -    | +       | -  | -  | -        | +       | +   | -                | +         |
| D79      | +                 | -      | +        | +       | +        | -    | +       | +  | -  | -        | -       | +   | -                | +         |
| D60      | +                 | +      | +        | -       | -        | -    | +       | -  | -  | +        | +       | +   | -                | +         |
| D80      | -                 | -      | +        | +       | +        | -    | +       | -  | +  | +        | -       | +   | -                | +         |
| D64      | -                 | -      | +        | -       | +        | -    | +       | -  | +  | +        | -       | +   | -                | +         |
| D63      | +                 | +      | +        | -       | +        | -    | +       | +  | -  | +        | +       | +   | -                | +         |
| D28      | +                 | +      | +        | -       | +        | -    | +       | -  | -  | +        | +       | +   | -                | +         |
| D66      | +                 | -      | +        | +       | +        | -    | +       | +  | -  | +        | +       | +   | -                | +         |
| C53      | +                 | +      | +        | -       | +        | -    | +       | +  | -  | -        | +       | +   | -                | +         |
| D69      | -                 | -      | +        | +       | +        | +    | +       | -  | -  | -        | +       | +   | -                | +         |
| S20      | -                 | -      | +        | +       | +        | -    | +       | -  | -  | -        | +       | +   | -                | +         |
| D77      | +                 | -      | +        | -       | +        | +    | +       | +  | -  | -        | +       | -   | -                | +         |
| S23      | +                 | -      | +        | +       | +        | -    | +       | -  | -  | -        | +       | +   | -                | +         |
| S50      | +                 | -      | +        | +       | +        | -    | +       | -  | -  | -        | +       | +   | -                | +         |
| D70      | +                 | -      | +        | +       | +        | +    | +       | -  | +  | +        | +       | +   | +                | +         |
| D2       | +                 | -      | +        | -       | +        | -    | +       | -  | +  | -        | +       | +   | +                | +         |
| D68      | +                 | +      | +        | -       | +        | -    | +       | -  | +  | +        | +       | +   | -                | +         |
| D62      | +                 | -      | +        | -       | +        | -    | +       | -  | -  | +        | +       | +   | -                | +         |
| D29      | +                 | +      | +        | -       | -        | -    | +       | -  | +  | -        | +       | +   | -                | +         |
| D51      | +                 | -      | +        | +       | +        | +    | +       | -  | +  | +        | +       | -   | +                | +         |
| S30      | +                 | -      | +        | +       | +        | +    | +       | -  | +  | -        | +       | +   | +                | +         |
| S31      | +                 | -      | +        | +       | +        | +    | +       | -  | +  | -        | +       | +   | +                | +         |
| S12      | +                 | -      | +        | -       | +        | +    | +       | -  | -  | +        | +       | +   | -                | +         |
| C5       | +                 | -      | +        | -       | +        | +    | +       | +  | -  | +        | +       | +   | -                | +         |
| S6       | +                 | -      | +        | -       | -        | -    | +       | -  | +  | +        | +       | -   | -                | +         |
| S42      | +                 | -      | +        | +       | +        | -    | +       | -  | +  | -        | +       | +   | -                | +         |
| D16      | +                 | -      | +        | +       | +        | +    | +       | -  | +  | +        | +       | +   | +                | +         |
| D3       | +                 | -      | +        | -       | +        | -    | +       | -  | -  | +        | +       | +   | -                | +         |
| D5       | +                 | -      | +        | +       | +        | +    | +       | -  | +  | +        | +       | +   | -                | +         |
| D61      | +                 | -      | +        | +       | +        | +    | +       | -  | +  | +        | +       | +   | +                | +         |
| D19      | +                 | -      | +        | +       | +        | +    | +       | -  | +  | +        | +       | +   | +                | +         |
| D72      | +                 | -      | +        | -       | +        | +    | +       | +  | -  | +        | +       | +   | -                | +         |

**Key:** (+) Positive, (-) Negative, MR: Methyl Red, VP: Voges-Proskauer, NFT: Nitrogen fixation test and H<sub>2</sub>S: Hydrogen Sulphide gas.



**Plate 3.4:** Voges- Proskauer test (A), Methyl red test (B), Nitrate reduction test(C) and Urease test (D) as part of biochemical tests carried out.

**Legend:** C-control, N- negative, P- positive, S- slightly positive

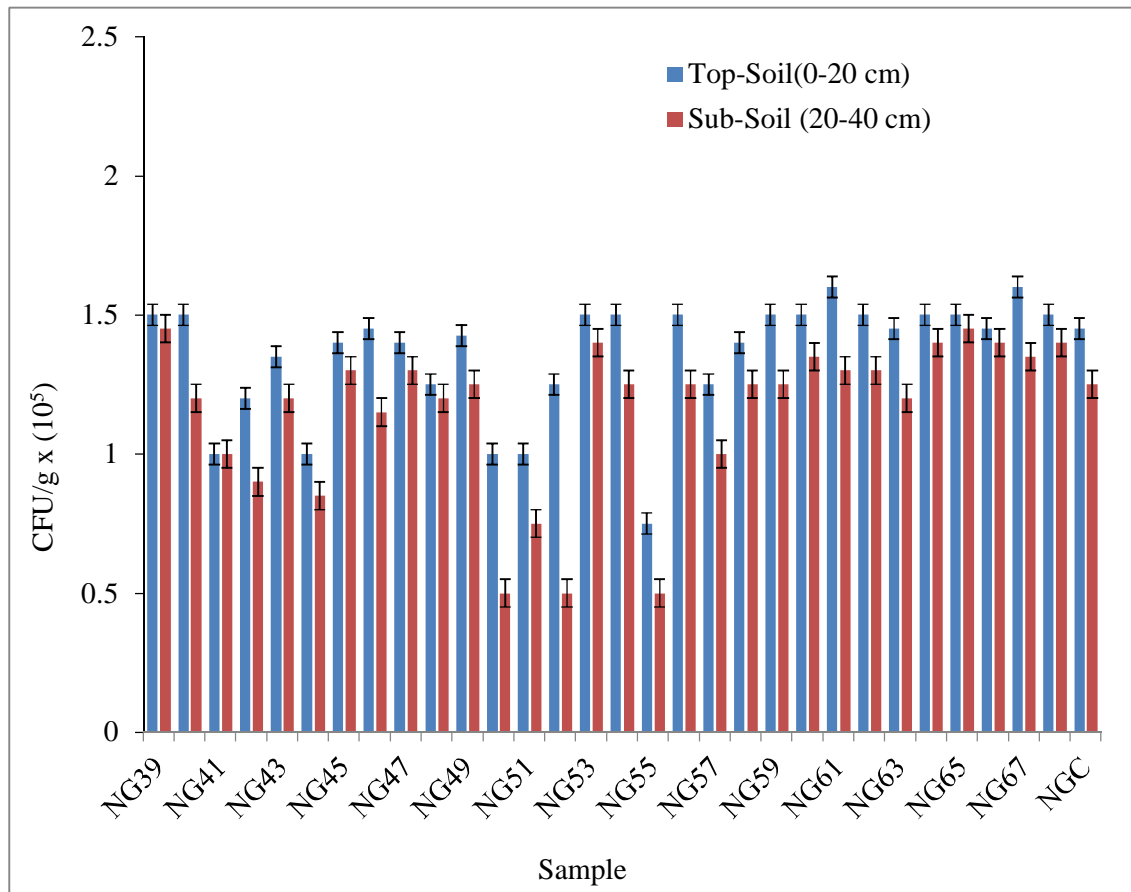
Indole generation by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid was tested for the isolates. Tryptophanase enzyme catalyzes the deamination reaction, during which the amine (-NH<sub>2</sub>) group of the tryptophan molecule was removed and final products of the reaction are indole, pyruvic

acid, ammonia ( $\text{NH}_3$ ) and energy. Isolates D60, D63, D28, S53, D68 and D29 were positive for the test (Table 3.2). The release of inorganic phosphate from organic phosphates is called mineralization and is caused by microorganisms breaking down organic compounds the ability of an organism to do this was determined by phosphate solubilization test which showed that all isolates were positive for this test. The starch molecule is a large one consisting of two constituents: amylose, a straight chain polymer of 200 to 300 glucose units, and amylopectin, a larger branched polymer with phosphate groups. Therefore bacteria that hydrolyze starch produce amylase enzyme that yield molecules of maltose, glucose and dextrin. Most isolates were positive for the test except isolate D13, D78, D80, D64, D68 and S20 were negative for the test (Table 3.2). Isolates D60, D80, D64, D63, D28, D66, D70, D62, D68, D51, S12, S5, S6, D3, D5, D61, D19 and D72 were positive for extracellular gelatinase enzyme. The ability of the organisms to fix atmospheric nitrogen which reduces nitrogen to ammonia was carried out by growing organisms in nitrogen free medium which showed the ability of an organism to possess the enzyme nitrogenase, which reduces nitrogen to ammonia most isolates were positive for the test except isolates S55, D77, D55 and S6 were negative for this test (Table 3.2). The ability of isolates to produce cytochrome oxidase which oxidizes transport molecule cytochrome C while reducing oxygen to form water in the final stage of cell was tested and most isolates were positive for the test (Table 3.2).

