ISOLATION AND CHARACTERIZATION OF PHOSPHATE ROCK-SOLUBILIZING MICROORGANISMS FROM SOILS AND ROCK PHOSPHATE SAMPLES OF PANDA HILL AND MINJINGU, TANZANIA

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ABSTRACT

Plants acquire P from soil solution as the phosphate anion. Phosphorus for plant can be obtained from soluble P fertilizers or from insoluble phosphate rocks. It is highly fixed by calcium and by Fe and Al oxides depending on soil pH. Phosphorus solubilizing microorganisms play a major role in P nutrition through solubilisation of insoluble phosphate rocks**.** The use of phosphate solubilizing microorganisms as inoculants increases P uptake by the plant and crop yield. This study was undertaken in order to isolate microorganisms which are capable of solubilizing rock phosphate. A total of 22 fungal and 39 bacterial isolates were isolated from soil and rock phosphate samples previously collected from Minjingu and Panda Hill phosphate deposits, Tanzania. The isolated strains were assessed for their ability to solubilize insoluble phosphorus from rock phosphate samples. Out of the isolated fungal or bacterial isolates, 10 isolates that gave larger diameters of the halo zones in media containing rock phosphateas sole source of P were selected for further studies. Selected isolates were quantitatively tested in broth containing either Panda Hill or Minjingu phosphate rock. Five isolates from each group that were selected as outstanding performers, based on their relative amounts of soluble P released, were identified using molecular techniques. The most efficient fungal isolates was *Aspergillus tamarii* which, while it solubilised only 12.774 mg P/kg of Panda Hill rock phosphate, was able to solubilize 80.39 mg P/kg of Minjingu rock phosphate. The most efficient bacterial isolate was *Stenotrophomonas maltophilia* which solubilised 27.45 mg P/kg of Panda Hill rock phospahte and 24.75 mg P /kg of Minjingu rock phosphate. Other isolates that performed well included *Aspergillus flavus* and *Aspergillus stellifer* fungal species and bacteria were *Bacillus safensis* and *Acenotobacter baumanii*. The outstanding performers are herein recommended as being potential inoculants for use in increasing crop yields.

DECLARATION

I, ESTER JIMMY SIMFUKWE, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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DEDICATION

This work is dedicated to my parents, Anastazia L. Kinyau and Jimmy P. Simfukwe who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my sister Grace Simfukwe, who helped me to understand that even the largest task can be accomplished if it is done one step at a time. I also dedicate this dissertation to my betrothed Simon Maeda for his continued and unfailing love, support and understanding during my pursuit of MSc. degree that made the completion of this dissertation possible. He had always been around and at the times I thought that it is impossible to continue, he helped me to keep things into perspective. I greatly value his contribution. Together we make a great team!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABREVIATIONS

ANOVA	Analysis of Variance
BNF	Biological Nitrogen fixation
BLAST	Basic Local Alignment Search Tool
CFU	Colony forming unit
DAP	Diammonium phosphate
DNMRT	Duncan's new multiple range test
HMPR	Hard Minjingu phosphate rock
HMRP	Hard Minjingu rock phosphate
LPCB	Lacto phenol cotton blue
NA	Nutrient agar
NBRIP	National Botanical Research Institute's phosphate
PRP	Panda hill rock Phosphate
PPR	Panda Hill phosphate rock
PSB	Phosphate solubilizing bacteria
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
PR	Phosphate rock
PSMs	Phosphate solubilizing microorganisms
rDNA	Ribosome deoxyribonucleic acid
RP	Rock phosphate
SUA	Sokoine University of Agriculture

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Phosphorus is the second most important essential element in plants nutrition, next to nitrogen (N) (Hamdali *et al*., 2012). It is involved in photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, and respiration (Sharma *et al*. 2013), all of which are necessary for plant growth and development. Despite its importance in plant growth and metabolism, phosphorus is the least accessible macro-nutrient and hence the most frequently deficient nutrient in most agricultural soils because of its low availability and its poor recovery from the applied fertilizers.

Extensive tracts of land in the tropical regions of Africa have soils that are highly weathered and of inherently low soil fertility; the main constraints are soil acidity and low inherent N and P fertility (Gweyi-Onyango *et al*., 2010). While N inputs can be obtained from sources such as biological nitrogen fixation (BNF), crop residues and external organic sources (Pereyra *et al*., 2015), this is not the case for P. Sufficient P inputs are mostly need to be brought into the soil from external sources such as P fertilizers, in order to improve the soil P status (Chien *et al*., 2011). However, phosphorus applied as inorganic fertilizers can easily be fixed in acid soils and thus made unavailable to plants unless applied in large amounts that may saturate the P fixing mechanisms in the soils (Balemi and Negisho, 2012), although, such large amount will be uneconomical. Phosphorus fixation in tropical soils is the major contributor to P deficiency, which makes applied soluble P to become insoluble.

In most soils, phosphorus is soluble around pH 6-7 where it is available in the forms of HPO_4^{-2} and $H_2PO_4^-$. At pH lower than 6, as is common in many tropical soils, P can be fixed by oxides of aluminium and iron. At pH above 7, which also occurs in some soils, P is fixed by calcium and magnesium (Marschner, 2012). In either pH extreme, even if soluble P fertilizers are used on the soils, some amount of P would be fixed.

The source of P fertilizers is rock phosphate, often times insoluble for direct use, which, after industrial processing gives rise to different soluble P fertilizers such as single super phosphate and triple superphosphate, with different levels of soluble P (Yingben *et al*., 2012). Farmers can then increase crop yields following the use of such fertilizers. However, one drawback for using these industrial fertilizers by small scale farmers is their high price. A 50 kg bag of diammonium phosphate fertilizer (DAP) costs Tanzanian shillings 66 000 and for Triple Superphosphate (TSP) it is 45 000 ex-godown's price of 2012-2013 season in Dar-es-salaam (Kamhabwa, 2014), and now prices are higher than these. Such fertilizer prices are beyond the reach of most small scale farmers, resulting in no or only minimal use of P fertilizers in general, which leads to low crop yields and poor quality of agricultural produce (Druilhe, 2012).

High fertilizer prices and the insolubility of raw phosphate rocks for direct use are problems that call for search for inexpensive and sustainable solutions. Tanzania has a wealth of rock phosphate deposits which are not directly utilized for agricultural purposes. Szilas (2000) reported on Minjingu deposit in Arusha which has both hard (insoluble) and soft (soluble) phosphate. The soft Minjingu rock phosphate has been used directly as fertilizers in acid soils while the hard Minjingu has been insoluble when tried on most soil types. Some other phosphate rocks are also highly insoluble for direct use and thus are not in use as source of P fertilizers. One such deposit is the Panda Hill

phosphate deposit in Mbeya, which is not yet exploited for the manufacture of soluble P fertilizers and is insoluble for direct use (Kula and Misra, 2000). Direct use of phosphate rocks is limited to soils of a lower pH that would chemically solubilize the phosphates. Partial acidulation to increase solubility has been attempted for some of these phosphates rocks and the results for hard Minjingu and Panda Hill phosphate rocks have not been encouraging (Appleton, 2002). The acidulation process was highly demanding in terms of amount of acid required and this increased the cost of production and therefore the price of the partially acidulated fertilizer. Hence, its use would also be limited.

1.2 Potential Role of Microorganisms in Solubilizing Phosphate Rocks

Some microorganisms are able to convert inorganic P present in phosphate rocks into the bioavailable form, thus facilitating uptake of P by plant roots. Some actinomycetes, bacterial and fungal species isolated from soils in the vicinity of different rock phosphate deposits around the world have shown positive results. Actinomycetes which have shown potential ability of solubilizing insoluble phosphorus are of the genera *Micromonospora* and *Streptomyces*, e.g. *S. griseus* and *S. coelicolor*. Similarly, members of the bacterial genus *Serretia* have been reported to display high P solubilizing ability (Hamdali *et al*., 2012). Other bacteria are from the genera *Bacillus, Rhodococcus* and *Arthrobacter*. Fungal species with the ability to solubilize phosphate rocks have also been reported. Omar (1997), for example, reported fungal species isolated from soil which have the ability to solubilize rock phosphate (RP) in agar plates; these include *Aspergillus niger* and *Penicillium citrinum*. It is probable that these and other microorganisms with potential to solubilize insoluble P compounds could be widespread in soils, especially those in and around rock phosphate deposits.

Isolation, testing and identification of efficient P-solubilizing microorganisms (PSMs) could lead to the development of inoculants to be used with insoluble rock phosphate as sources of P to plants, akin to the use of rhizobia inoculants in inoculating legume seeds for increased nitrogen fixation. Zaidi *et al*. (2009) observed that PSMs could be isolated from rhizosphere and non-rhizosphere soils, rhizoplane, phyllosphere, soils in contact with rock P and even from P stressed soils.

Historically, identification and classification of microorganisms were based on phenotypic characteristics. Currently, two fundamental molecular applications are being extensively utilized in identification and classification of microorganisms; these are based on hybridization and nucleotide sequencing molecular methods. These molecular methods are accurate in identifying organisms e.g. Chen *et al.* (2006), identified and phylogenetically analysed 36 phosphate solubilizing bacterial isolates using 16S rDNA sequencing.

The studies cited above (e.g. Hamdali *et al*., 2008; Rodríguez and Fraga, 1999), of microbial solubilization of P have been largely laboratory based. There is need to extend them to field testing. However, in Tanzania there is a lack of basic studies on phosphate solubilizing microorganisms (PSMs). We must, therefore, start with laboratory tests (isolation, testing, and characterization) and then try to extend the results to the field. This is important because these PSMs may be widely spread in soil. Such microorganisms could then be used to develop inexpensive inoculants that would render feasible the use of unprocessed phosphate rocks by small-scale farmers.

1.2 Justification of the Study

Tanzania is endowed with a wide variation of soils determined by differences in age, parent material, physiographic and climatic conditions (Kamhabwa, 2014). These soils differ in their inherent fertility, with soils most suitable for crop production covering the least proportion of the land area (MARI, 2006). Tropical soils in the sub-humid to humid areas, including Tanzania, are well drained, old, highly weathered and leached, with *Ultisols* and *Oxisols* dominating, which are deficient in phosphorus. Conventionally, large amounts of inorganic soluble P fertilizers such as Triple Superphosphate (TSP) are used to provide P which is in the form available to plants. The vast majority of domestic fertilizer demands that account for about 90% are currently met through importation (from USA, Europe and Asian countries) (Kamhabwa, 2014). This approach to correcting low soil P fertility using soluble P fertilizers has not been successful with small scale farmers because these fertilizers are expensive and largely beyond their reach (Chien and Hammond, 1978; Kamhabwa, 2014). The problem of farmers not affording high fertilizer prices could be resolved by employing soil microorganisms capable of solubilizing insoluble RP. Use of P solubilizing microorganisms would broaden the range of soils, acidic and or not acidic, in which rock phosphate could be used. The proposed study is thus aimed at isolating phosphate solubilizing microorganisms from soils within or in the vicinity of the rock phosphatedeposits in Panda Hill, Mbeya and Minjingu, Arusha both in Tanzania, which can solubilize insoluble rock phosphate. The isolates could eventually be used as phosphate solubilizing bio-fertilizers.

1.3 Objectives

1.3.1 Overall objective

To identify microbial isolates capable of solubilizing P from insoluble rock phosphate.

1.3.2 Specific objectives

i. To isolate bacteria and fungi from both RP and soils within the phosphate rock deposits areas at Minjingu and Panda Hill.

i. To test the isolates obtained in objective (i) above for ability to solubilize

ii. To characterize the microbial isolates having the greatest efficiency in solubilizing RP

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Types of Phosphate Rocks

Phosphates rocks are geologic materials having high content of phosphorus to the extent that they can be used as, or serve as, raw material for manufacturing of phosphate fertilizers. Some high grade RPs can be directly applied as phosphorus sources in agriculture where soils are highly weathered, very strongly acidic and deficient of phosphorus and calcium (Szilas, 2002). The main ingredient of phosphate rocks is apatite, Ca_3 $(PO_4)_2$. The actual formation of phosphate rock deposit is the product of the geological or biogeochemical transformation that prevailed during its formation. Phosphate rock deposits can be divided into four categories: igneous, marine sedimentary, guano and deposits derived from transformation of the first three categories (Mwambete 1991; Weil, 2000). Phosphate rocks have widely differing mineralogical, chemical and textural characteristics.

There are more than 200 known phosphate minerals; the main mineral group of phosphates are the apatites. Calcium-phosphates are mainly found in sedimentary, metamorphic and igneous rocks but also in weathering environments (Aissa *et al*., 2014). Apatite occurs as three different minerals depending on the predominance of either fluorine, chlorine or the hydroxyl groups in the mineral structure. The four categories can be fluoroapatite, chlorapatite, hydroxylapatite or carbornotoapatite, named due to the presence of hydroxide (OH^-), fluoride (F^-), carbonate (CO^{2-}) and chloride (Cl^-) ions which can be substituted freely in the crystal lattice (van Straaten, 2002). The generalised chemical formula of the apatite mineral has this form: Ca_5 $(PO_4)_3$(F, Cl, OH and CO_2) (Dutrow and Cornelis, 2007).