### **3.4 Bacterial viable cell count**

The viable cell counts were performed on the bacteria that were able to form visible colonies within 2- 3 days of inoculation. Soil samples were diluted in normal saline, and aliquots from dilutions were plated onto the media in replicates. The amount of variation in colony numbers between replicate plates in counting set decreased with increased dilution factor. The colony forming units were obtained by computing the average among the set of dilution factor ( $10^0$ ,  $10^{-1}$  and  $10^{-2}$ ) from different layers of soil. Top soil showed highest count ( $1.6 \times 10^5$ ) as compared to the sub-soil ( $1.42 \times 10^5$ ) (Figure 3.2). Each solid bar represents the mean of the three counting sets from the soil sample prepared from top layer and three counting sets prepared from sub- soil (Figure 3.2). The results also showed that there was statistical support for the differences between the mean counts obtained from top soil and sub-soil by one sample t test (Appendix 1).



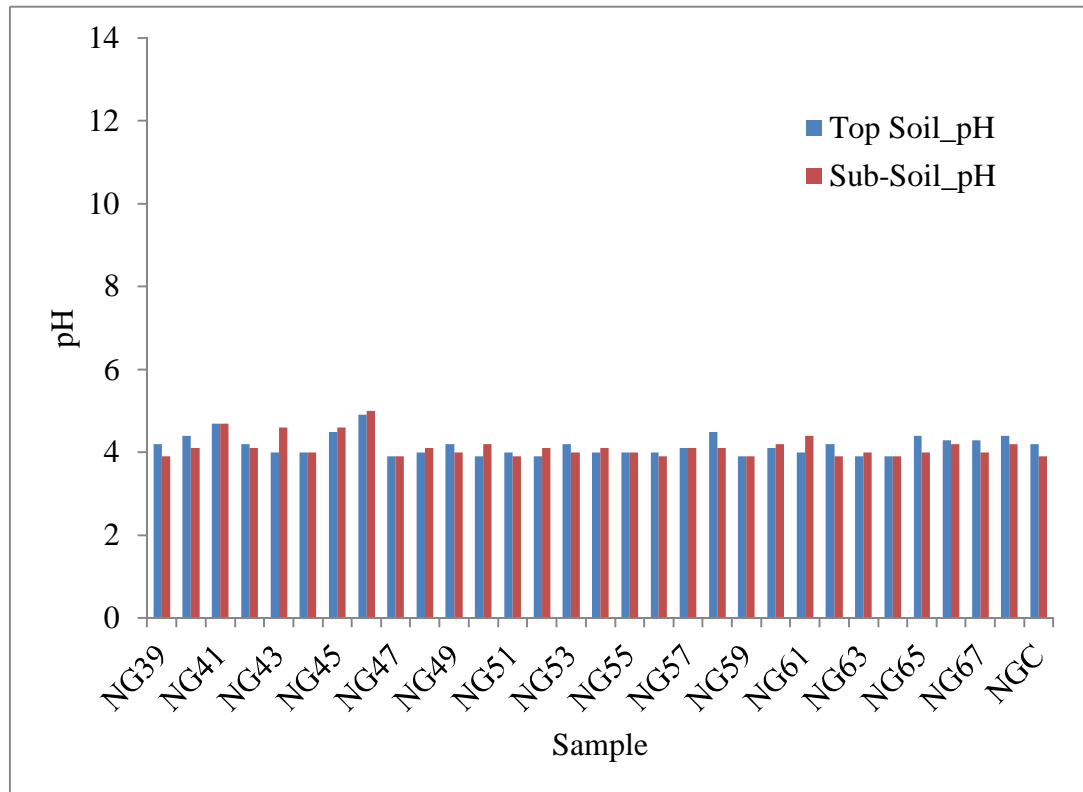


**Figure 3.3:** Showing bacteria viable cell counts from top-soil (0-20 cm) and sub-soil (20-40 cm)  
**Legend:** The error bars indicate standard errors.

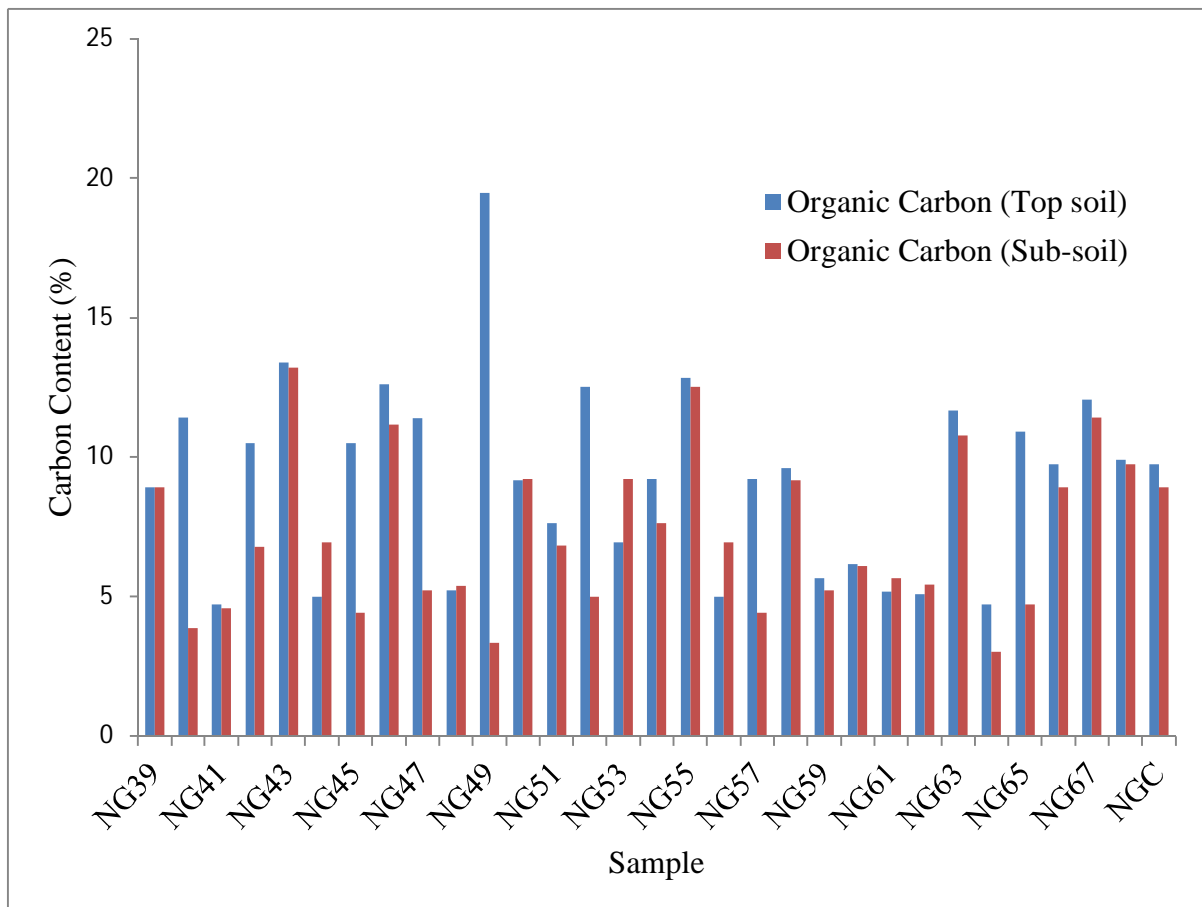
### 3.5 Relationship between organic carbon, pH and colony counts from top soil

The soil pH ranged from 3.9 to 5.0 (figure 3.4) with both tea soils and their adjacent Napier grass having the same pH values. The soil organic carbon concentration was higher in tea soils from top soils ranging from 4.7 to 19 than in the sub soils which ranged from 3.0 to 13.2 (Figure 3.5). In the tea soils studied, there was no correlation between soil pH and microbial biomass; however there was a linear correlation between organic carbon concentrations and soil microbial colony counts. Soil microbial biomass

increased significantly between pH 4 and 4.9 in top soils and between pH 3.9 and 5.0 in sub soils.



**Figure 3.4:** Relationship between the pH of the top (0-20cm) and sub-soil (20-40cm)

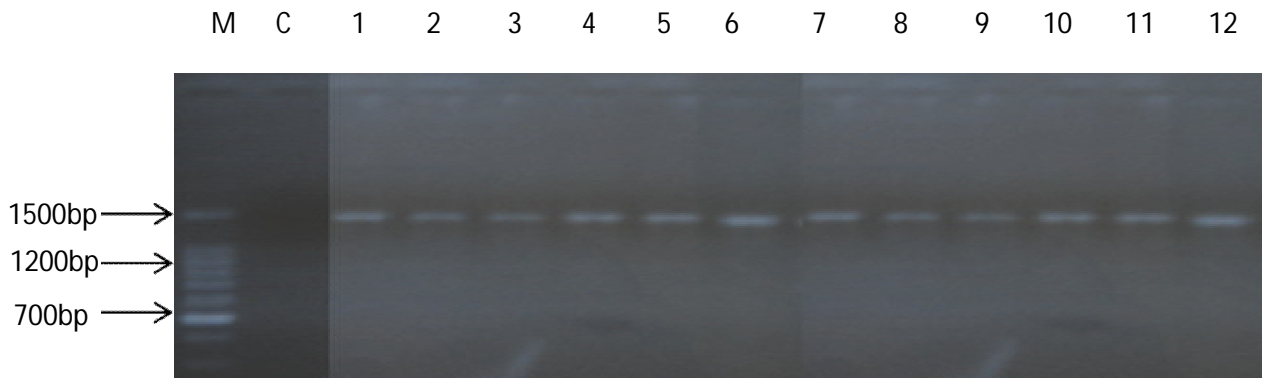


**Figure 3.5:** Relationship between the organic carbon content of the top (0-20cm) and sub-soil (20-40cm)

### 3.6 Molecular characterization

#### 3.6.1 PCR amplification of 16s rDNA genes from isolates

Genomic DNA was extracted from all the selected 38 isolates using the phenol/chloroform method. Amplification of 16S rDNA gene with bacterial universal primers bac 27F and bac 1392R (Embley and Stackebrandt, 1994; Lane, 1991) yielded an amplification product of approximately 1500 bp (Plate 3.5). The amplicons were then stained with ethidium bromide and visualised under UV light on 1 % agarose gel



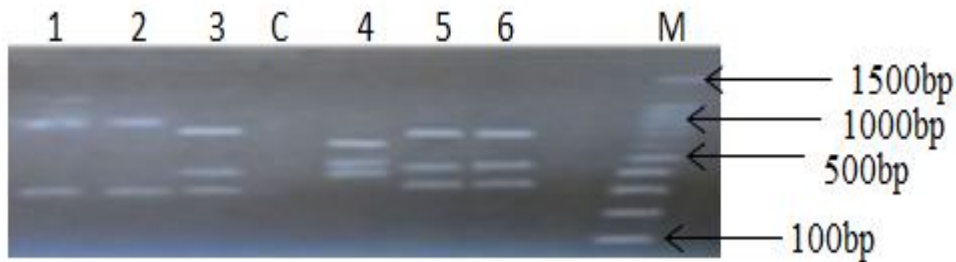
**Plate 3.5:** PCR amplified 16S rDNA products from representative isolates among the isolates from Ngere tea catchment area using universal primers bac 27F and bac 1392R.

**Legend:** Lanes 1(**D69\***), 2(**D79\***), 3(**D60\***), 4(**D61**), 5(**D28\***), 6(**D63\***), 7(**D2\***), 8(**S49\***), 9(**S49\***), 10(**D29\***), 11(**S20\***), 12(**S23\***), (**C\***) negative control and (**M\***) M-1500 bp Molecular marker size

\*The figures within the brackets are the isolate numbers

### 3.6.2 Restriction analysis

The restriction enzyme analysis provided an initial clustering of strains into six groups with three or more members with the same restriction profile. Fragment restriction using *RsaI* produced six clusters of the isolates which were generated based on fragment sizes (Plate 3.6).



**Plate 3.6:** Restriction products as generated by *Rsa I* digestion run in 1.5% (W/V) agarose gel.

**Legend:** Lanes 1(D60\*), 2(D61), 3(D28\*), 4(D63\*), 5(D2\*), 6(S49\*), (C\*) negative control and (M\*) M-1500 bp Molecular marker size

\*The figures within the brackets are the isolate numbers

### 3.6.3 Phylogenetic analysis of the sequences

Though a total of 25 isolates were sequenced, only 22 could be placed into the phylogenetic trees. The rest were not placed into the tree either because of they had too low an identity to allow for sensible alignment or they had sequence of less than 320 base pairs according to Rees *et al*, 2003. BLAST analysis of the partial sequences showed that 45 % were from the genus *Bacillus* within the Firmicutes in the domain bacteria (Table 4.3) with similarities between 83% and 99%. Among these were;

*Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* among others (Table 4.3). Five isolates had 23% and belonged to the genus *Pseudomonas* with similarities between 94% and 99 %. The genus *Enterobacter* represented 13% with similarities between 83% and 90%. Other genera such *Burkholderia*, *Chryseobacterium*, *Acinetobacter* and *Serratia* constituted 9 % with percentage similarities of 88% to 99 %. However, isolate D66 had sequence similarity of between 95-97 % and this could represent novel species. While isolates D69, D79, D63, S49, S48, D5, D51, D80, D70, S31 and D1 had sequence similarity of between 80-94 % these could represent novel genera (Table 4.3).

Phylogenetic analysis of the isolates from Ngere tea soils showed that ten isolates clustered into genus *Bacillus sp*, isolate D61, D19 and D51 were closely related to *Bacillus cereus* strain while isolates D5, D16, S31, D70 and D2 closely related to *Bacillus thuringiensis* isolate D29 was grouped together with *Bacillus mycoides* and isolate S30 clustered together with *Bacillus subtilis*. The study also shows that three isolates S49, S48 and D79 closely related to *Enterobacter sp*. Five isolate were clustered into genus *Pseudomonas sp* isolates S20 and D69 closely related to *Pseudomonas sp*, while isolates D28 and D63 were closely related to *Pseudomonas tolaasii* and isolate D66 to *Pseudomonas fluorescens*. Isolate S23 was grouped together with *Burkholderia sp* while isolate D60 closely related to *Chryseobacterium sp* (FR871430). Other groups were clustered into genus *Acinetobacter* and *Serratia*. However, isolates D60, D61, D28, D2, D29, S20, S23, D19, and S30 had sequence similarity of between 99 - 100 %.

Phylogenetic analysis of the isolates from Ngere tea soils showed that six isolates clustered into genus *Bacillus sp*, isolate D61 was closely related to *Bacillus cereus* strain (JF838294), while isolates D5, D19, S30, D29 and D2 closely related to *Bacillus sp* ((FJ654444), isolate D29 was grouped together with *Bacillus mycoides* (Figure 3.7). The study also shows that three isolates D60, S23 and D1 closely related to *Chryseobacterium sp* (FR871430), *Burkholderia sp* (AB636680), and *Acinetobacter sp* respectively. Other isolates were clustered into genus *Pseudomonas sp* isolates S20 closely related to *Pseudomonas sp* (JQ781589), while isolates D28 was closely related to *Pseudomonas tolaasii* (JN232076) (Figure 3.7)