2.1.1 Fluoroapatite

Fluoro-apatite, (Ca_{10} $(PO_4)_6F_2$), is found mainly in igneous and metamorphic environments, such as in carbonatite and mica-pyroxenites. Fluoro-apatite formation is divided into two-step process. First, formation of calcium phosphate as calcium and phosphate salts combined at neutral pH. This material then reacts further with fluoride sources (such as sodium monofluorophosphate (Na_2PFO_3) or calcium fluoride (CaF_2) to give $Ca_5(PO_4)_3F$. Fluoro-apatites are found mainly in igneous and metamorphic rocks, for example, in carbonatite, and mica-pyroxenites. Fluoro-apatites are relatively less soluble in high pH solvents as compared to hydroxyapatites (Van Kauwenbergh *et al*., 2003). The reactions for the formation of fluoro-apatite may be written as follows:

$3Ca^{2+} + 2PO_4^{3-} \rightarrow Ca_3(PO_4)_2$ ……..……. (i)

$3\ Ca_3(PO_4)_2 + CaF_2 \rightarrow 2\ Ca_5(PO_4)_3F$…….. (ii)

Overall equation:

$3Ca^{2+} + 2\ PO_4^{3-} + CaF_2 + 2\ Ca(PO_4)^2 \rightarrow 2\ Ca(PO_4)^3F$…….. (iii)

2.1.2 Hydroxyapatite

Hydroxyapatite has a chemical formula of $Ca_5(PO_4)_3(OH)$. Hydroxyapatite is a soluble salt due to its alkaline nature. Carbonate hydroxyapatite is soluble enough in acidic soils to be effective substitute for water-soluble P source thus can be applied directly. Hydroxyapatites are found in igneous and metamorphic but also in biogenic deposits. Francolite, (Ca_{10}-x-yNaxMgy $(PO_4)_6$-$z(CO_3)zF_{0.4}zF_2$), is a carbonate-substituted apatite found mainly in marine environments (Mar and Okazaki, 2012).

2.1.3 Chlorapatite

Chlorapatite ($Ca_{10}(PO_4)_6Cl_2$) is relatively much rarer than fluorapatite or hydroxyapatite and is formed in F-deficient environments (Ferraris *et al*., 2005). Chlorapatite is less soluble in acid as compared to fluoro-apatite due to its high lattice energy (Narasaraju *et al*., 1979).

2.1.4 Igneous phosphate rocks

Igneous phosphate rocks are deposits which occur mainly as sheets of intrusive alkaline hard crystalline rocks such as nepheline syenites, pyroxenites or carbonatites, with apatite content of up to 10-15% for carbonatites and up to 75% for apatite-nepheline ores (Szilas, 2002). One example of igneous RP is the Panda Hill RP. Several agronomic studies using Panda Hill rock phosphate concentrates from the Panda residual phosphate confirmed the low solubility of this igneous RP and hence low yield response when directly used as P fertilizer (Mnkeni *et al.,* 1994; Weil, 2000).

2.1.5 Sedimentary phosphate rocks

Sedimentary phosphates rocks are found in coastal areas where upwelling ocean currents driven by trade winds bring up previously trapped phosphorus from deep ocean waters back to the surface waters where it re-enters the biogeochemical P cycle (Tucker, 2009). Sedimentary deposits occur in North Africa, China, Middle East and USA (Cook and Shergold, 2005). In Tanzania, marine, sedimentary deposits are found as Mesozoic and younger marine sediments along the coasts (van Straaten, 2002).

2.1.6 Residual deposits

Residual deposits are found in connection with several of the carbonatites from around the world and in connection with some weathered limestone in North and South America

(Misra, 2012). The phosphate minerals of these deposits are mainly fluoro-apatite or slightly carbonated substituted fluoro-apatite, although Ca-Al-Fe phosphates such as crandallite and millisite and Al-Fe phosphates such as wavellite and variscite may also be associated with the apatites.

2.1.7 Guano deposits

Guano deposits occur in inland islands where upwelling of nutrient rich water or other favourable conditions support large colonies of sea bird (Szilas, 2002). Guano deposits are localized accumulations of excreta primarily produced by birds or bats. Some bat guano deposits have been found around the world in caves inhabited by bats whereas bird guano deposits typically are found along coasts. In Tanzania these deposits are found at Sukumawera near Mbeya, Amboni caves (Tanga) and Haitajwa and Manapwani caves in Zanzibar (van Straaten, 2002).

2.2 Chemistry of phosphate rocks

Solubility of phosphate rocks varies with origin of rock, resulting from degree of isomorphous substitution of phosphate by carbonate (McConnell, 2012). Solubility of sedimentary phosphate rocks is relatively higher than igneous and metamorphic phosphate rock. Solubility of carbonate substituted RP is higher than that of those with fluoroapatite with little or no carbonate substitution (Chien and Hammond, 1978; Chien *et al*., 2011). The P bearing mineral of the plutonic igneous rocks is normally fluoro-apatite and accessory minerals, typically quartz, calcite, micas, K-feldspars, amphiboles, pyroxenes and feldspathoids, making them less soluble (van Straaten, 2002).

The largest and commercially most important igneous deposits are those of the Kola Peninsula of Russia and Palabora in South Africa (Smith, 2005, Wall and Zaitsev, 2004).

In Tanzania, igneous phosphate rocks (carbonatites) with the highest phosphorus concentrations occur along the tectonically active Western rift valley. They include the carbonatites of Sangu-Ikola at Lake Tanganyika, Ngualla, carbonatites of Mbalizi, Songwe Scarp, Nachendezwaya, Sengeri Hill and Panda Hill. In West and Southwest Tanzania, several carbonatites are known to have intruded along the re-activated northwest striking shear and fault zone (van Straaten, 2002).

2.3 Chemical Solubility of Panda Hill and Minjingu Phosphate Rocks

Several agronomic studies using Panda rock phosphate (PRP) confirmed the low solubility of this igneous phosphate rock and hence low yield response when used as P fertilizer (van Straaten *et al.,* 1992; Mnkeni *et al.,* 1994; Weil, 2000). Panda Hill rock rock phosphate has total P_2O_5 of 24.8%; it is less reactive, with very low solubility of 2.1% in 2% citric acid (Chien *et al*., 2010). Panda Hill rock phosphate has very low reactivity due to little CO_3/PO_4 substitution in the apatite mineral structure, and therefore is not suitable for direct application (Chien *et al.,* 2009).

It is of guano-sedimentary origin, biogenic in nature, it was formed during the Pleistocene age from the remains and droppings of flamingo birds that inhabited the lake Manyara region millions of years ago. Some flamingo birds still inhabit the lake todate (Szilas *et al*., 2008). The deposit contains two ores which differ in consistency and fabric, the soft and hard ores located on the northern and southern sides of Minjingu hill respectively (Mwambete, 1991). Direct application of hard or soft MPR on acid soils low in available P and Ca in sub-humid to humid Tanzania has showed good results. Minjingu phosphate rock, which is of two types, hard (10.6% P) and soft MPR (13.3% P), showed comparable results after being tested in acidic soils, both in field and green house experiments (Msolla *et al*., 2005).

In order to increase the reactivity of Panda Hill phosphate rock several modification techniques were tested, including partial acidulation, blending with the soluble P fertilizer triple superphosphate (TSP) and pelletizing with Panda rock phosphate at a 50/50 ratio which showed significant increase in bioavailability of P to maize (van Straaten *et al.,* 1992). The soft Minjingu has been confirmed to have relatively higher solubility than the hard Minjingu. Studies have proved the use of soft Minjingu for direct applications in acidic soils (Msolla *et al*., 2005).

It is reported that both soft and hard MRP contain apatites which are the fluorine deficient francolites (Szilas *et al*., 2008). X-ray diffraction (XRD) analysis suggests that the soft MRP belongs to excess fluorine francolites with low carbonate substitution, thus low solubility. Hard and soft MRP have high neutral ammonium citrate (NAC) solubility of 4.2% and 6.2% P_2O_5, respectively the property that place hard MRP and soft MRP among the medium to highly reactive PRs (Mowo *et al*.,2014; Szilas *et al*., 2008; Shitindi, 2011). A simplified dissolution equation of apatite (Arcand, 2006) is shown here:

$Ca_{10}F_2(PO_4)_6 + 12\ H^+ \rightarrow 10\ Ca_2^+ + 6\ H_2PO_4. + 2F$ ……. (iv)

2.4 Occurrence of Phosphate Rocks in the World and in Tanzania

Cooper *et al*. (2011) described the distribution of world RP reserves, the largest ones being located in Morocco and China. The largest and commercially most important igneous phosphate rocks deposits are those of Kola Peninsula in Russia and Palabora in South Africa (Callaghan, 2013). Tanzania, as in the rest of the world, has the four types of phosphate rocks: igneous (carbonatite), lacustrine (rift valley sediments), metamorphic and guano phosphates. The igneous (carbonatite) phosphate with the highest phosphorus concentrations occur along the tectonically active Tanzanian Western Rift valley. They include the carbonatites of Sangu-Ikola at Lake Tanganyika, as well as those at

Ngualla, Mbalizi, Songwe Scarp, Nachendezwaya, Sengeri Hill, and Panda Hill all in Mbeya (Harris, 1981; Mchihiyo, 1991). In West and Southwest Tanzania, several carbonatites are known to have intruded along the re-activated northwest striking shear and fault zone (van Straaten, 1989). Metamorphic apatite-bearing limestone can be found in the Zizi area in Morogoro District (Stockley, 1946). Van Straaten *et al*. (1992) observed apatite-bearing limestone near Lupingu along Lake Nyasa in Mbeya. The lacustrine-biogenic phosphates are found in Minjingu, northern Tanzania, and Chali Hills (Dodoma) in central Tanzania. Kreuser *et al*. (1990) described sedimentary phosphate pebbles in the Mikumi area of eastern Tanzania.

Sedimentary deposits occur in North Africa, China, Middle East and the USA (Callaghan, 2013). Marine sedimentary phosphates are found in the coastal areas (Tanga, Dar es Salaam, Pwani, Lindi, Mtwara and Zanzibar) of Tanzania where upwelling ocean currents driven by trade winds bring previously trapped phosphorus from deep ocean waters back to the surface waters where it re-enters the biogeochemical P cycle. In Tanzania, as already mentioned marine sedimentary deposits are found as Mesozoic and younger marine sediments along the coast (van Straaten, 2002).

Guano deposits are localized accumulations of excreta primarily produced by birds or bats (Szilas, 2002). Several bat guano deposits have been found in caves inhabited by bats whereas bird guano deposits are found along coasts and on small oceanic or inland islands where upwelling of nutrients rich water or other factors provide favourable conditions which support roosting of large colonies of birds (Szilas, 2002). In Tanzania as already mentioned, these deposits are found at Sukumawera near Mbeya, Amboni caves (Tanga) and Haitajwa and Manapwani caves in Zanzibar (van Straaten, 2002).

2.5 The Panda Hill Rock Phosphate Deposit

Panda hill rock phosphate deposit contains about 300 million tons of phosphate which is considered insoluble in acidic soils due to its igneous nature (Kalumuna *et al*., 1998). Panda Hill rock phosphate containing P ranging from3.5% to 30% P_2O_5 has been mapped (Kalvig *et al*., 2012). This phosphate deposit is under exploration as a potential source of niobium and other rare-earth elements; it is not yet used as source of phosphate fertilizer. There are various constraints to the exploitation of Panda Hill rock phosphate deposit for P fertilizer production, the major one being its insolubility (Misra, 2012). Mchihiyo, cited by van Straaten (2002), reported on the chemistry and mineralogy of the apatites of Panda Hill, the mineralogy showing a unit-cell value of 9.387 to 9.397 Å, indicating a fluoro-apatite with very low rate of substitution and hence low solubility. Panda Hill RP has shown potential for agronomic uses, and promising results have been achieved using canola (rapeseed) as the test crop (Mnkeni *et al.,* 2000) due to ability of rapeseed roots to extract P from blended unreactive RPs. Weil (2000) reported on cabbage varieties' ability to effectively extract P from the unreactive Panda RP applied directly as P fertilizer.

2.6 The Minjingu Rock Phosphate Deposit

Minjingu is one of the most promising sources of RP in Tanzania and East Africa in general van Straaten (2002). The Minjingu deposit contains about 10 to 25 million tons of phosphate (Szilas, 2002) which is considered soluble under acidic conditions due to its guano nature. Minjingu RP is a biogenic type sedimentary deposit (van Straaten, 2002), which is believed to have been formed when Lake Manyara was an extensive alkaline lake and the current Minjingu Hill was an island. The deposit is thought to have resulted from the deaths of the large numbers of cormorants (*Phalacrocorax kuehnensis*) that used to roost on the island (Jama and van Straaten, 2006). Minjingu phosphate has 10-20% P_2O_5. A number of studies have highlighted the suitability of Minjingu RP as P source for

crops in P-deficient soils (Buresh *et al.,* 1997). For example, Bromfield *et al.* (1981) reported a relative agronomic effectiveness of 75% for Minjingu RP in the five seasons following application to maize in western Kenya. Based on results of 559 comparisons of phosphate rocks and fertilizers at equal levels of added P, Sanchez *et al.* (1997) observed higher maize yield increases with Minjingu RP than with some other RPs found in Africa. It would be desirable to find microorganisms that could solubilize the hard Minjingu RP upon direct application. While the soft Minjingu rock phosphate can be directly applied due to its relatively good solubility in acid soils, the hard Minjingu RP cannot

2.7 Phosphate Solubilizing Microorganisms

Phosphate solubilizing microorganisms (PSMs) have attracted the attention of agriculturists as soil inoculum to improve plant growth and yield (Chen *et al*., 2006). The selection of microorganisms capable of solubilizing phosphorus (P) from raw phosphate rocks (RP) may contribute to reduce the costs of agricultural production by reducing dependence on industrial P fertilizers. Some microbial species exhibit P solubilisation capacity; these include those of bacteria, fungi, actinomycetes and even algae (Sharma *et al*., 2013). It is generally accepted that the mechanism of mineral phosphate solubilisation by strains of PSMs strains is associated with the release of low molecular weight organic acids, whose hydroxyl and carboxyl groups chelate the cation bound to phosphate, thereby converting phosphorus into soluble forms (Khan *et al*., 2009).