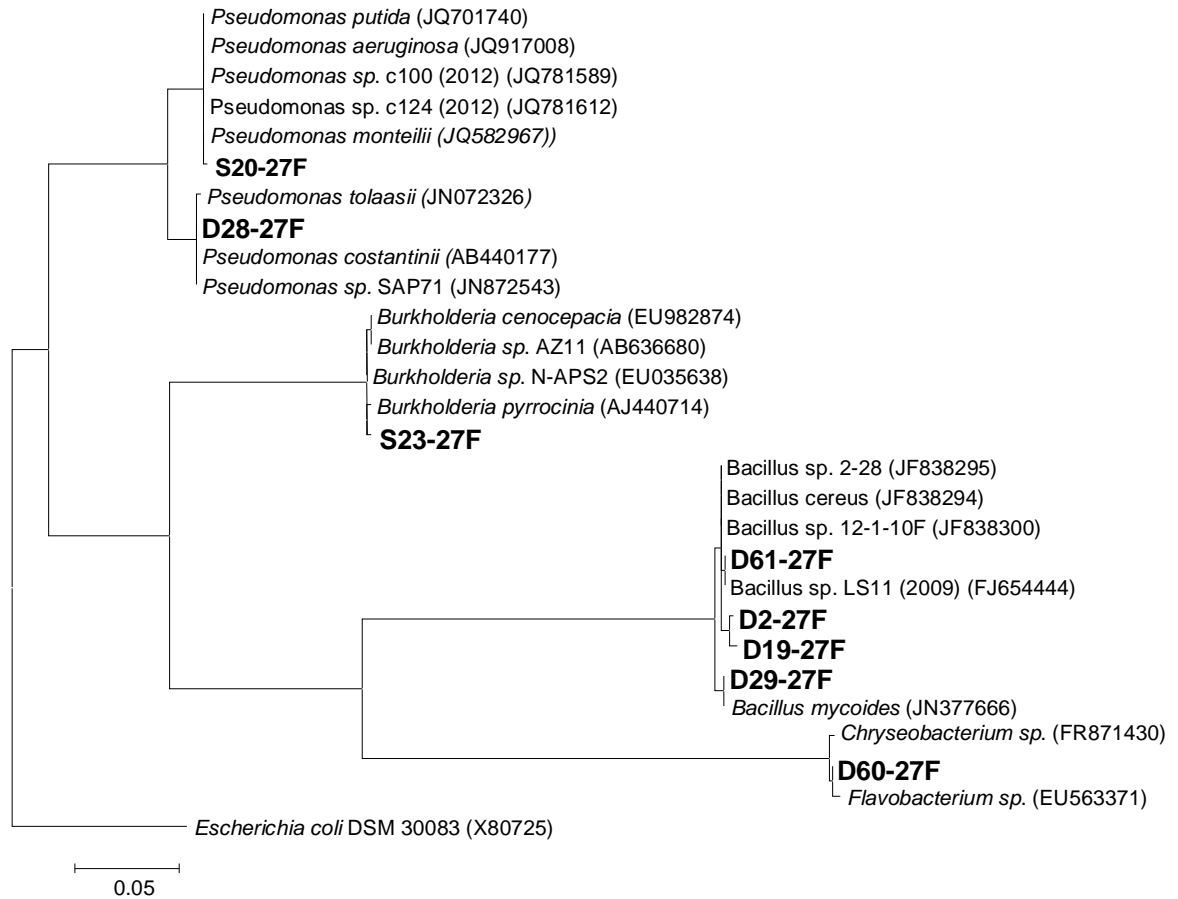
**Table 3.3:** BLAST analysis results of the isolates from Ngere tea soils area nearest neighbours in the data bank and their percentage relatedness.

| <b>Isolate</b>       | <b>Next Neighbour</b>                             | <b>Accession Number</b> | <b>Base pairs</b> | <b>% similarity</b> | <b>E- Values</b> |
|----------------------|---|-------------------------|-------------------|---------------------|------------------|
| <b>D60-(bac 27F)</b> | <i>Chryseobacterium sp.</i> 1095B-08 strain 1095B | FR871430.1              | 594               | 99                  | 0                |
|                      | <i>Flavobacterium sp.</i> PH1                     | EU563371.1              |                   | 99                  | 0                |
| <b>D61-(bac 27F)</b> | <i>Bacillus sp.</i> LS11(2009)                    | FJ654444.1              | 770               | 99                  | 0                |
|                      | <i>Bacillus cereus strain 1-8-10F</i>             | JF838294.1              |                   | 99                  | 0                |
|                      | <i>Bacillus cereus strain BC-1</i>                | JF838295.1              |                   | 99                  | 0                |
| <b>D63-(bac 27F)</b> | <i>Pseudomonas sp.</i> N26                        | JN055435.1              | 706               | 93                  | 0                |
|                      | <i>Pseudomonas tolaasii strain 6264</i>           | JN187439                |                   | 93                  | 0                |
|                      | <i>Pseudomonas sp</i> 4AG6                        | HQ849997                |                   | 93                  | 0                |
| <b>D28-(bac 27F)</b> | <i>Pseudomonas palleroniana strain Y1</i>         | JQ770187.1              | 600               | 99                  | 0                |
|                      | <i>Pseudomonas palleroniana strain POT2</i>       | JN872543.1              |                   | 99                  | 0                |
|                      | <i>Pseudomonas costantinii</i>                    | AB440177.1              |                   | 99                  | 0                |
|                      | <i>Pseudomonas tolaasii strain IPPBC Pt01</i>     | JN232076.1              |                   | 99                  | 0                |
| <b>D2-(bac 27F)</b>  | <i>Bacillus sp.</i> BE506                         | JQ764997.1              | 636               | 99                  | 0                |
|                      | <i>Bacillus cereus strain SAc</i>                 | JQ410792.1              |                   | 99                  | 0                |
|                      | <i>Bacillus sp.</i> 13839                         | JN874756.1              |                   | 99                  | 0                |
|                      | <i>Bacillus cereus strain B10</i>                 | JN252060.1              |                   | 99                  | 0                |
| <b>S49-(bac 27F)</b> | <i>Enterobacter sp.</i> NCCP-280                  | AB641889.1              | 736               | 90                  | 0                |
|                      | <i>Enterobacter cloacae strain JNVU TW3</i>       | JN832584.1              |                   | 90                  | 0                |
|                      | <i>Enterobacter kobei strain JMC 8580</i>         | AB616140.1              |                   | 90                  | 0                |
| <b>D29-(bac 27F)</b> | <i>Bacillus sp.</i> WYT034                        | JQ807859.1              | 751               | 100                 | 0                |
|                      | <i>Bacillus sp.</i> WYT007                        | JQ807855.1              |                   | 100                 | 0                |
|                      | <i>Bacillus cereus</i>                            | AB679980.1              |                   | 100                 | 0                |
|                      | <i>Bacillus weihenstephanensis</i>                | FR848411.1              |                   | 100                 | 0                |
|                      | <i>Bacillus mycoides strain B20</i>               | JN377666.1              |                   | 100                 | 0                |
| <b>S20-(bac 27F)</b> | <i>Pseudomonas sp.</i> c124(2012)                 | JQ781612.1              | 541               | 99                  | 0                |



|                      |  |             |     |    |   |
|----------------------|--|-------------|-----|----|---|
|                      | <i>Pseudomonas sp.</i> c100(2012)              | JQ781589.1  |     | 99 | 0 |
|                      | <i>Pseudomonas sp.</i> 211B                    | JF732909.1  |     | 99 | 0 |
|                      | <i>Pseudomonas aeruginosa</i> strain IRLM.4    | JQ917008.1  |     | 99 | 0 |
| <b>S48-(bac 27F)</b> | <i>Enterobacter sp.</i> BIHB1401               | JF766696.1  | 635 | 86 | 0 |
|                      | <i>Enterobacter ludwigii</i> strain BXCC-36    | JF431271.1  |     | 86 | 0 |
|                      | <i>Enterobacter cloacae</i> strain Bru-1       | HQ231214.1  |     | 86 | 0 |
|                      | <i>Enterobacter ludwigii</i> strain PSB1       | HQ242714.1  |     | 86 | 0 |
|                      | <i>Enterobacter ludwigii</i> strain PSB1       | HQ242714.1  |     | 86 | 0 |
| <b>S23-(bac 27F)</b> | <i>Burkholderia sp.</i> isolate N-APS2         | EU035638.1  | 683 | 99 | 0 |
|                      | <i>Burkholderia cenocepacia</i> strain BK6     | EU982874.1  |     | 99 | 0 |
|                      | <i>Burkholderia pyrrocinia</i> strain R13058   | AJ440714.1  |     | 99 | 0 |
|                      | <i>Burkholderia sp.</i> T132                   | GU191167.1  |     | 99 | 0 |
|                      | <i>Burkholderia sp.</i> O1a_RA002              | FJ800561.1  |     | 99 | 0 |
| <b>D16-(bac 27F)</b> | <i>Bacillus thuringiensis</i> clone C05        | GU003833.1  | 745 | 93 | 0 |
|                      | <i>Bacillus thuringiensis</i> serotype H4a4b   | DQ286358.1  |     | 93 | 0 |
|                      | <i>Bacillus thuringiensis</i> 41205            | HM032789.1  |     | 93 | 0 |
|                      | <i>Bacillus sp.</i> JH2-2                      | JN880445.1  |     | 93 | 0 |
| <b>D5-(bac 27F)</b>  | <i>Bacillus cereus</i> strain HCY02            | EF126181.1  | 533 | 92 | 0 |
|                      | <i>Bacillus thuringiensis</i> strain KU4       | JF895480.1  |     | 92 | 0 |
|                      | <i>Bacillus thuringiensis</i> strain Z8B-46    | HQ238660.1  |     | 92 | 0 |
|                      | <i>Bacillus thuringiensis</i> strain S512Ba-14 | HQ238549.1  |     | 92 | 0 |
| <b>D51-(bac 27F)</b> | <i>Bacillus sp.</i> PL49                       | HQ536223.1  | 503 | 86 | 0 |
|                      | <i>Bacillus cereus</i> strain SK               | FJ2267613.1 |     | 86 | 0 |
| <b>D69-(bac 27F)</b> | <i>Pseudomonas sp.</i> AR-356                  | HE603898.1  | 721 | 94 | 0 |
|                      | <i>Pseudomonas putida</i> strain NBRC 102092   | AB681703.1  |     | 94 | 0 |
|                      | <i>Pseudomonas monteilii</i> strain SY-A2      | JN656162.1  |     | 94 | 0 |
| <b>D80-(bac 27F)</b> | <i>Serratia sp.</i> AS12                       | CP002774.1  | 553 | 88 | 0 |
|                      | <i>Serratia sp.</i>                            | CP002773.1  |     | 88 | 0 |
|                      | <i>Serratia proteamaculans</i> 568             | CP000826.1  |     | 88 | 0 |

|                      |   |            |     |    |   |
|----------------------|---|------------|-----|----|---|
|                      | <i>Serratia sp</i>                            | CP002773.1 |     | 88 |   |
| <b>D19-(bac 27F)</b> | <i>Bacillus sp.</i> MBK-s5                    | JQ729676.1 | 607 | 99 | 0 |
|                      | <i>Bacillus thuringiensis</i> strain R5-331-1 | JQ659733.1 |     | 99 | 0 |
|                      | <i>Bacillaceae bacterium</i> b6_i2            | JN392825.1 |     | 99 | 0 |
|                      | <i>Bacillaceae bacterium</i> b1               | JN392787.1 |     | 99 | 0 |
| <b>D66-(bac 27F)</b> | <i>Pseudomonas sp.</i> PsK                    | AF105389.1 | 733 | 96 | 0 |
|                      | <i>Pseudomonas syringae</i> NBRC 1407         | AB682254.1 |     | 96 | 0 |
|                      | <i>Pseudomonas syringae</i> NBRC 12656        | AB680306.1 |     | 96 | 0 |
|                      | <i>Pseudomonas fluorescens</i> NBRC 12568     | AB680296.1 |     | 96 | 0 |
|                      | <i>Pseudomonas pavonaceae</i> NBRC 12052      | AB680222.1 |     | 96 | 0 |
| <b>S30-(bac 27F)</b> | <i>Bacillus thuringiensis</i> strain Pp14     | JQ861546.1 | 748 | 99 | 0 |
|                      | <i>Bacillus cereus</i> strain 367             | JQ860095.1 |     | 99 | 0 |
|                      | <i>Bacillus sp.</i> MBK-s5                    | JQ729676.1 |     | 99 | 0 |
| <b>D79-(bac 27F)</b> | <i>Enterobacter aerogenes</i> KCTC 2190       | CP002824.1 | 649 | 90 | 0 |
|                      | <i>Enterobacter gergoviae</i>                 | AF072440.1 |     | 90 | 0 |
|                      | <i>Enterobacter sp.</i> 638                   | CP000653.1 |     | 90 | 0 |
| <b>D1-(bac 27F)</b>  | <i>Acinetobacter sp.</i> SP1 strain SP1       | FR773879.1 | 709 | 91 | 0 |
|                      | <i>Acinetobacter xiamenensis</i>              | EF030545.1 |     | 91 | 0 |
|                      | <i>Acinetobacter sp.</i> A1PC16 strain A1PC16 | FN298236.1 |     | 91 | 0 |
|                      | <i>Acinetobacter sp.</i> Q2T1-88              | HM246140.1 |     | 91 | 0 |
| <b>D70-(bac 27F)</b> | <i>Bacillus thuringiensis</i> KNUC2103        | JN846923.1 | 634 | 94 | 0 |
|                      | <i>Bacillus thuringiensis</i> KNUC2102        | JN084030.1 |     | 94 | 0 |
|                      | <i>Bacillus thuringiensis</i> RG17-11         | AB677944.1 |     | 94 | 0 |
|                      | <i>Bacillus sp.</i> EE10m15                   | JN846923.1 |     | 94 | 0 |
| <b>S31-(bac 27F)</b> | <i>Bacillus sp.</i> SAP02-1                   | JN872500.1 | 554 | 83 | 0 |
|                      | <i>Bacillus thuringiensis</i> DSB8            | JQ342872.1 |     | 83 | 0 |
|                      | <i>Bacillus thuringiensis</i> DSB4            | JQ342868.1 |     | 83 | 0 |
|                      | <i>Bacillus thuringiensis</i> DSR5            | JQ342656.1 |     | 83 | 0 |



**Figure 3.6:** The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al., 2011). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 nucleotide sequences. Evolutionary analyses were conducted in MEGA5. The scale bar indicates approximately 5% sequence difference. The gene sequence of *Escherichia coli* DSM 30083 (X80725) was used as an out-group.

## CHAPTER FOUR

### 4.0 DISCUSSION

The aim of this study was to isolate and identify soil bacterial species from Ngere tea catchment area. The study contributes to the understanding of bacteria diversity and their possible role in tea soils. Identification was based on morphological, biochemical characteristics and molecular analysis using 16S rDNA.

A wide range of different media have been used to estimate the size of the bacterial community of soil and to isolate representatives of this community (Balestra and Misaghi, 1997; Olsen, and, Bakken, 1987). However, it has been known for a long time that the number of bacteria that are able to form colonies on microbiological media is generally only a small part of the total number of bacteria in the soil (Jensen, 1968). Recently, the use of non-traditional media has allowed the isolation of members of some of these previously uncultured groups (Sait, *et al.*, 2002). One of these media was dilute nutrient broth agar (DNBA) was formulated to mimic the low concentrations of nutrients and inorganic ions in soils, to allow sufficient biomass formation to produce visible colonies. The pH was adjusted to the pH of the soil at the site being studied (*Singh et al.*, 1999). Based on the results, DNBA was chosen as the growth substrate as it yielded the highest mean viable counts in this study and it has been successfully used in earlier studies (*Janssen et al.*, 2002), this medium was compared with 0.1 X TSA which is commonly used to grow soil bacteria.

The enumeration of bacteria viable cell count against two layers of soil i.e. top-soil (0-20 cm) and sub-soil (20-40c m) (Figure 3.3) showed that the viable cell counts were significantly different between the two soil layers. The effect of two different soil layers was investigated by determining the number of colonies visible on plates after 24 to 72 hours. The viable counts after 72 hours of incubation showed that the counts with top layer reached their maximum of  $1.6 \times 10^5$  while the counts reached  $1.42 \times 10^5$  for sub-soil (Figure 3.3). Using student's t- test, the results showed significant difference between the mean of the soil layers in relation to viable counts. The counts were higher for the top layer as compared to those obtained with sub-soil (Table 3.3). The top soil consists of high amount of organic matter which is produced originally by living organisms such as plant or animals (Torsvik *et al.*, 2002). This organic matter is returned to the soil and goes through decomposition process by soil microorganisms. The composition of soil microorganisms depends on the food source. Each species and groups exists where it can find appropriate nutrient supply, space, nutrients and moisture. Soil microorganisms occur where organic matter occurs (Ingham, 2000). Therefore, soil microorganisms are concentrated around roots, in litter, on humus, on the surface of soil aggregates and in spaces between aggregates. For this reason, they are most prevalent in forested areas and cropping systems that leave a lot of biomass on the surface. Furthermore top soils are well aerated which is ideal for aerobic and facultative anaerobic bacteria.

The soil microbial biomass is a significant component of terrestrial ecosystems. Its activity contributes to the regulation of soil carbon sequestration, carbon mineralization, nutrient recycling and ecosystem productivity. Soil pH is a significant controlling parameter for the microbial biomass in both laboratory and field studies (Anderson and Donsch, 1993; Motavali *et al.*, 1995; Blagodatskaya and Anderson, 1998; Kemmitt *et al.*, 2006). The biomass, microbial activity and microbial community structure have been shown to change significantly in soils under tea plants (Yao *et al.*, 2000; Yu *et al.*, 2003). Nioh *et al.*, (1993) found that, despite low pH, tea soils contained more microbial biomass concentrations than in cultivated soil of pH 7. This study agrees with Yan *et al.* (2003) and Tokuda and Hayastu (2002) found no significant differences in microbial biomass concentrations between tea soils and forest or arable soils as controls.

Total carbon was significantly high in this soil probably because the carbon and nitrogen inputs might be large. Also, it is possible that the pH had declined to threshold where mineralization were seriously impeded, allowing accumulation of organic matter Pansombat *et al.* (1997a) demonstrated that long term tea cultivation resulted in accumulation of organic carbon and total nitrogen. This relationship may have been caused by the different rates of fertilizer application which affected both nutrient availability to plants thus influencing the amount and quality of litter inputs to the soil and the soil pH. According to Martin and Nolin (1991), soils are distributed among eight organic matter content classes as follows; less than 3% are classified as very low, 3-4% low, 4-5% moderately low, 5-7.5% moderate, 7.5-9% high, 15-30% very high and those equal or above 30% are extremely high. However, according to this study Ngere tea

soils had moderately high carbon content; this could be due to the fact that most farmers use both plant and animal materials as manure this in turn promotes biological and microbial activities that facilitates the formation of soil humus which is suitable for uptake by plants (Wang *et al.*, 2007b).