2.7.1 Bacteria

Phosphate solubilizing bacteria are a group of beneficial bacteria capable of hydrolysing not only organic but also inorganic phosphorus from insoluble compounds (Liu *et al.,* 2015). Soil bacteria that are capable of enhancing P availability include the following

species of bacteria genera *Alcaligenes, Arthrobacter, Acinetobacter, Flavobacterium Pseudomonas, Azotobacter, Burkholderia Erwinia, Serratia, Bacillus and Rhizobium* (Rodríguez and Fraga, 1999; Bashan *et al*., 2013).

2.7.2 Actinomycetes

The P-solubilizing ability of actinomycetes has attracted interest in recent years because this group of soil organisms is not only capable of surviving in extreme environments (e.g. drought and fire) but also possesses other potential benefits (e.g. production of antibiotics and phytohormone-like compounds) that could simultaneously benefit plant growth (Hamdali *et al*., 2008). Numerous P-solubilizing actinomycete species have been isolated from the rhizosphere of *Theobroma cacao* (Barreto *et al.,* 2008) and their presence in soil has been linked to enhanced efficiency of P use (El-Tarabily *et al*., 2008). Hamdali *et al*. (2008) has reported that about 20% of actinomycetes can solubilize P, including those in the common genera *Streptomyces* and *Micromonospora*. These microbes release large quantities of organic acid anions (e.g. citrate, formiate, lactate, malate, succinate), which are implicated in the P dissolution process (Jones and Oburger, 2010).

2.7.3 Fungi

A range of non-mycorrhizal soil fungi have been screened and selected for their P solubilizing capacity. Fungal species such as *Penicillium spp*, *Mucor spp* and *Aspergillus spp*, have shown ability to solubilize insoluble P (Sharma *et al*., 2013). In addition, a range of *Trichoderma spp* have also been identified and found to stimulate plant growth both in the laboratory and field in Mexico and Bangalore, India, using chickpea (*Cicer arietinum* L) as test plant (Contreras-Cornejo *et al*., 2013; Rudresh *et al.,* 2005). Generally, many P-solubilizing ectomycorrhizal fungi, non-mycorrhizal fungi

(e.g. *Emericella rugulosa, Penicillium spp*.) appear to employ three strategies for mobilizing soil P, namely acidification of the soil, the release of organic acid anions (e.g. citrate, oxalate, gluconate) and the release of acid and alkaline phosphatases and phytase which hydrolyse the C-O-P ester bond (Yadav and Verma, 2012). These strategies occur upon phosphate starvation (Reddy, 2014).

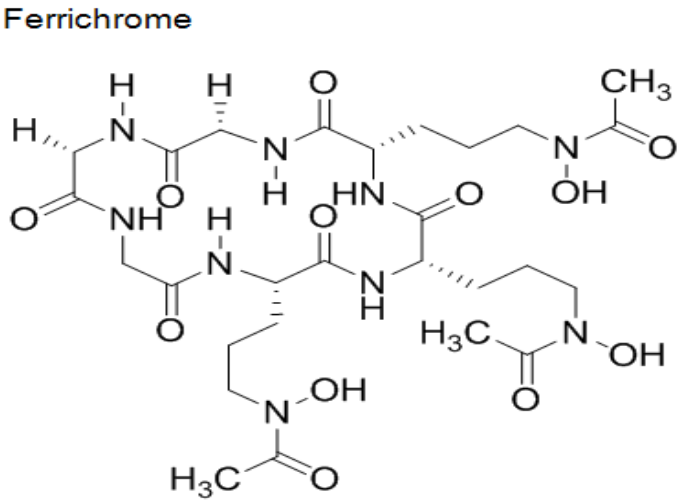
2.8 Mechanisms of RP Solubilization by Phosphate Solubilizing Microorganisms

Mechanisms for rock phosphate solubilization are explained by production of organic acids and siderophores by soil microorganisms. The potential mechanism for phosphate solubilization might be acidification either by proton extrusion associated with ammonium assimilation or by organic acids production and proton extrusion (Yadav and Verma, 2012). Microorganisms tend to secrete acid phosphatases and phytase which play an important role in phosphate solubilization (Richardson *et al*., 2000). On the other hand, inorganic P is solubilized by the action of organic and inorganic acids secreted by PSMs in which the hydroxyl and carboxyl groups of the acids chelate cations (Ca, Al and Fe) and decrease the pH in basic soils (Kpomblekou and Tabatabai, 1994). Several cases of insoluble P solubilization by PSMs have been reported; including compounds secreted by PSMs which help in solubilization of insoluble P include siderophores, organic acids and enzymes.

2.8.1 Siderophores

Siderophores are complexing agents that have a high affinity for iron and are produced by almost all microorganisms in response to iron deficiency. Thus, siderophores act as solubilizing agents for insoluble P from minerals or organic compounds under conditions of iron limitation. Studies have reported the release of siderophores from PSMs (Vassilev *et al*., 2006; Caballero-Mellado *et al*., 2007; Hamdali *et al*., 2008). Considering the

complexing ability of siderophores, the potential role of siderophores in enhancing P availability should be obvious. Siderophores can bind a variety of metals thereby functioning as biological control, biosensor, bio-remediation and chelating agents. Moreover, siderophores can weather soil minerals and render nutrient elements available to plants. Microorganisms produce a wide range of siderophores; most of the microbial siderophores include enterobactin, rhizobactin and hydroxamates (ferrioxamine B) (Matzanke, 1991). An example of chemical structure of one of the siderophores is shown below.



Source: Hider and Kong, 2010.

Figure 1: Structure of Ferrichrome, a hydroxamate siderophore.

2.8.2 Organic acids

Generally, it is accepted that the mechanism of mineral phosphate solubilisation by phosphate solubilizing bacteria (PSB) is associated with the release of low molecular weight organic acids, which through their carboxyl groups chelate the cation bound to phosphate, thus releasing the P and making it soluble (Kpomblekou and Tabatabai, 1994; Bolan *et al*., 2003).

According to Pradhan and Sukla (2005), in most soils, proton substitution reactions are driven by microbial production of organic acids, represented generally by the equation:

$$(Ca^{2+})_m (PO_4^{3-})_n + (HA) = (H^+) (PO_4^{3-}) + (Ca^{2+}) (A^-) \quad \text{(v)}$$

Microorganisms play an important role in the major components of the soil P cycle which are dissolution–precipitation and sorption–desorption (Sharma *et al*., 2013). Adsorption is the chemical binding of plant available P to soil particles, which makes it unavailable to plants. Desorption is the release of adsorbed P from its bound state into the soil solution. Adsorption occurs quickly whereas desorption is usually a slow process. Adsorption is reversible chemical binding of P to soil particles while precipitation involves a more permanent change in the chemical properties of the P as it is removed from the soil solution (Hyland *et al.,* 2005).

2.8.4 Enzymes

Insoluble phosphate compounds can be solubilized via enzyme-mediated reactions such as those mediated by phosphatase enzymes produced by plants and microorganisms during phosphate solubilization (Rodríguez and Fraga, 1999). Enzymes produced by PSMs can be substrate dependant (inducible enzymes) or constitutive which are secreted

without regarding substrate concentration (Nahas, 2007). Phosphate solubilizing bacteria have been shown to enhance the solubilisation of insoluble P compounds through the release of phosphatase enzymes (Sharma, 2005). These enzymes tend to catalyse different metabolic activities which lead to production of chemicals (organic acids and siderophores) which are important in phosphate solubilisation.

2.9 Inoculation of Phosphate Solubilizing Microorganisms in Fields

Inoculation is the transfer of microbial isolates or preparations from laboratory apparatus such as test tubes and petri dishes to a desired medium, e.g. soil or seeds, and using them for performing a desired process. A classic example of this is the mixing of rhizobia preparations with legume seeds so as to improve the process of biological nitrogen fixation by plants obtained from germinated inoculated seeds (Burns and Hardy, 2012). There are various ways of inoculating PSMs; these include direct application of PSMs to soil, and this method of inoculating directly to the soil has been quite effective (Kyei-Boahen *et al*., 2002). Seed inoculation is the other way of inoculating PSMs (Hartley *et al*., 2013; Bennett and Lloyd, 2015), whereby the inoculum to be mixed with seed before planting can be delivered using a variety of carriers; the most common carrier is peat which is used for rhizobia inoculants. Peat has proven to be better than most other carriers in preserving live bacteria under unfavourable conditions (Albareda *et al*., 2008). Peat could also be evaluated as carrier of PSMs.

Ehteshami *et al*. (2007) reported on maize seed inoculation with *Glomus intraradices* (arbuscular mycorrhiza) and planted in a furrow containing apatite so as to ensure release of P from apatite. The Phosphate-Solubilizing Bacteria (PSB) *Pseudomonas tolaasii* and *Pseudomonas koreensis* when used for seed inoculation demonstrated a remarkable increase in maize weight by, 45% and 40% respectively, P content compared to the

uninoculated control (Viruel *et al*., 2014). Dwivedi *et al*. (2004) reported pre-plant inoculation of rice seedlings with P-solubilizing *Aspergillus awamori* in a field experiment in India to increase rice yields as compared to yields of un-inoculated seedlings.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Soils and rock phosphate samples were collected from Panda Hill and Minjingu rock phosphate deposits in Mbeya and Arusha, respectively. Media for isolating bacteria, fungi and actionomycetes were Nutrient agar, Potato dextrose agar and Starch casein agar, respectively.

3.2 Soil and Rock Phosphatesampling

Sampling was done for soils in contact with PR, PR itself and soils located away from RP. Sampling was done at the depth of 0-5 and 5-10 cm. rock phosphatewas sampled by taking samples of weathered (as source of P solubilizing microorganisms) and hard (unweathered) PR. Panda Hill, rock phosphatesamples were picked following determination of P content by X-ray fluorescence (XRF) where samples having P% of above 0.443% were chosen. Collected samples were brought to SUA Soil Science Laboratory for physicochemical and microbiological analyses.

3.3 Soil Analysis

Portions of the soil samples were dried, ground and sieved through a 2 mm sieve for physio-chemical characterization. Parameters measured were: soil pH, texture, organic carbon and available P.

3.3.1 Soil pH

Soil pH was determined electrochemically in 1:2.5 (weight/volume) soil:water suspensions in accordance with the procedure described by Okalebo (1993). To 10g of

soil sample, 25 ml of distilled water was added and shaken on a reciprocating mechanical shaker for 30 minutes. The pH values of soil samples were determined using a pH meter.

3.3.2 Particle size distribution

The hydrometer method (Bouyoucos, 1962; Okalebo *et al*., 1993) was used to determine the particle size distribution of soils. Briefly, 50 g of oven-dry soil was placed into a soil dispersion cup and filled up with distilled water. Then, 125 ml of 5% sodium hexametaphosphate (calgon) was added. The mixture was the allowed to soak for up to 15 minutes. The cup was then attached to a mixer and the contents mixed for 5 minutes for sandy (course-textured) soils and 15 minutes for fine textured soils. Then, the suspension was transferred into a sedimentation cylinder and filled up to the 1000 ml mark with distilled water. A plunger was used to mix the contents and hydrometer and temperature readings were taken after 5 minutes and again after 5 hours. The percentages of sand, silt and clay were determined. The USDA textural class triangle was used to determine the textural classes of soils.

3.3.3 Organic carbon

Determination of organic carbon was done by the wet digestion (oxidation) method of Walkely-Black (Nelson and Sommers, 1996), as follows: To 0.5 g of soil 10 ml of 1 M $K_2Cr_2O_7$ and 25 ml of concentrated H_2SO_4 were added and allowed to stand for 30 minutes. After 30 min. incubation, 200 ml of water were added, allowed to cool, followed by addition of 10 ml of phosphoric acid. The amount of $K_2Cr_2O_7$ reduced was used to estimate the organic carbon content of the soil following the titration of excess dichromate against a 0.5 N ferrous sulphate solution by using diphenylamine indicator. In this and subsequent sections, all reagents used in chemical analysis are of analytical grade.

3.3.4 Extractable phosphorus

Extractable phosphorus was determined according to procedures described by Okalebo (1993). Due to differences in soil pH, available P in Panda Hill and Minjingu was determined by Bray No 2 and Olsen's method, respectively. In the Bray method three grams of soil were weighed and placed in 50 ml plastic bottle. Twenty ml of extracting solution containing 0.03 M NH_4F and 0.025 M HCl were added, shaken vigorously by hand for one minute and immediately the suspension was filtered using Whatman No. 2 filter paper into a dry plastic vial. Five ml of extract were transferred into 50 ml volumetric flask; 30 ml distilled water and 4 ml of ammonium molybdate reagent were added and mixed and made volume to the mark. After 15 minutes absorbance of solution was measured using a Spectrophotometer at the wavelength of 884.

3.4 Analysis of Elemental P composition in Panda Hill RP

Rock phosphate from Panda Hill elemental composition was determined using XRF. Ten readings were taken at 30 seconds time interval from 10 points of a rock phosphatecrystal sample.

3.5 Microbiological Studies

3.5.1 Isolation of bacteria, fungi and actionomycetes from both RP and soils in contact with RP at Minjingu and Panda hill

3.5.1.1 Preparation of microbiological media

Three types of media namely, nutrient agar, starch casein agar and potato dextrose agar were prepared for isolating bacteria, actinomycetes and fungi, respectively. Nutrient agar was prepared based on formulations by Downes and Ito (2001) as follows: Five g of peptone, 3 g of beef extract, and 1 g of yeast extract, and 15 g of agar were added into 1000 ml of distilled water. Similarly, starch-casein agar was be prepared according to

Kuster and Williams (1966) as follows: Ten g of starch, 0.3 g of casein (vitamin-free), 2.0 g of KNO_3, 2.0 g of NaCl, 2.0 g of K_2HPO_4, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_2O$, and 18 g of agar were added into 1000 ml of distilled water. To prepare The Potato Dextrose Agar (Potato Glucose Agar), 39 g of PDA was dissolved into one litre of distilled water, its pH adjusted to 6. All media were sterilized by autoclaving at 1.05 kg/cm^2 and 121^{o}C for 20 minutes (Curry *et al*., 1993).

3.5.1.2 Isolation of microorganisms and determination of total microbial counts of bacteria, fungi and actinomycetes

Collected soil and PR samples were subjected to serial dilutions (Usha *et al.*, 2011) as briefly described here. Ten grams of soil or PR were added into a bottle containing 90 ml of sterile water and shaken vigorously to suspend the soil particles, thus making the 10^{-1} suspension. One ml of the above suspension was aseptically transferred to a bottle carrying 9 ml of distilled sterile water and shaken to mix well, making a 10^{-2} suspension. Using a fresh sterile pipette, one ml of the 10^{-2} was transferred to make a 10^{-3} suspension. Thus, serial dilutions were made up to the 10^{-8}. One ml aliquots from 10^{-3} to 10^{-6} was placed into separate petri dishes following the pour plate technique (Frankhauser, 2009) using starch casein, nutrient and potato dextrose agar for actinomycetes, bacteria and fungi, respectively. Each dilution was replicated four times. The plates were incubated, up-side down, at 30°C for 3 to 7 days for bacteria and fungi, and up to 14 days for actinomycetes or until visible colonies were seen. Colonies were counted from plates showing a good distribution of the colonies, and expressed as colony forming units (CFU). Some of the developed colonies were purified by repeated sub-culturing and pure cultures were preserved in slants of respective media with 30% glycerol at -80^{o}c.

3.5.1.3 Enumeration of total microbial population

Microbial colony counts were done depending on the nature of growth. For bacteria and fungi colony counts were done on the third day of incubation while for actinomycetes it was on the 14^{th} day when colonies were clearly seen. Microbial count was done only on those plates with colonies ranging from 30-300. Microbial counts were presented as colony forming unit per gram of soil (CFU/g of soil).

3.5.2 Testing the ability of isolates obtained in section 3.5.1 above to solubilize RP

Rock phosphate samples were ground with mortar and pestle and passed through a 500 µm sieve. The fine powder obtained was used to prepare the synthetic minimum medium (SMM) containing $NaNO_3$ (2 g/L), $MgSO_4$ (0.5 g/L), KCl (0.5 g/L); $FeSO_4$ (0.01g/l), RP (0.5 g/L) as sole phosphate source, and 15 g agar/L (Hamdali *et al*. 2008). Special medium, Pikovskaya (PVK) containing: $C_6H_{12}O_6$ 10, $CaHPO_4$ 5, $(NH_4)_2SO_4$ 0.5, NaCl 0.2, $MgSO_4.7H_2O$ 0.1, KCl 0.2, yeast extract 0.5, $MnSO_4.H_2O$ 0.002 and $FeSO_4.7H_2O$ 0.002 (Roy et al., 2013) was used for comparison. Both PVK and SMM were sterilized by autoclaving at 1.05 kg/cm^2 and 121^{o}C for 20 minutes, poured into petri dishes and used for RP solubilization ability test of isolates.

3.5.3 Qualitative and quantitative assessment of the ability of microbial isolates to solubilize rock phosphate

Sterile inoculation wire loop was used to aseptically transfer actionomycetes, fungi or bacteria to a petri dishes of appropriate media (section 3.5.1.1) and incubated for 3-10 days at 28^{o}C. Clear halo zones around the developing colonies were taken as a qualitative index of phosphorus solubilization. The diameter of the halo (including colony) was measured. Microbial isolates with high ability to solubilize RP were subsequently characterized by their micromorphology using a microscope and by their macro-

morphology using the naked eye (Guarro *et al.,* 1999), followed by molecular characterization (section 3.5.3).

Rock Phosphate (RP) samples from Panda hill and Minjingu were washed using sterile water, dried and ground using a mortar and pestle and passed through a 100 mesh sieve. Sterilization of RP powder was done by using UV irradiation. Sterile RP powder (0.5 g) was mixed with 50 ml SMM without agar; the mixture was inoculated with either bacteria, fungi or actinomycetes isolates, replicated four times. The mixture was then incubated for 10 days at 28^{o}C. Controls contained the P sources with no inoculation. The experiment was laid out as split plot, with treatments arranged in the randomized complete block design (RCBD). Amount of soluble phosphate (P) released from RP was determined by the chlorostannous reduced molybdo-phosphoric acid blue colour method (Olsen and Sommers, 1982).The abilities of different isolates to solubilize P were compared based on amount of P solubilised.

3.6 Identification of Microbial Isolates with the Greatest Efficiency in Solubilizing Rock Phosphate

3.6.1 Macro- morphology of phosphate solubilizing microorganisms

Representatives of each group of microorganisms which showed great ability to solubilize RP were selected and described morphologically with aid of naked eyes. The image of each preventative was capture by digital camera (Nikon, 20.1 MEGAPIXELS COOLPIX).

3.6.2 Micro-morphology of phosphate solubilizing microorganisms

The representative isolates were described micro-morphologically by using microscope. Bacteria smears were prepared using standard Gram stain procedure (Carter and Cole,

2012). On the other hand, fungal isolates were observed under microscope through fungal slides prepared by using lacto phenol blue solution for fungal identification under light microscope as described by Alfred (2012).

3.6.3 Staining fungi using LPCB

Lactophenol cotton blue is the mounting medium used during fungi microscopic examination (Sudan and Sharma, 2003; Lakshmi and Anuradha, 2008). Lactophenol cotton blue was prepared by mixing 20 g of phenol crystals, 20 ml of lactic acid, 20 ml of distilled water and 0.075 g of methyl blue. The solution was shaken to mix the contents. During staining, a drop of LPCB was placed on a slide using wire loop, fungal culture was careful spread to obtain a thin preparation on a slide. The slide was covered by a coverslip by lowering it slowly to avoid trapping air bubbles under and left for 5 minutes. The slides were observed under a light microscope with 40 magnifications.

3.6.4 Gram staining of bacteria

Bacterial smears were stained based on Gram staining procedure as described by Chapelle (2001). A loopful of the bacterial culture was placed on the slide and spread by means of circular motion using inoculating loop to about one centimetre in diameter. The underside of a slide was marked by drawing a circle fixation of bacterial cells to the surface of the microscope slide was done by heating. Primary stain crystal violet was applied which stains cells blue or purple. Iodine solution was added to form a crystal violet iodine complex; all cells continue to appear blue. The decolourization process was done using ethanol so as to distinguish gram-positive from gram-negative bacterial cells. The red dye safranin stained the decolorized gram-negative cells red or pink; the gram-positive bacteria remained purple.

3.7 Molecular Identification of Microbial Isolates with Greatest Efficiency in Solubilizing RP

Five fungal and five bacteria isolates with outstanding ability of solubilizing RP were selected for identification using ITS1-5.8S-ITS2 and 16S rDNA nucleotide sequencing, respectively (Nelson and Cox, 2008). Fungal and bacterial DNAs were extracted from pure cultures by heating at 90^{o}C for 15 minutes followed by extraction using silica columns prior to amplification. Amplification of the ITS1-5.8S-ITS2 rDNA of fungi was done using primers ITS-F and ITS4. Bacterial isolates were identified by amplification and nucleotide sequencing of the 16S rDNA using universal bacteria primers 27F and 1492R (Balajee *et al*., 2007). Amplification of bacterial rDNA included an initial denaturation at 95ºC for 10 minutes followed by 40 cycles each for 30 seconds at 95ºC, 30 seconds at 55ºC, and 1 minute at 72ºC, and a final extension at 72ºC for 10 minutes. Amplification of fungi 5.8S rDNA included an initial denaturation at 95ºC for 10 minutes followed by 40 cycles each for 45 seconds at 95ºC, 30 seconds at 55ºC, and 1 minute at 72ºC, and a final extension at 72ºC for 10 minutes.

The amplified rDNA fragments were then separated by electrophoresis through 1.5% agarose gel and visualized using a gel documentation system after staining with GelRed (Biotium, Phenix, USA), a nucleic acid gel stain. Afterwards, PCR products were directly sequenced using dideoxynucleotide cycle sequencing (ABI 3500 Genetic Analyser, Applied Biosystems, Foster City, CA). After treatment with exonuclease I and shrimp alkaline phosphatase, sequencing PCR was conducted using Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were visualised using a sequence scanner software (Applied Biosystems, Foster City, CA). Obtained nucleotide sequences were input at GeneBank using BLASTn in order to find identities with sequences at GeneBank database.

3.8 Data Analysis

Data of P solubilized were obtained in the quantitative experiment (section 3.5.2.3) and subjected to analysis of variance (ANOVA) to evaluate the efficiency of different microbial isolates in solubilizing PR. Treatment means separation was done using Duncan's New Multiple Range Test (DNMRT) at the 0.05 level of significance.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Some Physico-chemical Properties of the Soils

The properties of soils collected for microbial isolation are presented in Table 1. The textural class of the soils was sandy loam according to the USDA textural class triangle (Brady and Weil, 2002). Soil texture influences microbial population in an indirect way, influencing soil moisture content and aeration. These would influence different microbial metabolic activities including respiration. On the other hand, soil texture can influence distribution of microorganisms in soils due to aerobic or anaerobic conditions; when there is more water than air anaerobic conditions prevail and when water and air are at balance aerobic conditions will prevail.

Soil pH values were 8.8 and 6.83 for Minjingu and Panda Hill, respectively (Table 1). According to Landon (2014) the soil of Panda Hill was rated as being neutral and that of Minjingu as being slightly alkaline. Soil pH influences microbial types and activities in the soil; different microorganisms have different pH range preferences. Most fungal species thrive over a wide range of pH while some bacteria are neutrophilic, acidophilic or alkaliophilic, and actinomycetes are sensitive to acidity (optimum pH range 6.5 to 8.0) Alexander (1980).

The percentage organic carbon levels were 0.804% and 0.810% for Minjingu and Panda Hill soils, respectively (Table 1), which were rated as being very low (Landon, 1991). Very low organic carbon (%OC) reflects low organic matter content. Organic matter is one of the nutrient-rich components of soil. Microorganisms use organic matter as their source of carbon and energy. Very low organic matter does not adequately support

microbial growth due to lack or low substrate thus decreasing in their number and biomass (Feng and Schaefer, 2009). Very low %OC negatively influence the availability of nutrients e.g. phosphorus and nitrogen due to poor soil structure. Available phosphorus of Minjingu and Panda Hill were 31.36 mg/kg and 8.068 mg/kg respectively. Phosphorus is one of the major essential macronutrients for microbial growth and development (Rodríguez and Fraga, 1999).

Table 1: Selected properties of the soils

Soil from	pH in H_2O	OC %	P (mg/kg)	Textural class
Minjingu	8.8 (SA)	0.804 (VL)	31.36 (M)	Sandy loam
Panda Hill	6.83 (N)	0.81 (VL)	8.068 (L)	Sandy loam

N=Neutral, SA= slightly alkaline, VL= Very low, L=Low, M=medium
Ratings of soil parameters were according to Landon (1991).

4.2 Metal Contents of the Rock Phosphate

The elemental composition rock phosphate from Panda Hill was analysed using XRF (Appendix 1 and 2). The RP contained Uranium and Thorium which are radioactive; heavy metals were also present (Table 2). Rock phosphate samples from Panda hill contained detectable amounts of Zinc (Zn), Iron (Fe) and Manganese (Mn) that were found at all sampling points while Arsenic (As), Lead (Pb), Copper (Cu), Nickel (Ni) and Titanium (Ti) where detected in only some points. These heavy metals could affect microbial growth in media containing Panda Hill RP. Gold (Au), Silver (Ag), Cadmium, Palladium (Pd), Cobalt and Chromium (Cr) were less than the limit of detection (LOD).