The taxonomic classification of the isolates performed using morphological characteristics, biochemical tests and 16S ribosomal DNA sequences of their genomic DNA placed the isolates to the genera *Bacillus*, *Pseudomonas*, *Burkholderia*, *Chryseobacterium*, *Acinetobacter*, *Enterobacter*, *Serratia* and *Micrococcus*.

The nitrogen fixation test was performed to establish the ability of the isolates to fix nitrogen. Organisms that are able to fix atmospheric nitrogen possess the enzyme nitrogenase, which reduces nitrogen to ammonia (Cappuccino and Sherman, 2002). Nitrogenase enzyme catalyzes the reduction of not only nitrogen but also a variety of other substrates (Cappuccino and Sherman, 2002). This test showed that most isolates were positive thus their potential to fix nitrogen into the soil. This is a crucial aspect for tea soils as nitrogen is one of key component in soil fertility.

The ability of the isolates to reduce nitrate indicates their ability to produce enzyme nitrate reductase which reduces nitrates that the cell uses as a final hydrogen acceptor during anaerobic respiration to nitrites or free nitrogen gas and water (Harold, 2002). Most isolates were positive for this test This is an important factor to help maintain the nitrogen cycle in the three phases namely the atmosphere, water, and soil. This

respiratory process reduces oxidized forms of nitrogen in response to the oxidation of an electron donor such as organic matter.

The release of inorganic phosphate from organic phosphates is called mineralization and is caused by microorganisms breaking down organic compounds. The ability of bacteria to solubilize insoluble Phosphorous minerals has been attributed to their capacity to reduce pH by the excretion of organic acids (for example gluconate, citrate, lactate and succinate) and protons during the assimilation of ammonia (Mullen, 2005). The *Bacillus*, *Enterobacter*, *Serratia*, *Micrococcus*, *Burkholderia*, *Chryseobacterium*, *Acinetobacter* and *Pseudomonas* genera identified in this study were also shown to have potential to solubilize phosphorous. Phosphorus is an essential plant nutrient with low availability in many agricultural soils (Wakelin *et al.*, 2004). Today many agricultural soils have a high total Phosphorous content due to the application of Phosphorous fertilizers over long periods of time. On the other hand, much of this Phosphorous is in mineral forms and is only slowly available to plants (Richardson *et al.*, 2009).

The urease test was done to determine the ability of the isolates to break down urea, to simple forms of nitrogen which can be readily absorbed by the plants to promote growth. The positive implication is an important aspect in growth and development of tea in the case where fertilizers are applied, as the bacteria have shown potential to convert urea to simpler forms of nitrogen which are readily absorbed by plants. For plants to absorb nitrogen from urea it must first be broken down. Urease catalyzes the hydrolysis of urea to unstable carbamic acid. Rapid decomposition of carbamic acid occurs without enzyme catalysis to form ammonia and carbon dioxide (Tisdale *et al.*, 1985). The ammonia will



likely escape to the atmosphere unless it reacts with water to form ammonium ( $\text{NH}_4^+$ ). This is important because ammonium is a plant available source of nitrogen while ammonia is not. The urease test performed in the process of this characterization of bacterial isolates showed that most isolates have potential to break down urea to simpler forms that can be readily available to the host plant.

*Bacillus* are described as aerobic or facultative anaerobic, Gram positive, rod shaped, flagellated motile bacteria, catalase positive belonging to the division Firmicutes with a wide ecological diversity mostly saprophytic they are commonly found in soil, dust, milk, plant surfaces, a few are animal or insect parasites or pathogens. Many *Bacillus* species have been described including *Bacillus anthracis*, causative agent of anthrax, *Bacillus cereus* which causes food poisoning, *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus coagulans* notable food spoilers, causing ropiness in bread and related food. *Bacillus thuringiensis* is an important insect pathogen, and is sometimes used to control insect pests. Other species include; *Bacillus mycoides*, *Bacillus weihenstephanensis* among others (Turnbull and Kramer, 1991). *Bacillus* is the most abundant genus in the rhizosphere of soil, are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Gutierrez *et al.*, 2003).

Phylogenetic analysis of isolate D61, D19 and D51 suggested that it was closely related to *Bacillus cereus* with 86% -99% rDNA sequence analysis similarity while isolates D5, D2, S30, D70 and D19 were closely related to *Bacillus thuringiensis* with 92 - 99% rDNA sequence analysis similarity. Isolate D29 was closely related to *Bacillus mycoides*

with 100% rDNA sequence analysis similarity (Table 3.4). Morphological and biochemical assignments of the isolates D16, D61 and D51 also suggested their close relatedness with *Bacillus cereus*; they are catalase positive, liquefied gelatine, citrate positive, Voges Proskauer positive and motile and had the ability to reduce nitrate to nitrite, and oxidase positive. Most isolates were negative with indole, methyl red test and hydrogen sulphide gas production. Isolates D5, D2, S30, D70, S31 and D19 were biochemically and morphologically closely related to *Bacillus thuringiensis* with the same characteristics as *Bacillus cereus* only that they had large rods upon gram staining. Isolate S31 was closely related to *Bacillus subtilis* it was catalase positive, motile and did not produce hydrogen sulphide gas. Isolate D29 closely related to *Bacillus mycoides* and it was a non- motile rod all isolates were phosphate solubilization and nitrogen fixation tests positive (Table 3.2). Some of the *Bacillus* species have been classified as plant growth promoting Rhizobacteria (Probanza *et al.*, 2002). There are a number of metabolites that are released by these strains (Charest *et al.*, 2005) which strongly affect the environment by increasing availability of the plants nutrients (Barriusso *et al.*, 2008). Naturally present in the immediate vicinity of plant roots, *Bacillus subtilis* is able to maintain stable contact with higher plants and promote their growth. *Bacillus megaterium* is very consistent in improving different root parameters (rooting, root length and dry matter content of roots) in mint (Kaymak *et al.*, 2008). *Bacillus pumilus* can be used as a bio-inoculant for biofertilizer production to increase the crop yield of wheat variety in Mongolia (Hafeez *et al.*, 2006). Soil bacteria isolates including *Bacillus cereus* UW 85 produces Siderophore and they can be used as efficient PGPR to increase

the yield of the crop (Husen, 2003). *Bacillus megaterium* from tea rhizosphere is able to produce siderophore and thus it helps in the plant growth promotion and reduction of disease intensity (Chakroborty *et al.*, 2006). Many soil microorganisms are able to solubilize phosphate through their metabolic activities exuding organic acids, which directly dissolve the rock phosphate, or chelating calcium ions that release P to the solution. About 95% of Gram-positive soil bacilli belong to the genus *Bacillus* (Garbeva *et al.*, 2003). Members of *Bacillus* species are able to form endospores and hence survive under adverse conditions; some species are diazotrophs such as *Bacillus subtilis* (Timmusk *et al.*, 1999),

Phylogenetic analysis showed that isolates D28, D66, D69, D63 and S20 are closely related to the genus *Pseudomonas*. *Pseudomonas* are described as aerobic, rod shaped, Gram negative bacteria with one or more polar flagella providing motility. The genus demonstrates great deal of metabolic diversity, and consequently is able to colonize a wide range of niches. Consequently, they are ubiquitous in soil and water ecosystems and important as plant, animal and human pathogens (Schroth *et al.*, 1992). The best studied species include; *P. aeruginosa* in its role as an opportunistic human pathogen, the plant pathogen *P. syringae*, the plant growth promoting *P. fluorescens* and the soil bacterium *P. putida* has a very diverse aerobic metabolism. *Pseudomonas sp* is ubiquitous bacteria in agricultural soils and has many traits that make them well suited as plant growth promoting Rhizobacteria. The presence of most *Pseudomonas sp* helps in the maintenance of soil health (Lata *et al.*, 2002). The presence of *Pseudomonas*

*fluorescence* plays an effective role in stimulating yields and growth of various plants (Rokhzadi *et al.*, 2008). Specific strains of *Pseudomonas putida* have recently been used as seed inoculants on crop plants to promote growth and increase yields (Johri, 2001). A secondary metabolite produced commonly by rhizosphere *pseudomonads* is Hydrogen Cyanide (HCN), a gas known to negatively affect root metabolism and root growth (Schippers *et al.*, 1990) and is a potential and environmentally compatible mechanism for biological control of weeds (Heydari *et al.*, 2008). The HCN production is found to be a common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%) in the rhizospheric soil and plant root nodules (Ahmad *et al.*, 2008) and is a serious environmental pollutant and a biocontrol metabolite in *Pseudomonas* species. Morphological and biochemical signatures of isolate D28 and D63 indicated that they were highly closely related to *Pseudomonas tolaasii*, it was slightly indole positive, did not reduced nitrate to nitrite, liquefied gelatine, hydrolysed starch and showed positive results with Voges- Proskauer while isolate S20 and D69 were closely related to *Pseudomonas putida*, they were negative with indole, starch gelatine and urea and isolate D66 was closely related to *Pseudomonas fluorescence*, and was positive with citrate, oxidase, nitrate and gelatine tests and negative with starch, indole and Voges – Proskauer tests. They were positive with nitrate all isolates were phosphate solubilization and nitrogen fixation tests positive (Table 3.2). The isolates had multiple polar flagella for motility and grew optimally at a temperature of 25° C to 35° C. The 16SrDNA sequence analysis suggested that isolate D28 was phylogenetically closely related to *Pseudomonas tolaasii* with 99% sequence

similarity while isolate S20 is phylogenetically closely related to *Pseudomonas putida* with over 99% sequence similarity (Table 3.4).