Minjingu RP contains both heavy metals and radioactive elements Szilas (2002) cited the contents of uranium in Minjingu RP to be ranging from 110-370 mg U kg^{-1} to 210-850

mg U kg^{-1} with an average of 380 mg U $kg^{-1}$for both soft and hard Minjingu RP ores. These levels are considered high as compared to other RP around the world. It was found that during production of soluble phosphate most of the radionuclides were transferred from the MRP to the soluble fertilizer end product (Makweba and Holm, 1993). During the acidulation process, the RP solution was reported to contain high amounts of Uranium, Radium and Lanthanum (Habashi, 1994; in van Straaten, 2002). Presence of heavy metals and radioactive elements may have implication on their uptake by plants and effects on soil microorganisms (Giller *et al*., 2009) and animals/humans. However, effects on animals or humans, e.g. Minamata disease in the case of Mercury poisoning, or cancers as results of radiations from Uranium, are beyond the scope of the present study and are not discussed herein. The present study did not characterize elemental composition for Minjingu rock phosphate due to the fact that there are several studies reporting on its composition e.g. van Strateen (2002) and Szilas (2002).

Table 2: Metal and radioactive elemental composition of Panda Hill RP

Point ID	Time(Sec)	U	Th	Pb	Zn	Cu	Ni	Fe	Mn	Ti	As %
C1	30.14	< LOD	0.007	0.003	0.024	< LOD	0.026	14.786	0.38	0.613	0.004
C2	29.94	< LOD	0.019	0.009	0.018	< LOD	0.063	11.198	0.434	< LOD	< LOD
C3	32.5	0.002	0.03	0.011	0.013	0.01	0.02	9.275	0.201	0.171	< LOD
C4	26.17	0.007	0.04	0.045	0.027	0.03	< LOD	12.847	0.196	0.491	< LOD
C5	28.25	< LOD	0.025	< LOD	0.04	< LOD	0.073	18.322	0.283	< LOD	0.029
C6	26.98	0.005	0.013	< LOD	0.02	< LOD	< LOD	5.883	0.253	0.187	< LOD
C7	25.72	< LOD	< LOD	< LOD	0.012	< LOD	< LOD	8.809	0.303	< LOD	< LOD
C8	27.52	< LOD	0.024	0.16	0.021	< LOD	0.045	8.831	0.882	< LOD	< LOD
C9	27.12	< LOD	< LOD	< LOD	0.029	< LOD	< LOD	9.544	1.509	< LOD	< LOD
C10	25.87	< LOD	< LOD	< LOD	0.015	< LOD	0.049	8.5	0.788	< LOD	< LOD

4.3 Microbial Populations in the Soils Adjacent to the Phosphate Rocks and PRs

The results present in Table 3 relatively high microbial populations ranging from 9 x 10^7 to 1.3 x 10^8 CFU per g bacteria, 8 x 10^7 to 1.5 x 10^8 CFU per g for fungi and 4 x 10^7 to 1.25 x 10^8 CFU per g actinomycetes in both soils (Minjingu and Panda Hill) (Table 3) and this could be due to its relatively neutral to slightly alkaline pH range which favors microbial growth and development. Soil microbial populations are also influenced by soil particle size where clayey soils are reported to have higher populations of up to 10^{10} bacterial cells per gram of soil while sandy soils have lower populations (Chenu and Stotzky, 2002). Lee *et al*. (1994) reported on dynamics of fungi and bacterial populations in soils, whereby bacterial populations ranged between 1.175x10^6 to 5.88x10^8 CFU per 1 g dry soil; fungi from 1.23 x 10^4 to 3.09 x 10^4 CFU per 1 g dry soil) while clay loam and silt soils had the highest bacterial populations 5.888 x 10^8 and 1.072 x 10^8 CFU per 1 g dry soil, respectively. Soil adjacent to PRs and PRs contain high populations of bacteria, fungi and actinomycetes than soils from far form PRs,

Table 3: Microbial numbers in soils adjacent and in contact to PR and PR itself

Sample	Log CFUg^{-1}Sample		
	Bacteria	**Fungi**	**Actinomycetes**
Soil MRP Composite	1.16 x10^8	1.25 x 10^8	1 x 10^8
Soil MRP in contact	1.2 x 10^8	1.4 x 10^8	9.9 x 10^7
Soil MRP far	1.3 x 10^8	1.5 x 10^8	1.21 x 10^8
Soft MRP	1.19 x10^8	1.21 x 10^8	9.5 x 10^7
Hard MRP	1.01 x10^8	1.25 x 10^8	1.19 x 10^8
Soil Panda Hill Composite	1.15 x10^8	1.19 x 10^8	1.25 x 10^8
Soil Panda Hill in contact	1.29 x10^8	1.05 x 10^8	1.11 x 10^8
Soil Panda Hill far	1.15 x10^8	1.35 x 10^8	1.22 x 10^8
Panda Hill RP	9 x 10^7	8 x 10^7	4 x 10^7

4.4 Qualitative Assessment of Ability of Microorganisms to Solubilize Phosphate Rocks

The phosphate-solubilizing activity of the isolates was qualitatively tested by measuring diameter of clear zones around the colonies growing on three solid media (PVK and SMM containing Minjingu or Panda hill rock phosphate as phosphorus source). Ten fungal and ten bacteria isolates with the largest diameter of clear zone were subsequently selected for quantitative assessment in their ability to solubilize phosphate rock, five of each group presenting their place of origin (Panda Hill or Minjingu).

4.4.1 Solubilization of rock phosphate by fungal isolates as determined by diameter of clear zones

The diameter of clear zones around microbial colonies indicated the ability to solubilize phosphate rocks, and these are presented in appendix 5 for fungal isolates. Of more than 22 fungal isolates, 18 displayed different degrees of rock phosphate solubilizing activity.

4.4.1.1 Minjingu fungal isolates

When Minjingu fungal isolates were tested in PVK and in SMM media containing PRP or MRP, larger clear zone diameter were measure in PVK followed by SMM (HMRP) and most of SMM -PRP gave smaller diameters (Appendix 7). Fungal isolates performed better in PVK and SMM -HMRP media and this could be due to their less complex nature, PVK is a standard media that contains less complex and relatively pure $Ca_2(HPO)^{4.}$ Minjingu rock phosphate is sedimentary in nature (medium to high reactivity); is less complex as compared to Panda Hill carbonatite which is igneous in origin making it less reactive (Szilas, 2002 and Okalebo *et al.,* 2007). Thus, the reactivity of the phosphates sources was in the order of PVK>HMRP>PRP, which was reflected in the same order of solubilization of the phosphate sources.

4.4.1.2 Panda Hill fungal isolates

Panda Hill fungal isolates, when cultured in PVK and SMM (PRP or HMRP) media, gave the same trend as was observed with Minjingu isolates. Fungal isolates which were inoculated in PVK and SMM (HMRP) gave larger diameters than those in SMM (PRP). Generally, Panda Hill fungal isolates gave large diameters of clear zones than those of Minjingu, but with few exceptions. Each of the fungal isolates was efficient to its particular rock phosphate, and this is probably due to adaptive mechanisms built by these fungi to survive in such conditions. Fungi can secrete siderophores, enzymes or organic acids depending on the prevailing conditions; fungi secrete siderophore only if there is iron deficiency (Ahmed and Holmstrom, 2014).

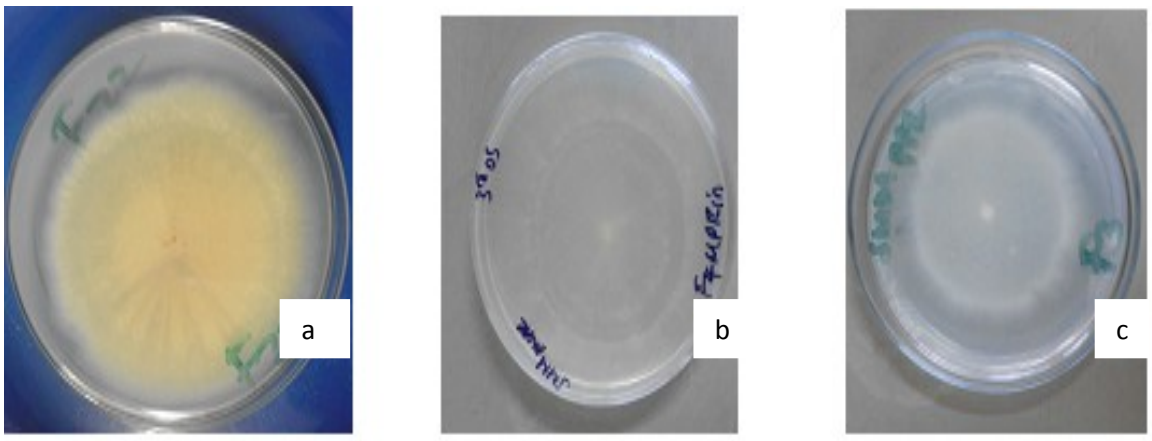


Figure 2: Clear zone (Rock phosphate solubilization) surrounding a fungi colony (a) PVK (b) HMRP and (c) PRP media

4.4.2 Solubilization of rock phosphate by bacterial isolates as determined by diameters of clear zones

In the qualitative experiment 39 bacterial isolates from soils and phosphate rocks samples of panda hill and Minjingu were tested into PVK, SMM (PPR) and SMM (MRP) media. Out of 39 bacterial isolates only 35 isolates showed clear zones. Ten out of 35 had outstanding results and were the candidates for quantitative test.

4.4.2.1 Minjingu bacterial isolates cultured on PVK and SMM (PRP or HMRP)

When Minjingu bacterial isolates were tested with media containing RP, only 2 out of 16 did not show clear zone while most of the isolates had clear zones and their respective diameter were of 2 mm and above. Generally, the PVK medium gave larger diameters followed by SMM -MPR and lastly PRP, with few exceptions. The trend is due difference in origin of phosphate rocks and ability of bacterial isolates to secrete and release organic acids for insoluble P solubilization. PVK medium on the other hand had the least chemically complex source of P as compared to Minjingu and Panda Hill phosphate rocks in SMM media (Szilas, 2002 and Roy *et al*., 2013).

4.4.2.2 Panda hill bacterial isolates cultured on PVK and SMM (PRP or HMRP)

Panda hill isolates were tested in three media(PVK and SMM(PRP or MRP) and out of 23 only 2 isolates failed to show clear zones while 21 did by giving the largest diameter of 25, 12 and 10 mm in PVK, SMM-MPR and SMM-PRP respectively (Appendix 6). Generally, Minjingu and Panda Hill bacterial isolates gave outstanding results when treated with PVK and MPR containing media than in media containing PRP. The trend of these diameters is influenced by nature of phosphate rocks and bacterial isolates abilities to solubilize insoluble P (Sharma *et al*., 2013 and van Straaten, 2002). The MRP, being a sedimentary rock is more reactive as compared to the more complex igneous PRP, hence

greater solubilization by bacterial for MPR than for PRP, as was also observed in the case of fungal solubilization (section 4.3.1.1).

4.5 Quantitative Assessment of Rock Phosphate Solubilization by Microorganisms

The best ten isolates each from fungi and bacteria group were quantitatively tested for their ability to solubilize PR under SMM broth containing PRP or HMPR. The candidates were tested based on amount of mg soluble P/kg of PR released at the end of 10 day incubation period. Both fungi and bacterial isolates differed in the amount of soluble P released when treated with it original or with foreign PR. Table 4 present best candidates which were tested in quantitative assessment.

Table 4: Selected fungal and bacterial isolates capable of solubilizing Minjingu and Panda Hill PR

S/N	Fungal Isolates	Bacteria Isolates	Origin of Isolation
1	FI	B1	Panda Hill
2	F5	B25	Panda Hill
3	F14	B8	Panda Hill
4	F6	B12	Panda Hill
5	FI8	B16	Panda Hill
6	FI9	B40	Minjingu
7	F2I	B21	Minjingu
8	F22	B35	Minjingu
9	FI3	B18	Minjingu
10	F17	B6	Minjingu

4.5.1 Fungi isolates

Out of 18 fungal isolates, 10 isolates showed active growth and larger clear zones on SMM and PVK media were selected for quantitative experiment.

4.5.1.1 Effectiveness of Panda Hill fungal isolates in solubilizing Panda Hill rock phosphate

The effectiveness of some fungal isolates from Panda Hill in solubilizing Panda Hill rock phosphate is presented in Table 5. Panda Hill fungal isolates F6 and F14 were significantly ($P<0.05$) more effective in solubilizing Panda Hill rock phosphate as compared to Panda Hill fungal isolate F1, but were similar to F5 and F18 (Table 5). However, the rates of solubilization were not very high. Semi qualitative results gave (Appendix 7) demonstrated outstanding diameter, but these are not reflected in the quantitative assessment results (Table 5), although general trends were comparable. Plate halo detection was similarly used as preliminary test to characterize PSMs; this is considered reliable in assessing microorganisms for the ability to solubilize insoluble P (Gupta *et al*, 1994; Rodríguez and Fraga (1999).

Table 5: Effectiveness of Panda Hill isolates on Panda Hill RP

Fungal Isolates	Soluble P (mg/kg PRP)
F1	2.471 a
F5	6.469 ab
F18	8.376 ab
F6	11.774 b
F14	12.774 b

Mean separation by DNMRT at 5% level of significance.