*Burkholderia* was first described by W. H Burkholder in 1950 (Burkholder, 1950). The genus is described as aerobic, Gram negative, rod-shaped, motile with multitrichous polar flagellated bacteria (Gilligan and Whittier, 1999). The genus *Burkholderia* contains organisms that are important causes of human, animal and plant disease, as well as organisms useful in promoting plant growth and bioremediation. Many *Burkholderia* species have been described including *Burkholderia cepacia* originally identified as a plant pathogen that caused soft rot in onions (Burkholder, 1950). *B. cepacia* and the related species are soil bacteria that are found most commonly on plant roots or the immediately adjacent area (the rhizosphere) and in moist environments (Nijhuis *et al.*, 1993). *Burkholderia vietnamiensis* originally was identified as a nitrogen-fixing organism isolated from the rice rhizosphere (Gillis *et al.*, 1995). *B. cenocepacia* has been isolated as the causative agent of fingertip rot disease of banana (Lee and Chang, 2007). In addition, a variety of *Burkholderia* spp. have been characterized as important environmental strains, with phenotypes that include biological control of plant root-infecting fungi, plant growth promotion, nitrogen fixation, and biodegradation of recalcitrant compounds in soil (Parke and Gurian Sherman, 2001). The environmental distribution of *B. cepacia* complex strains includes the rhizosphere of several agricultural-crop plants, soil in woodland and urban habitats, and river water (Balandreau *et al.*, 2001, Coeyne and Vandamme, 2003).

Sequence analysis by BLAST search system on the NCBI website showed that the isolate S23 was phylogenetically most closely related to *Burkholderia sp* strain AZ11 (AB636680) with 99% rDNA sequence similarity (Table 3.4). Isolate S23 adheres to the entire signature phenotypic and biochemical characteristics of *Burkholderia sp* which is a rod-shaped, gram negative, oxidase positive, motile, positive with Phosphate solubilization, nitrogen fixation, and citrate, nitrate, and starch tests. It is indole, urease and methyl-red negative and unable to reduce gelatine (Table 3.2).

This genus *Enterobacter* is described as facultative anaerobe, rod-shaped, Gram negative bacteria with peritrichous flagella providing motility. *Enterobacter* species are found in the natural environment in habitats such as water, sewage, vegetables, and soil. Before the widespread use of antibiotics, *Enterobacter* species were rarely found as pathogens, but these organisms are now increasingly encountered, causing nosocomial infections such as urinary tract infections and bacteraemia (Eichhoff *et al.*, 2001). Strains of the *E. agglomerans* complex have been used for the biological control of plant pathogens such as *Erwinia amylovora* or *Xanthomonas oryzae*; eliciting their effect either by competition for nutrients, production of acid, or by bacteriocin or phage production (Slade and Tiffin, 1984). Various species of *Enterobacter* plays an important role in nitrogen fixation this has been shown to be performed by *E. cloacae* and *E. agglomerans* (Curl and True, 1986. Recent studies have indicated that the genus *Enterobacter* is associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth (Tilak *et al.*, 2005 and Egamberdiyeva, 2005). The important role

is played by plants in selecting and enriching the types of bacteria by the constituents of their root exudates. Thus, the bacterial community in the rhizosphere develops depending on the nature and concentrations of organic constituents of exudates, and the corresponding ability of the bacteria to utilize these as sources of energy (Curl and True, 1986). The morphological, and biochemical signatures for isolates D79, S48, and S49 indicated that they are closely related to genus *Enterobacter cloacae* they were citrate positive, nitrate positive were positive with Voges-Proskauer, phosphate solubilization, nitrogen fixation and showed negative results with indole, urea and hydrogen sulphide gas production. The isolates were motile (Table 3.2) and grew optimally at a temperature range of 25°C to 35°C. The 16S rDNA sequence analysis suggested that the D79, S48 and S49 are closely related to *Enterobacter sp* with 86-90% sequence similarity (Table 3.4)

*Chryseobacterium* are described as Gram- negative, non-motile rods, catalase, gelatine hydrolysis and oxidase positive (Bernardet *et al.*, 2002) Many *Chryseobacterium* strains occur in soil, freshwater, and marine environments, while others are found in dairy products; others are opportunistic pathogens of humans and animals (Jooste and Hugo 1999). Recent works suggest that *Chryseobacterium sp* would be used as Plant growth promotion Rhizobacteria are universal symbionts of higher plants, which enhance the adaptative potential of their hosts through a number of mechanisms, such as the fixation of molecular nitrogen, the mobilization of recalcitrant soil nutrients and the synthesis of phytohormones and the control of phytopathogens (Weller and Thomashow, 1994).

*Chryseobacterium balustinum* CECT 5399 with other groups of bacteria have shown synergistic effect on growth promotion and biocontrol on tomato and pepper against *Fusarium* wilt and *Rhizoctonia* damping off (Duffy *et al.*, 1996). *Chryseobacterium indologenes* strains recently recognized from their 16S rRNA gene sequence in soil samples in Indonesia and Spain have been shown to degrade various toxic compounds (Radianingtyas *et al.*, 2004) Some *Chryseobacterium* strains were also recovered from particular industrial environments. For instance, *C. defluvii* was described from the bacterial flora in activated sludge (Kämpfer *et al.*, 2003) and two *Chryseobacterium sp.* strains were detected among the complex filamentous microflora that occurs in paper mill slimes and frequently affects machine efficiency and paper quality (Oppong *et al.*, 2003). Some *Chryseobacterium* strains have potential applications in the clean-up of various environmental contaminants. For instance, the clean-up of pentachlorophenol (PCP), a pesticide that has been associated with wood preservation and a disinfectant in the food industry, is essential since PCP poses significant health hazards (Yu and Ward, 1996).

Isolate D60 displayed the typical characteristics and biochemical properties of members of the genus *Chryseobacterium*. They were Gram- negative, non-motile rods, oxidase, catalase, indole and gelatine positive and were negative for urease, nitrate reduction and hydrogen sulphide gas production (Table 3.2) with an optimal growth at temperatures 25<sup>o</sup> C to 35<sup>o</sup>C. sequence analysis by BLAST search systems on NCBI website showed that the isolate D60 was phylogenetically most closely related to *Chryseobacterium sp* strain 1095B with 99% rDNA sequence similarity (Table 3.4)



*Acinetobacter* can be described as strictly aerobic, Gram negative, rod-shaped, oxidase negative and non-motile bacteria (Bergogne-Bérézin and Towner, 1996). The species of *Acinetobacter* are common, free-living saprophytes found in soil, water, sewage and foods. They are also ubiquitous organisms in the clinical environment, where they can be isolated as commensals from the skin of hospital staff and patients. They have been increasingly recognized as important nosocomial pathogens involved in outbreaks of hospital infection, particularly in high-dependency or intensive care units, where they rapidly develop resistance to even the most potent antimicrobials (Bergogne-Bérézin and Towner, 1996). Species of *Acinetobacter* have been attracting increasing attention in both environmental and biotechnological applications. Some strains of this genus are known to be involved in biodegradation of a number of different pollutants such as biphenyl and chlorinated biphenyl, amino acids (aniline), phenol, benzoate, crude oil, acetonitrile, and in the removal of phosphate or heavy metals. *Acinetobacter* strains are also well represented among fermentable bacteria for the production of a number of extra- and intracellular economic products such as lipases, proteases, cyanophycin, bioemulsifiers and several kinds of biopolymers (Adams and Ribbons, 1988). A number of studies have focused on the biodegradation of phenol by various microorganisms. Among phenol degraders are several strains of *Acinetobacter*, which can use it as a sole energy and carbon source (Briganti *et al.*, 1997). Biological phosphate removal from wastewater is an efficient cost-effective alternative to chemical phosphorus precipitation. This biological process is obtained by recycling the sludge through

anaerobic and aerobic zones. It is dependent on the enrichment of activated sludge with polyphosphate accumulating strictly aerobic *Acinetobacter sp.* which could absorb phosphate up to 100 mg phosphorus per gram of dry biomass during aerobic conditions and release it anaerobically (van Groenestijn *et al.*, 1989; Timmerman, 1984). It was confirmed that *Acinetobacter* are primarily responsible for biological phosphate removal (Wagner *et al.*, 1994). *Acinetobacter* strains also play an important role in the removal of heavy metals (Francisco *et al.*, 2002). The morphological and biochemical signatures for isolates D1 and S55 indicated that they are closely related to *Acinetobacter sp.* They were oxidase negative, were catalase positive, nitrate reduction negative, were positive with phosphate solubilization, nitrogen fixation tests and showed negative result with Voges- Proskauer. The isolates were non- motile and grew optimally at a temperature range of 25° C to 35° C. The 16S rDNA sequence analysis suggested the isolate D1 is phylogenetically closely related to *Acinetobacter sp* SP1 with 91% sequence similarity (Table 3.4)