4.4.1.2 Effectiveness of Panda Hill fungal isolates in solubilizing Minjingu rock phosphate

Table 6 shows the effectiveness of Panda Hill fungal isolates in solubilizing hard Minjingu rock phosphate. Fungal isolate F14 and F18 were more effective than F5. Fungal isolate F14 which was ranked rather high for Panda Hill, did much better for hard Minjingu phosphates also (Table 5 and 6). Performance of Panda hill fungal isolates in solubilizing rock RP was not consistent; some did well in both phosphate rocks but others

did better in one rock but rather poorly in the other. Panda Hill fungal isolate performed better in the hard Minjingu than in Panda Hill phosphate due to simpler chemical nature of the sedimentary HMPR as opposed to Panda Hill which is of igneous nature and thus more complex chemically (Szilas, 2002 and van Straaten, 2002). The rationale for testing the effectiveness of Panda Hill isolates in solubilizing Minjingu rock phosphate is that, once an effective isolate is obtained from a particular source, it will be desirable for such an isolate to be of wider application. Thus, it would be desirable that, Panda Hill isolates, or other isolates, should not be limited not only to Panda Hill rock phosphate but to be also of use on other phosphate rocks. Thus, the isolates F14 and F18 could be effective in solubilizing different types of phosphate rocks. They could be candidates for preparing inoculants for treating crop seeds before planting them on soil into which insoluble rock phosphate has been applied. Upon proliferation in the soil the fungi would then solubilize the rock phosphate for plant uptake to improve plant growth and yields (Killham, 1994).

Table 6: Effectiveness of Panda hill fungal isolates performance Hard Minjingu rock phosphate

Fungal Isolates	Soluble P (mg/kg HMPR)
F5	29.57 a
F6	55.36 ab
F1	56.8 ab
F18	75.29 b
F14	80.39b

Mean separation by DNMRT at 5% level of significance.

4.5.1.3 Effectiveness of Minjingu fungal isolates in solubilizing Hard Minjingu rock phosphate

Hard Minjingu rock phosphate solubilization by fungal isolate from Minjingu was generally high (Table 7). There was no significant difference ($P<0.05$) among the Minjingu fungal isolates. Minjingu isolates performed well in HMRP due to adaptive nature of microorganisms. Exposure of microbes to such low soluble P conditions might have triggered secretion of inducible enzymes so that they survive (Sharma *et al*., 2013). Sometimes, long exposure of microorganisms to substrate lead to production of inducible enzymes to maximize the efficient use of the substrate, in this case solubilization of rock phosphate (Allison and Vitousek, 2005).

Table 7: Effectiveness of Minjingu fungal isolates performance on HMRP

F. Isolates	Soluble P (mg/kg HMPR)
F22	46.81a
F21	68a
F19	74.79a
F13	77.49a
F17	77.84a

Mean separation by DNMRT at 5% level of significance.

4.5.1.4 Effectiveness of Minjingu fungal isolates in solubilizing Panda hill rock phosphate

The effectiveness of Minjingu fungal isolates in solubilizing Panda hill rock phosphate is presented in Table 8. Minjingu isolates did relatively poor in solubilizing Panda hill rock phosphate as compared to HMPR though fungal isolate F13 was rather better. Reasons for Minjingu fungal isolates poor performance in solubilizing Panda hill rock phosphate is due to the more complex nature of PR as already explained (Msolla *et al*., 2005).

Table 8: Effectiveness of Minjingu fungal isolates against Panda hill RP performance

F. Isolates	Soluble P(mg/kg PRP)
F19	5.01a
F17	5.47a
F21	10.37ab
F22	10.77ab
F13	20.97b

Mean separation by DNMRT at 5% level of significance.

4.5.1.5 Effectiveness of Minjingu fungal isolates in solubilizing Panda Hill and Minjingu rocks phosphate

The effectiveness of Minjingu fungal isolates across phosphate rocks, and there was no statistical difference among the isolates. Minjingu fungal isolates were statistically more efficient ($P<0.05$) in solubilizing both Panda Hill and Minjingu RP. Minjingu fungal isolates gave 68.986 mg of P $kg^{-1}$of HMRP and 10.519 mg of P $kg^{-1}$of PRP amount of soluble P released.

4.5.1.6 Effectiveness of Panda hill fungal isolates in solubilizing Panda Hill and Minjingu rock phosphates

Panda Hill fungal isolates were statistically more efficient ($P<0.05$) in solubilizing both Panda Hill and Minjingu PR. The effectiveness of Panda Hill fungal isolates across phosphate rock, and there was no statistical difference among the isolates. The Panda Hill fungal isolates gave 59.481 mg of P $kg^{-1}$of HMPR and 8.373 mg of P $kg^{-1}$of PRP amount of soluble P released.

Difference in performance of fungal isolates on solubilizing Panda Hill and Minjingu rock phosphate can also be due to difference in abilities of fungi in producing organic acids and siderophores which can be inhibited by rock phosphate. Panda hill rock

phosphate contains heavy metal and radioactive elements, which can affect microorganisms and contribute to low rates of microbial RP solubilization (Appendix 1 and 2). de Oliveira Mendes *et al*. (2013) reported on the contribution of Cu, Fe, Mn, and Zn accompanied by RP, even at low concentrations, in inhibiting enzymes and production of organic acids and siderophores by fungi. The Panda Hill rock phosphate contains radioactive elements e.g. Uranium, Radium and Thorium, which can reduce the rate of rock phosphate solubilization (Szilas, 2002; van Strateen, 2002). Heavy metals can also reduce or inhibit fungal growth (Lema *et al*., 2014). Fungi are diverse and thus differ in production of siderophore and organic acids; some produce more or relatively low efficient chemicals depending on strain (Sharma *et al*., 2011, Benítez *et al*., 2010 and Johnson, 2008), and this may also account for the observed differences.

4.5.2 Bacterial isolates

Of the 39 bacterial isolates that were qualitatively tested for their ability to solubilize RP 35 isolates formed clear zones when plated on the solid SMM containing the phosphate rocks and PVK as sole phosphate source. Out of these 35 isolates, 10 isolates showed active growth and larger clear zone on SMM and PVK media and were selected for quantitative experiment (Table 4).

4.5.2.1 Effectiveness of Panda hill isolates in solubilizing PRP

The ability of Panda Hill bacterial isolate in solubilizing PRP was statistically not different ($P<0.05$) among the isolates (Table 11). This poor performance is due to the complex nature of the igneous PRP as mentioned above (section 4.3.2.2).

Table 11: Panda Hill bacterial isolates performance on Panda Hill RP

Bacteria Isolates	Soluble P(mg/kg PRP)
B12	3.76 a
B40	3.78 a
B8	20.32a
B1	23.4 a
B25	27.45a

Mean separation by DNMRT at 5% level of significance.

4.4.2.2 Effectiveness of Panda Hill bacterial isolates in solubilizing HMRP

Panda Hill isolates were statistically not different ($P<0.05$) in solubilizing HMRP, and they did poorly as compared to their performance on PRP (Table 12). Panda Hill bacterial isolates' better performance is solubilizing its original rock phosphate (PRP) may be contributed by adaptive mechanisms of the isolates since they have been exposed to it for a longer time. The adaptive mechanisms include secretion of inducible enzymes, special siderophores and organic acids (Rodríguez and Fraga, 1999).

Table 12: Effectiveness of Panda hill bacterial isolates against Minjingu rock phosphate performance

Bacteria isolates	Soluble P(mg/kg HMRP)
B1	5.46 a
B8	9.84 a
B40	9.95 a
B12	12.43 a
B25	24.75 a

Mean separation by DNMRT at 5% level of significance.

4.5.2.3 Effectiveness of Minjingu isolates insolubilizing HMRP

Table 13 shows the effectiveness of Minjingu bacterial isolates in solubilizing HMRP. Minjingu isolates had a similar solubilizing efficient on HMRP. The isolates were statically not different ($p<0.05$) in terms of their abilities to solubilize P from HMRP. The similarities of these bacterial isolates performance is probably due to their similar adaptation by being exposed to same type of P source (Schimel and Mikan, 2005).

Table 9: Effectiveness of Minjingu isolates performance on hard Minjingu phosphate rock

Bacteria isolates	Soluble P (mg/kg HMRP)
B16	6.007a
B6	7.261a
B21	7.32 a
B18	7.375a
B35	8.469a

Mean separation by DNMRT at 5% level of significance.

4.5.2.4 Effectiveness of Minjingu bacterial isolates against Panda Hill phosphate rock

The effectiveness of Minjingu bacterial isolates in solubilizing PRP is showed in Table 14. There was no significant difference ($P<0.05$) among the Minjingu bacterial isolates in solubilizing Panda Hill rock phosphate. Minjingu isolates performed better with hard Minjingu RP (Table 13) than with Panda Hill RP (Table 14). This trend was the same with qualitative only data with few exceptions, and this could be due to reasons on nature of RP and adaptability of microorganisms, as already discussed. Therefore, an isolate from one location may not always solubilize insoluble PR from a different location.

Table 10: Effectiveness of Minjingu bacterial isolates against Panda hill RP

Bacteria isolates	Soluble P (mg/kg PRP)
B35	0.123a
B16	0.244a
B6	0.39 a
B18	3.217a
B21	5.948a

There was no significant difference among the Minjingu bacterial Isolates on solubilization of PRP at $P\leq0.05$ level of significance.

4.5.2.5 Effectiveness of Minjingu bacterial isolates in solubilizing Panda Hill and Minjingu rock phosphates

The effectiveness of Minjingu fungal isolates between Panda Hill and Minjingu rock phosphate 5.There was significance difference ($p<0.05$) in the amount of soluble P released. Minjingu bacterial isolates solubilized 7.286 mg of P kg of HMRP and 1.984 mg of P kg of PRP.

4.5.2.6 Effectiveness of Panda Hill bacterial isolates in solubilizing Panda Hill and Minjingu phosphate rocks

The effectiveness of Panda Hill bacterial isolates Panda Hill and Minjingu phosphate rocks. There was no significance difference ($p<0.05$) in the amount of soluble P released among phosphate rocks. Panda Hill bacterial isolates solubilized 15.528 mg of P kg of PRP and12.486 mg of P kg of HMRP.

4.6 Micro and Macro Morphology of Rock Phosphate Solubilizing Microorganisms

Macro and micro morphological features both of the fungi and bacterial isolate were examined by naked eye and under light microscope. Generally, fungi colonies were plentiful with significant sporulation. The microscopic features showed the fungi to be filamentous. On the other hand bacteria were slimy and shiny on the surface, whitish, creamy, yellow to orange in colour. Bacteria were observed as single celled entities under microscope.

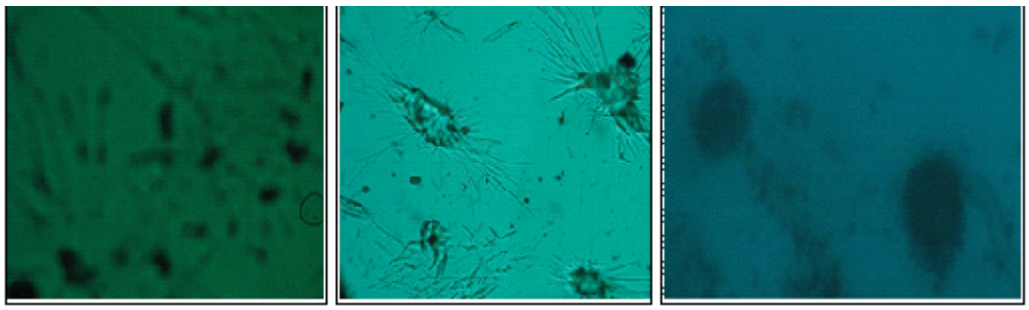


Figure 3: Fungal micrographs under light microscope, stained by LPCB total magnification of 400

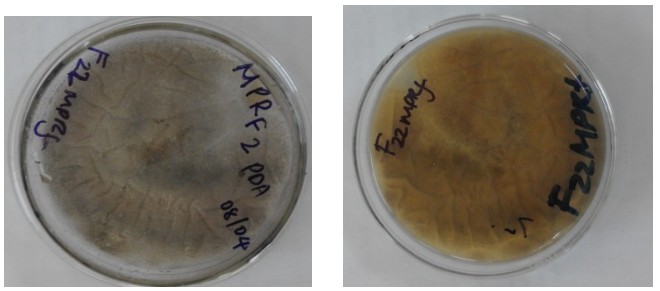
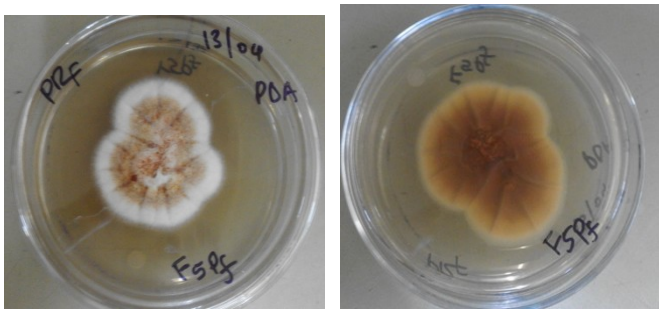
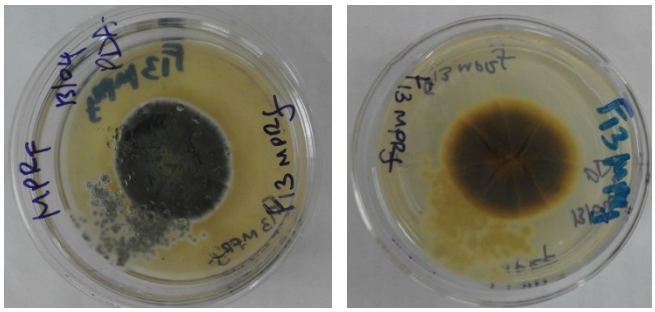
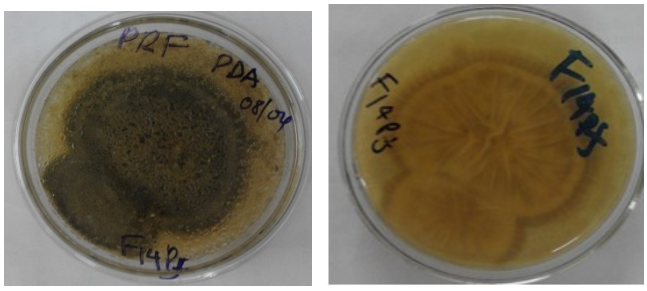
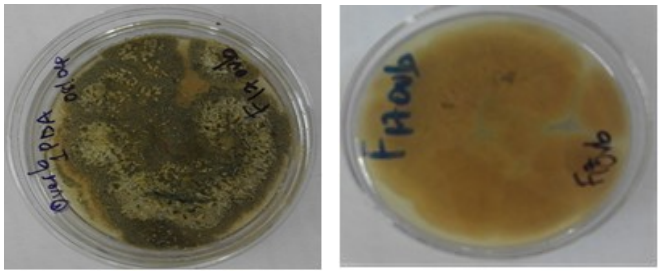
Fungi Isolates	Colony Description
	Isolate 22. Irregular in form has elevation w is flat, with undulate margin. It is large in size with wrinkled surface, opaque white to grey coloured.
	Isolate F5 which is same as F19 is irregular in form with umbonate elevation, margin entire. It is large fungus with wrinkled opaque surface; centre is brown middle with brown new gold and edge as white.
	Isolate F 13 is circular in form with convex elevation. Its margin is entire, large size fungus with smooth surface which is opaque. Its centre is olivaceous green (upper) with white lower surface.
	Isolate F 14 is circular in form with raised elevation. Its margin is filiform, large size fungus with rough surface which is opaque. It is green with white ring before it edges.
	Isolate F 17, aggregates of long branching filamentous hyphae. It is irregular in form with Nmbonate elevation. Its margin is filiform, large size fungus with rough surface which is opaque. It is white (upper) and green lower surface.

Figure 4: Fungal isolates growing in PDA media

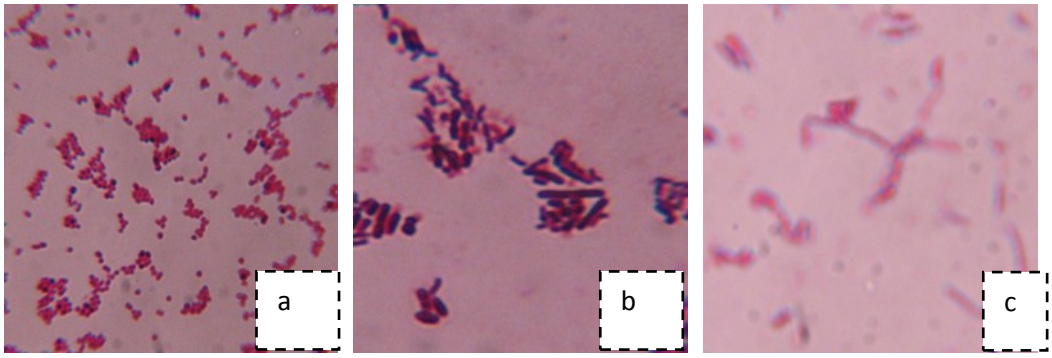


Figure 5: Rock phosphate solubilizing bacterial micrograph, Gram staining. X100 magnification (a) pink for Gram negative cocci and (b) purple for Gram positive large rods bacteria (c) Gram negative rod shaped bacteria.

Table 11: Macro and micro morphology of rock phosphate solubilizing bacteria PSB

No.	Bacteria	Lactose fermentation	Micromorphology	Gram stain
1	B6 (Minjingu)	NG	Large rods	Positive
2	B35	NG	Short Rods	Positive
3	B21	NLF	Cocco-rods shaped	Negative
4	B18	NLF	Small cocci	Negative
5	B40	NG	Rods in branches	Negative
6	B16 (Panda Hill)	LLF	Rods in chains	Positive
7	B8	NLF	Cocci in groups	Negative
8	B25	NLF	Large rods in chains	Negative
9	B12	NLF	Rods in short chains	Negative
10	B1	NLF	Cocci in singly	Negative

NG No growth,
NLF Not lactose fermenter
LF lactose fermenter

4.7 Molecular identification of rock phosphate solubilizing microorganisms

The 16S rDNA for bacteria and ITS1-5.8S -ITS4 rDNA for fungi were amplified, produced PCR products with approximately1400 base pairs (bp). Obtained PCR amplicons were of sufficient quality and quantity for DNA sequencing (Fig. 6; Table 18).

a) Fungi b) Bacteria

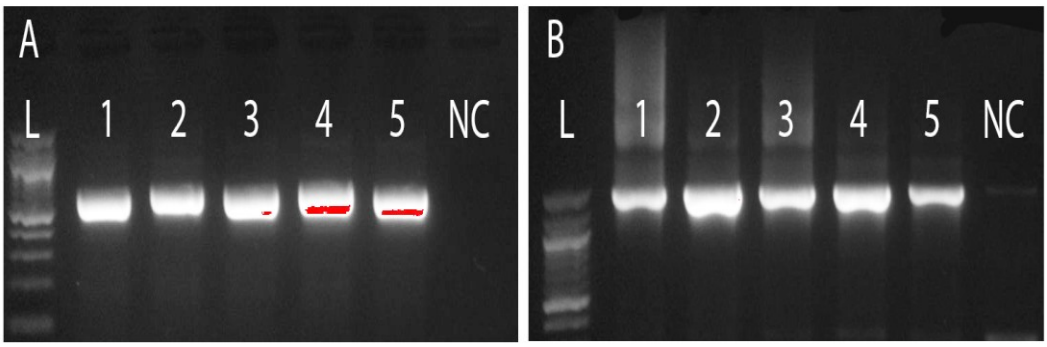


Figure 6: Amplification of ribosomal DNA (rDNA) for identification of bacteria and fungi. Agarose gel electrophoresis showing (A) amplified of 5.8S and its flanking intergenic spacer regions (ITS1 and ITS2) and (B) amplified of 16S rDNA of bacteria.

Note: L, Molecular marker; 1-5, bands for rDNA samples, NC for negative control.

After DNA sequencing of PCR products (section 3.7), the nucleotide sequences were compared with other sequences at GenBank using BLASTn. The identity of bacteria and fungi with the ability to solubilize RP are presented in Table 18. The results showed different bacterial and fungal species that had 100% nucleotide identity to other known species at the American National Institutes of Health (NIH) NCBI genetic sequence database (GenBank) using BLASTn.

The identified bacterial species were *Stenotrophomonas maltophilia*, *Bacillus safensis*, *Acinetobacter nosocomialis* and *Acinetobacter baumanii*. Identified fungal species included *Aspergillus stellifer, Aspergillus tamarii, Aspergillus flavus, Aspergillus terreus strain and Aspergillus brunneoviolaceus* as shown in Table 18.

4.8 Potential of the Presently Identified Microorganisms as Bio-Fertilizer

Some fungal and bacterial species, e.g. *Aspergillus terreus* (fungus) and *Stenotrophomonas maltophilia* (bacterium), are similar to the ones identified in the present studies (section 4.6). Those similar microorganisms have elsewhere been reported to solubilize insoluble P sources *in vitro* (Reddy *et al*., 2002; Vassilev *et al*., 1997), as was similarly observed with the ones presently identified. Similarly, *Acinetobacte*r spp. has been reported to solubilize insoluble phosphate by production of gluconic acid (Ogut *et al*., 2010). *Acinetobacter nosocomialis* and *Acinetobacter baumanii* identified in the present studies also have been reported to solubilize insoluble rock phosphates (Peix *et al*., 2009). Dwivedi *et al*. (2004) reported that pre-plant inoculation of rice seedlings with P-solubilizing *Aspergillus awamori* in a field experiment in India resulted in yield increases as compared to un-inoculated seedlings. Rashid *et al*. (2004) reported on the ability of *Aspergillus flavus* to solubilize rock phosphate in vitro, amount of soluble P released was 0.1417%. Xiao *et al*. (2009) reported on the potential of *Stenotrophomonas maltophilia* in solubilize insoluble P in NBRIP growth medium by production of gluconic acid. Vassilev *et al*. (2006) described the potential of *Acinetobacter baumanii* as biological control of most of soil borne pathogens synthesis and release of pathogen-suppressing metabolites including siderophores, phytohormones, and lytic enzymes which are also involves in phosphate solubilization mechanisms. Therefore, there exists potential to use the microorganisms identified in the present study to develop inoculants and to apply them in the field. So far no P inoculants of identified fungal or bacteria strains have been develop and reported in the literature.

Table 12: Identity of isolated RP-solubilizing species to base on nucleotide identity at GeneBank species

Isolate	Species	Accession Number	Nucleotide Identity	Country	Source
B25	*Stenotrophomonas maltophilia*	KU726005	1095/1095 (100%)	Bulgaria	Seed coat of *Solanum lycopersium*
B35	*Bacillus safensis*	KX694275	1305/1305 (100%)	Dubai	Date palm oil
B18	*Acenotobacter nosocomialis*	LC014122	1353/1353 (100%)	Japan	Rice seeds
B21	*Acenotobacter baumanii*	KX242271	222/222 (100%)	India	Gut
F13	*Aspergillus stellifer*	AB248984	575/575 (100%)	Japan	Industrial area
F14	*Aspergillus tamarii*	KP784375	604/604 (100%)	Brazil	Coffee beans
F17	*Aspergillus flavus*	HQ340108	600/600 (100%)	Portugal	*Zea mays* field bulk soil
F19	*Aspergillus terreus*	KC119206	620/620 (100%)	India	
F22	*Aspergillus brunneoviolaceus*	FR727129	540/540(100%)	Czesh Republic	Industrial material

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

All microorganisms isolated were effective, to different extents, in solubilizing both Panda Hill and Minjingu RP, giving possibilities for field use of some isolates in solubilizing applied insoluble RP. Some isolates were able to efficiently solubilize both phosphate rocks while other isolates were more efficient on the phosphate rocks of their geographical origin. Therefore, widespread use of a particular isolate cannot be assumed always.

Microorganisms were diverse in terms of their morphology; most bacterial isolates were creamy to white coloured, and there were represented members of cocci and rods groups as well as Gram positive and Gram negative phosphate solubilizing bacteria. Fungi colours were green, grey, white and brown with deep brown and black reverse containing long conidiophores with presence of conidia.

Bacterial isolates were identified to be *Stenotrophomonas maltophilia*, *Bacillus safensis*, *Acenotobacter nosocomialis* and *Acenotobacter baumanii* and fungi were *Aspergillus stellifer, Aspergillus tamarii, Aspergillus flavus, Aspergillus terreus and Aspergillus brunneoviolaceus*.

The most efficient isolates were fungal isolates *Aspergillus stellifer*, *Aspergillus tamarii* and *Aspergillus flavus* and bacterial isolates *Bacillus safensis*, *Acenotobacter baumanii* and *Stenotrophomonas maltophilia*.

5.2 Recommendations

In the view of the results obtained herein, the following are recommended:

i. In view of the fact that fungal isolates *Aspergillus stellifer*, *Aspergillus tamarii* and *Aspergillus flavus* and bacterial isolates *Bacillus safensis*, *Acenotobacter baumanii* and *Stenotrophomonas maltophilia* were the most potential candidates for P bio-fertilizer production, further studies be undertaken, including field studies, that will lead to production of inoculants.

ii. Due to difference of isolate abilities to solubilize rock phosphate, a cocktail of compatible candidates could increase efficiency in solubilizing RP. Further research on these identified isolates' compatibility in a cocktail should be undertaken.

iii. Further studies should be undertaken to understand the mechanisms involved during RP solubilization by these isolates.