Members of the genus *Serratia* are Gram-negative, non-spore forming rods belonging to the family Enterobacteriaceae. These facultative anaerobes typically are motile by means of peritrichous flagella. They ferment glucose and other carbohydrates with the production of acid and gas and are, catalase positive. With a wide ecological diversity, found in the natural environment such as water, soil, plant surfaces or as opportunistic human pathogens, they are also isolated from small mammals and their territories (Gavini *et al.*, 1979). Many *Serratia* species have been described including; Strains

producing the non-diffusible red pigment prodigiosin seem to be toxic to protozoa (Groskop and Brent, 1964), and this may be an ecological advantage in water and soil. In soil, *S. marcescens* might play a role in the biological cycle of metals by mineralizing organic iron and dissolving gold and copper (Parès, 1964). A mineralization role has also been attributed to cold-tolerant *Serratia* associated with low-moor peat (Janota-Bassalik, 1963). Recent studies indicates that a number of bacterial species among them *Serratia* play an important role in biological nitrogen fixation where they are associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth. (Egamberdiyeva, 2005). Sequence analysis by BLAST search system on NCBI website showed that the isolate D80 was closely related to *Serratia sp* AS12 with 88% rDNA sequence similarity (Table 3.4). Isolate D80 adheres to all the signature phenotypic and biochemical characteristics of *Serratia sp* which is rod-shaped, gram negative, utilize citrate as a sole carbon source, reduces nitrate to nitrite, it is negative with oxidase, indole and hydrogen sulphide gas production and positive with Voges- Proskauer, gelatine, nitrogen fixation, phosphate solubilization and catalase test (Table 3.2). The isolates had multiple flagella for motility and grew optimally at a temperature range of 25° C to 35° C. The ability of the isolates to reduce nitrate indicates their ability to produce enzyme nitrate reductase which reduces nitrates that the cell uses as a final hydrogen acceptor during anaerobic respiration to nitrites or free nitrogen gas and water (Harold, 2002)

*Micrococcus* can be described as Gram positive, non-motile, coccus-shaped, with colonies circular, Smooth, convex, entire and pale yellow in colour bacteria. These organisms occur in a wide range of environments including dusts, water, milk, dairy products and soil. Many *Micrococcus* species have been described including *M. ureae*, *M. flavus*, *M. luteus* and other related modern members of the genus have numerous genetic adaptations for survival. This includes extreme, nutrient-poor conditions. These phenotypes have assisted the microbe in persistent and prevalent dispersal within the environment. This species has an ability to utilize succinate and terpene related compounds to enhance and ensure its survival in oligotrophic environments (Greenblatt, *et al.*, 2004). It is also mobile in soil and is considered an environmental teratogen (Sims *et al.*, 1986). Microorganisms play an important role in effecting the availability of soil phosphorous to plant roots, and increasing P-mobilization in soil. The ability of soil microorganisms to convert insoluble forms of phosphorus to a soluble form is an important trait in plant growth-promoting bacteria for increasing plant yields a potential *Micrococcus* strain NII-0909 isolated from Western *ghat* forest possessed multiple plant growth traits, like P-solubilisation, and Siderophore production (Berleth and Sachs, 2001). Isolates D13 and D78 adheres to all the signature phenotypic and biochemical characteristics of genus *Micrococcus* both isolates formed circular, Smooth, convex, entire cream colonies and were gram positive non-motile coccus, catalase and citrate positive, bacteria negative with indole production, starch hydrolysis, hydrogen sulphide gas production, nitrate reduction, methyl red and Voges Proskauer negative. However

isolate D78 was urease positive which suggested it was *Micrococcus ureae* while isolate D13 did not hydrolyse urea hence *Micrococcus luteus* (Robert *et al.*, 1957) (Table 3.2). Fragment restriction using *RsaI* produced six clusters of the isolates which were generated based on fragment sizes. This enzyme was used to perform an amplified ribosomal DNA restriction analysis (ARDRA). Cluster analyses of the restriction fragment profiles obtained from isolates showed patterns with distinct similarities allowing distinguishing six different groups. These were isolates 1, 2, 3, 5 and 6; which clustered together generating fragments ranges 300bp-400bp; isolates 3, 4, 5, and 6 clustered to generate fragments ranges 400bp-500bp; isolate 4 generated fragment range 500bp-600bp; isolates 4, 5 and 6 were clustered together generating fragments range 600bp–700bp. Isolate 3 had one restricted fragment of 800bp-900bp. Finally isolates 1 and 2 were clustered together generating fragments range 900bp–1000bp Sequences of PCR fragments from isolates were in close agreement with the phylogenetic correlations predicted with the ARDRA approach. ARDRA thus provided a quick assessment of the diversity in a strain collection.

## CHAPTER FIVE

### 5.0 CONCLUSIONS AND RECOMMENDATIONS

#### 5.1 CONLUSSIONS

Soil microorganisms are important components of ecosystem functioning as they determine the mineralizing of soil organic matter and energy flow. Microbial recycling of crop residues provides an important component to improve the soil organic matter pool and soil productivity in agricultural management systems.

The study has demonstrated that Ngere tea soils harbour diverse bacteria species. 38 isolates were obtained; 22 isolates were characterized and identified.

Biochemical properties of some isolates like ability to reduce nitrate to nitrite, nitrogen fixation, starch hydrolysis, urease test and phosphorous solubilization suggests their involvement in the nutrient recycling within the tea soils.

Molecular characterization of the bacterial isolates indicates that 45 % belong to genus *Bacillus* within the Firmicutes in the domain bacteria, 23 % belong to the genus *Pseudomonas* The genus *Enterobacter* represented 13 % while other genera such *Burkholderia*, *Chryseobacterium*, *Acinetobacter* and *Serratia* constituted 9 % .

One (1) isolates showed identity of 95 - 97 % similarity with the previously known sequences in the GenBank database. This represents novel species of organisms within Ngere tea soils. Ten (10) isolates showed identity of 80 -93 % similarity, representing novel genera of organisms within the tea soils.

## 5.2 RECOMMENDATIONS

- i. Presence of isolates D61, D19, S30, D1, D78, D13, S55, D60, D79 and S48 can be used in enhancing soil fertility. Therefore more research is required to assess the applicability of these isolates as indicators of soil health or biofertilizers.
- ii. More research is required to design studies that would compare the diversity of Bacteria in different seasons of the year such as the dry seasons since this study took place in wet season.
- iii. Further analysis of bacteria is necessary for total characterization and identification of more strains from other tea regions across the country; this will help in understanding the role of these microorganisms in promoting soil fertility.
- iv. The novel organisms need to be further confirmed by methods such as fatty acid analyses and DNA-DNA hybridization.
- v. Further molecular characterization should be carried out using more restriction enzymes to establish on any genetic diversity. Molecular characterization can also be performed for the isolates that were not subjected to the same process



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## APPENDIX

**Appendix 1:** The mean difference of Top soil and sub soil determined by one- sample T test using Portable\_PASW\_Statistics\_18 (SPSS) at 95% confidence interval

|                     | Test Value = 0 |    |                 |                 |   |        |
|---------------------|----------------|----|-----------------|-----------------|---|--------|
|                     | T              | Df | Sig. (2-tailed) | Mean Difference | 95% Confidence Interval of the Difference |        |
|                     |                |    |                 |                 | Lower                                     | Upper  |
| Top-Soil(0-20 cm)   | 36.047         | 30 | .000            | 1.3605          | 1.283                                     | 1.438  |
| Sub-Soil (20-40 cm) | 23.286         | 30 | .000            | 1.15645         | 1.0550                                    | 1.2579 |

**Key:** T: test, df: Degrees of Freedom