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APPENDICES

Appendix 1: Geochemical data taken by XRF Panda Hill showing composition of radioactive and important carbonatite metals

S/N	Time(Sec)	Units	Th	U	Nb	Ca	Al	P	Si	Mg
C1	0.14	%	0.007	< LOD	0.075	15.235	< LOD	2.179	18.087	< LOD
C2	29.94	%	0.019	< LOD	0.6	19.211	1.301	2.699	17.037	< LOD
C3	32.5	%	0.03	0.002	0.298	13.655	< LOD	2.304	28.669	< LOD
C4	26.17	%	0.04	0.007	0.368	9.058	0.962	1.461	27.36	< LOD
C5	28.25	%	0.025	< LOD	0.387	9.779	< LOD	2.355	6.424	< LOD
C6	26.98	%	0.013	0.005	0.153	8.31	< LOD	2.418	36.461	< LOD
C7	25.72	%	< LOD	< LOD	0.024	15.516	< LOD	4.813	18.464	< LOD
C8	27.52	%	0.024	< LOD	0.656	27.261	< LOD	1.984	8.083	< LOD
C9	27.12	%	< LOD	< LOD	0.051	23.003	< LOD	0.899	4.66	< LOD
C10	25.87	%	< LOD	< LOD	0.215	17.296	< LOD	2.223	14.848	< LOD

Appendix 2: Geochemical data taken by XRF showing Panda Hill heavy metals composition

S/N	Time (Sec)	Units	As	Au	Pb	Zn	Cu	Ni	Cd	Pd	Ag	Co	Fe	Mn	Cr	Ti
C1	30.14	%	0.004	< LOD	0.003	0.024	< LOD	0.026	< LOD	< LOD	< LOD	< LOD	14.786	0.38	< LOD	0.613
C2	29.94	%	< LOD	< LOD	0.009	0.018	< LOD	0.063	< LOD	< LOD	< LOD	< LOD	11.198	0.434	< LOD	< LOD
C3	32.5	%	< LOD	< LOD	0.011	0.013	0.01	0.02	< LOD	< LOD	< LOD	< LOD	9.275	0.201	< LOD	0.171
C4	26.17	%	< LOD	< LOD	0.045	0.027	0.03	< LOD	< LOD	< LOD	< LOD	< LOD	12.847	0.196	< LOD	0.491
C5	28.25	%	0.029	< LOD	< LOD	0.04	< LOD	0.073	< LOD	< LOD	< LOD	< LOD	18.322	0.283	< LOD	< LOD
C6	26.98	%	< LOD	< LOD	< LOD	0.02	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	5.883	0.253	< LOD	0.187
C7	25.72	%	< LOD	< LOD	< LOD	0.012	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	8.809	0.303	< LOD	< LOD
C8	27.52	%	< LOD	< LOD	0.016	0.021	< LOD	0.045	< LOD	< LOD	< LOD	< LOD	8.831	0.882	< LOD	< LOD
C9	27.12	%	< LOD	< LOD	< LOD	0.029	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	9.544	1.509	< LOD	< LOD
C10	25.87	%	< LOD	< LOD	< LOD	0.015	< LOD	0.049	< LOD	< LOD	< LOD	< LOD	8.5	0.788	< LOD	< LOD

< LOD – Less than limit of detection by XRF

Appendix 3: Sequence results of bacterial isolates

B2==>CTTGCTACTGGACCTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCTAATACCGCAT
ACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGA
CGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG
GCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGATAATACCTAGAGA
TAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAA
GCGCGCGTAGGCGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATGGTA
GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGTGCGAAAGCA
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGAT
AAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAAC
GCGAAGAACCTTACCTGGCCTTGACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCGAGTAATGTCGGGAACTTTAAGGATACTGCCAGTGACA
AACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACACAGCG
ATGTGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCC
GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGT

B3==>AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTT
TACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGA
TCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAA
CTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGC
CCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGG
GGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT
AAGCAT

B4==>CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAA
AGAAATCCAGCCGGCTAATACCTGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCG
GAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAG
GGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGC
CTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCT
TGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC
CTTACGGCCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCA
ACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGC
ACCAGAAGC

B5==>AGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCG
CGGCGCATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAAC
AAGTGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATT
ATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGA
GTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAG
CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGAC
ATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCAAGACCGCAAGGTTTAGCCAATCCCATAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAG
T

Appendix 4: **Sequence results of fungal isolates**

F1==>AGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCTGCCTCCGGGCGCCCAACCTCCCACCCGTGAATA
CCTAACACTGTTGCTTCGGCGGGGAGCCCTCTCGGGGGCGAGCCGCCGGAGACCACTGAACTTCATGCCTGTAGTGATGAGTCTGAGCCTAAATGAAAATTTAGTC
AAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAA
CGCACATTGCGCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTTCAAGCCCGGCTTGTGTGTTGGGTCGTCGTCCCCCCCCGGGGGACGGG
CCCGAAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGATTAGGGCCGGCCGGGCGCCAGCCGGCGTCTCCAACCTTATTT
TTCTCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT

F2==>AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTA
ACCTTAGTTGCTTCGGCGGGCCCGCCTTTAAGGCCGCCGGGGGGCATCAGCCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTC
TGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAA
TTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGG
GTCGTCGTCCCCTCTTCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGG
CGCTTGCCGAACGCAAAACAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT

F3==>>AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGT
ACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCT
GAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAAT
TCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGG
TCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGC
GCTTGCCGAACGCAAATCAATCTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT

F4==>AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGTCTTTATGGCCCAACCTCCCACCCGTGACTATTG
TACCTTGTTGCTTCGGCGGGCCCGCCAGCGTTGCTGGCCGCCGGGGGGCGACTCGCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACATGAACCCTGTTCTGAAAG
CTTGCAGTCTGAGTGTGATTCTTTGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGT
GTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCTTCCGCTCCGTAGGC
CCGGCCGGCGCCCGCCGACGCATTTATTTGCAACTTGTTTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA

F5==>AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCTGGGTCCTTCGGGGCCCAACCTCCCACCCGTGCTTACCGTAC
CCTGTTGCTTCGGCGGGCCCGCCTTCGGGCGGCCCGGGGCCTGCCCCCGGGACCGCGCCCGCCGGAGACCCCAATGGAACACTGTCTGAAAGCGTGCAGTCTGAGT
CGATTGATACCAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGT
GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTCCCCTCCAGCCCCGCTGGTTGTTGGGCCGCGCC
CCCCCGGGGGCGGGCCTCGAGAGAAACGGCGGCACCGTCCGGTCCTCGAGCGTATGGGGCTCTGTCACCCGCTCTATGGGCCCGGCCGGGGCTTGCCTCGACCCCC
AATCTTCTCAGATTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT

Appendix 5: Possibilities for molecular identification of fungal and bacterial isolates with outstanding ability of solubilizing Minjingu and Panda hill Phosphate Rock

Sample	BLASTN	Accessetion No.	Nucleotide Identity	Country	Source
B4,B25	*Stenotrophomonas maltophilia* strain gc-N3	KU726005	1095/1095(100%)	Bulgaria	Seed coat of *Solanum lycopersium*
	Pseudomonas geniculata strain ZJY-693	KP282737	1095/1095(100%)	China	Crude oil
	Gammaproteobacterium bacterium S4 AAI	KT907027	1095/1095(100%)	USA	Flow cytometry sheath fluid
	Uncultured gamma proteobacterium	KM978218	1095/1095(100%)	Mexico	
B5,35	*Bacillus safensis*	KX694275	1305/1305(100%)	Dubai	Date palm oil
	Bacillus pumilus	KT371465	1305/1305(100%)	Turkey	soil
	Bacillus altitudinis	LT221254	1305/1305(100%)	Pakistan	Saline Lake soil
	Bacillus invictae	Kt720238	1305/1305(100%)	USA	Viking space craft teflon ribbon surfaces
	Endophytic bacterium SV 715	KP757595	1305/1305(100%)	USA	Deep water horizon oil spill
B2,B18	Uncultured bacterium clone 958	KT386363	1353/1353 (100%)	China	Rice seeds
	Acenotobacter nosocomialis	LC014122	1353/1353 (100%)	Japan	
	Acenotobacter sp.	JQ863378	1353/1353 (100%)	China	Tunnel slugde
	Bacterium P2-20-9	HM583879	1353/1353 (100%)	Colombia	Sols from former garbage camp
	Acenotobacter calcoaceticus	KT878384	1353/1353 (100%)	China	Petrochemical waste water
B3,21	*Acenotobacter baumanii* strain OS 5.1	KX242271	222/222 (100%)	India	Gut
	Acenotobacter sp RRNFB-6	KU531586	222/222 (100%)	China	
	Bacterium QAT11	KU354252	222/222 (100%)	China	Mucus and tissue of *Galaxea fascularis*
	Bacillus Sp RFANFB-5	KT725621	222/222 (100%)	China	coe rumen fluid
	Uncultured bacterium clone SYN201307-75	Kx508903	222/222 (100%)	China	Rain water

Sample	BLASTN	Accessetion No.	Nucleotide Identity	Country	Source
	Aspergillus stellifer	AB248984	575/575(100%)	Japan	
	Aspergillus variecolor	EF652571	532/532 (100%)	USA	
	Emericella appendiculata	AB248997	575/576 (99%)	Japan	
	Emericella qingixianii	AB249008	575/576 (99%)	Japan	
F2,F14	*Aspergillus tamarii 122 strain*	KP784375	604/604 (100%)	Brazil	Coffee beans
	Aspergillus caelatus	JQ676205	582/582 (100%)	China	
	Aspergillus spp. BAB 5683	KX160452	568/568 (100%)	India	Plant leaves
	Asperillus oryzae SVP 01	KP256849	595/604 (99%)	India	
	Aspergillus parasiticus- voucher RIFA 68A	KF 624769	595/604 (99%)	USA	Soil
F3,F17	Uncultured Aspergillus genome DNA	HG936504	600/600(100%)	Germany	*Zea mays* field bulk soil
	Aspergillus flavus MUM 10.220	HQ340108	600/600 (100%)	Portugal	
	Fungal endophyte	FJ 378069	600/600 (100%)	China	Air
	Aspergillus minIslerotigenes	JX292091	600/600 (100%)	Morroco	White pepper
	Aspergillus oryzae	KX527867	600/600 (100%)	China	Water reservor
F4,F19	*Aspergillus terreus* strain KAML04	KC119206	620/620(100%)	India	
	Aspergillus terreus strain Wb464	AF455426	611/611(100%)	India	
	Aspergillus Sp. BAB-2916	KM066553	591/591(100%)	India	
	Aspergillus hortai strain CBS 124230	KP987087	619/620(99%)	Neitherlands	
	Aspergillus alabamensis	KP 987071	618/621(99%)	India	Soil
F5,F22	*Aspergillus brunneoviolaceus*	FR727129	540/540(100%)	Czesh Republic, Prague	Industrial material
	Fungal sp. SNB-CN119	KJ023746	538/538(100%)	France	Termite cuticle
	Aspergillus aculeatus strain A1.9	EU833205	582/582(100%)	Mexico	leaf litter from cave
	Aspergillus japonicus strain VIT-B1	KC128815	580/582(99%)	India	
	Aspergillus fijiensis strain ATCC 20611	KU729079	564/564(100%)	USA	

Appendix 6: Bacterial isolate performance on qualitative experiment

No.	Isolate	Description	Diameter of clear zone (mm) in different media		
			PVK	SMM+MRP	SMM+PRP
1	B17	Minjingu overburden	5	2	2
2	B35	Soft MRP	10	10	7
3	B13	Soft MRP	-	-	-
4	B21	Soft MRP	25	10	5
5	B11	Soft MRP	10	5	3
6	B16	Soft MRP	15	6	5
7	B9	Soils in contact with MRP	9	9	8
8	B1	Hard MRP	17	10	9
9	B6	Minjingu overburden	5	2	1
10	B27	Minjingu overburden	9	6	5
11	B22	Minjingu overburden	4	5	3
12	B7	Hard MRP	-	-	-
13	B36	Minjingu overburden	-	-	-
14	B15	Hard MRP	9	5	5
15	B39	Minjingu overburden	7	5	4
16	B40	Minjingu overburden	25	16	10
17	B25	Panda Hill RP	20	12	9
18	B8	Panda Hill RP	17	10	5
19	B40	Panda Hill RP	25	16	10
20	B19	Soils far from Panda Hill RP	5	3	2
21	B12	Composite soil of Panda Hill	20	12	10
22	B20	Panda Hill RP	12	9	5
23	B37	Soils far from Panda Hill RP	-	-	-
24	B23	Composite soil of Panda Hill	15	12	9

No.	Isolate	Description	Diameter of clear zone (mm) in different media

			PVK	SMM+MRP	SMM+PRP
25	B24	Panda Hill RP	15	7	7
26	B29	Panda Hill RP	12	12	10
27	B38	Panda Hill RP	8	7	5
28	B10	Composite soil of Panda Hill	15	8	5
29	B28	Panda Hill RP	7	6	4
30	B34	Composite soil of Panda Hill	7	6	4
31	B31	Panda Hill RP	9	3	2
32	B9	Panda Hill RP	15	12	10
33	B2	Panda Hill RP	7	6	5
34	B3	Composite soil of Panda Hill	11	11	10
35	B2	Soils far from Panda Hill RP	2	1	1
36	B1	Panda Hill RP	3	2	1
37	B10	Composite soil of Panda Hill	15	10	8
38	B26	Panda Hill RP	-	-	-
39	B33	Panda Hill RP	11	10	9

Appendix 7: Fungal isolate performance on qualitative experiment

No.	Isolate	Description	Diameter of clear zone (mm) in different media		
			PVK	$SMM^{+}MRP$	$SMM^{+}PRP$
1	F13	Soils far from Minjingu	26	24	33
2	F8	Soils far from Minjingu	35	10	22
3	F7	Soils in contact with MRP	39	24	22
4	F16	Soils far from Minjingu	40	34	32
5	F20	Soils in contact with MRP	42	24	25
6	F12	Soils far from Minjingu	-	-	-
7	F4	Soils in contact with MRP	43	44	17
8	F3	Soils far from Minjingu	45	15	24
9	F19	Soils in contact with MRP	47	20	30
10	F21	Soils in contact with MRP	50	40	38
11	F10	Soils far from Minjingu	-	-	-
12	F22	Soils far from Minjingu	50	35	31
13	F2	Composite soil of Minjingu	40	50	30
14	F17	Minjingu overburden	53	32	30
15	F23	Soils in contact with MRP	55	34	33
16	F18	Soils far from Panda Hill RP	34	25	20
17	F1	Soils far from Panda Hill RP	43	35	35
18	F5	Soils far from Panda Hill RP	40	40	22
19	F6	Composite soil of Panda hill	58	33	30
20	F14	Soils far from Panda Hill RP	55	46	30
21	F11	Soils far from Panda Hill RP	-	-	-
22	F9	Panda Hill RP	-	-	-