Prospects of *Bacillus spp.* as consortium in bio-fertilizer for phosphate solubilization

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Glossary Acronyms

ATP	Adenosine Tri Phosphate
mg	Microgram
ml	Microliter
µM	Micromolar
dNTP	Deoxyribonucleotide triphosphate
DMSO	Dimethylsulfoxide
EDTA	Ethylene Diamine Tetra Acetic Acid
L	Litre
LB	Luria Bertani
m	Milli
M	Molar
Min	Minute
NADPH	Nicotinamide Adenosine Diphosphate(H)
NCBI	National Center For Biotechnology Information
NEB	New England’s Biolabs
OD	Optical Density
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
Rpm	Revolutions Per Minute
RT	Room Temperature
Tm	Melting Temperature
Ta	Annealing Temperature
UV	Ultraviolet
EDTA	Ethylenediaminetetraaceticacid
TE	Tris EDTA
DDW	Double Distilled Water
16S rRNA	Svedberg’s unit ribosomal Ribonucleic Acid
TCA	Tricarboxylic Acid

LB	Luria Bertani
ET	Electron transfer
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
g/l	gram per liter
PGPR	Plant Growth Promoting Rhizobia
PBAT	Polybutylene adipateterepthlate
MCC	Microcrystalline cellulose

List of Tables

List of Figures

Table of Contents

ABSTRACT

The matter of degrading agricultural land resource and their desertification has been a subject of concern all over the world. This has led a peer pressure on farmers to increase agricultural production within a limited space. So, for these many years the use of chemical fertilizers has been extensive in the agricultural community which can even double the production. But these fertilizers are also associated with serious health and environmental problems like soil pollution. Chemical fertilizers are also responsible for degrading the quality of soil by increasing acidity, lacking soil micronutrients eventually leading to desertification of soil. It is known that soil naturally contains several microorganisms including bacteria and fungi which are beneficial to plant. They are associated with the plant growth and its productivity. Many of them; especially bacteria are responsible for providing the essential macronutrients like nitrogen, phosphate, sulfur etc. by converting their insoluble forms into soluble forms. Phosphate is the second most important key nutrient required by plants. It plays a key role in plant growth and development. On average, soil contains 400–1000 mg kg−1of total P, of which only 1.00–2.50% is available to plants for uptake. Phosphorus is found in soil in both mineral and organic forms. Most of the organic phosphate (20 to 80%) has been found to be inert. This study is an approach to learn the effects of phosphate solubilizing bacteria on insoluble phosphate and as a bio-fertilizer by integrating it with residual biochar. The bacteria used in this study was isolated and indentification was done by morphological, biochemical and molecular techniques. Thus isolated bacteria was subjected to phosphate solubilization test and its effects as biochar on plants. Our study finds out that the isolated bacteria of *Bacillus s*pp. have certain ability to solubilize insoluble phosphate present in hydroxyapatite into soluble forms. The concentration of soluble phosphate in the media was found in average of 7.93µg/ml in case of *B. subtilis* and 13.4µg/ml in case of *B. megaterium*. These bacteria along with others when integrated with biochar showed positive results on plant development. Our study also suggests that biochar could be used as artificial soil in marginalized land and deserted areas.

Keywords: *Soil macronutrients, phosphate, soil microorganisms, bio-fertilizer, residual biochar, hydroxyapatite, artificial soil*

CHAPTER 1

INTRODUCTION

1.1 Background

Currently, our world is facing threats from nature in the form of global warming, pollution, overpopulation etc. and from the mankind as well in the form of wars and political atrocity. According to United Nations, “The current world population of 7.3 billion is expected to reach 8.5 billion by 2030, 9.7 billion in 2050 and 11.2 billion in 2100” (World Population Prospects: The 2015 Revision, 2015) . Nevertheless, the rapid increase in human population would increase further anthropogenic activities that would possibly result in greater greenhouse gas emission that could highly contribute to global warming, pollution and food scarcity as well. Overpopulation has also increased demand for resources such as clean drinking water, food, housing, energy and over exploitation of these resources can cause serious impacts on the environment.

Scarcity of food has led a huge pressure on the agricultural community to increase the production in a limited land resource. This has led to the extensive use of chemical fertilizers whose hazardous effects are now continuing to emerge. The ill effects of excessive chemical fertilizer include leaching out, polluting water basins, destroying flora and fauna including friendly organisms, making the crop more susceptible to the attack of diseases, reducing the soil fertility and thus causing irreparable damage to the eco system (Rodríguez H. a., Phosphate solubilizing bacteria and their role in plant growth promotion, 1999). Although the use of chemical fertilizers has been credited earlier with nearly fifty percent increase in agricultural production but they are closely associated with environmental pollution and health hazards (Gaur A. C., Phosphate solubilizing microorganisms-An overview, 1999). Major strategy till date to maintain and improve agricultural productivity is exclusively through the use of chemical fertilizers. Many synthetic fertilizers contain acids, such as sulfuric acid and hydrochloric acid, which tend to increase the acidity of the soil, reduce the soil's beneficial organism population and interfere with plant (Rodriguez H. T., 2004)growth and is thought to reduce soil fertility. Thus the global necessity to increase agricultural production from a steadily decreasing and degrading land resource base has placed considerable strain on agro ecosystems (Tilak, 2005). Also due to excessive use of chemical fertilizers the quality of agricultural land is degrading day by day converting them to marginalized land.

Generally, for example; healthy soil contains enough nitrogen-fixing bacteria to fix sufficient atmospheric nitrogen to supply the needs of growing plants. However, continued use of chemical fertilizers may destroy these nitrogen-fixing bacteria. Furthermore, chemical fertilizers may affect plant health. For example, citrus trees tend to yield fruits that are lower in vitamin C when treated with synthetic fertilizer (Subba-Rao, 1993). Lack of trace elements in soil regularly dosed with chemical fertilizers is not uncommon that makes for plants to be devoid of trace elements (. Ranjan, 2003) This lack of vital micronutrients can generally be attributed to the use of chemical fertilizers because of which the soil becomes highly less fertile. On the other hand biofertilizer augments nutrients in soil as they can mineralize micronutrients embedded in soil (Gerhardson B. , 2002). Taking consideration of bio-fertilizers as potential substitutes for chemical fertilizers it can be envisaged that the environmentally friendly biotechnological approaches could offer alternatives to chemical fertilizers (Dobbelaere, 2003). Given the negative environmental impacts of chemical fertilizers and their increasing costs, the use of PGPB (Plant Growth Promoting Bacteria) is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture(Welbaum, 2004).

1.1.1 Phosphate Availability in the Soil

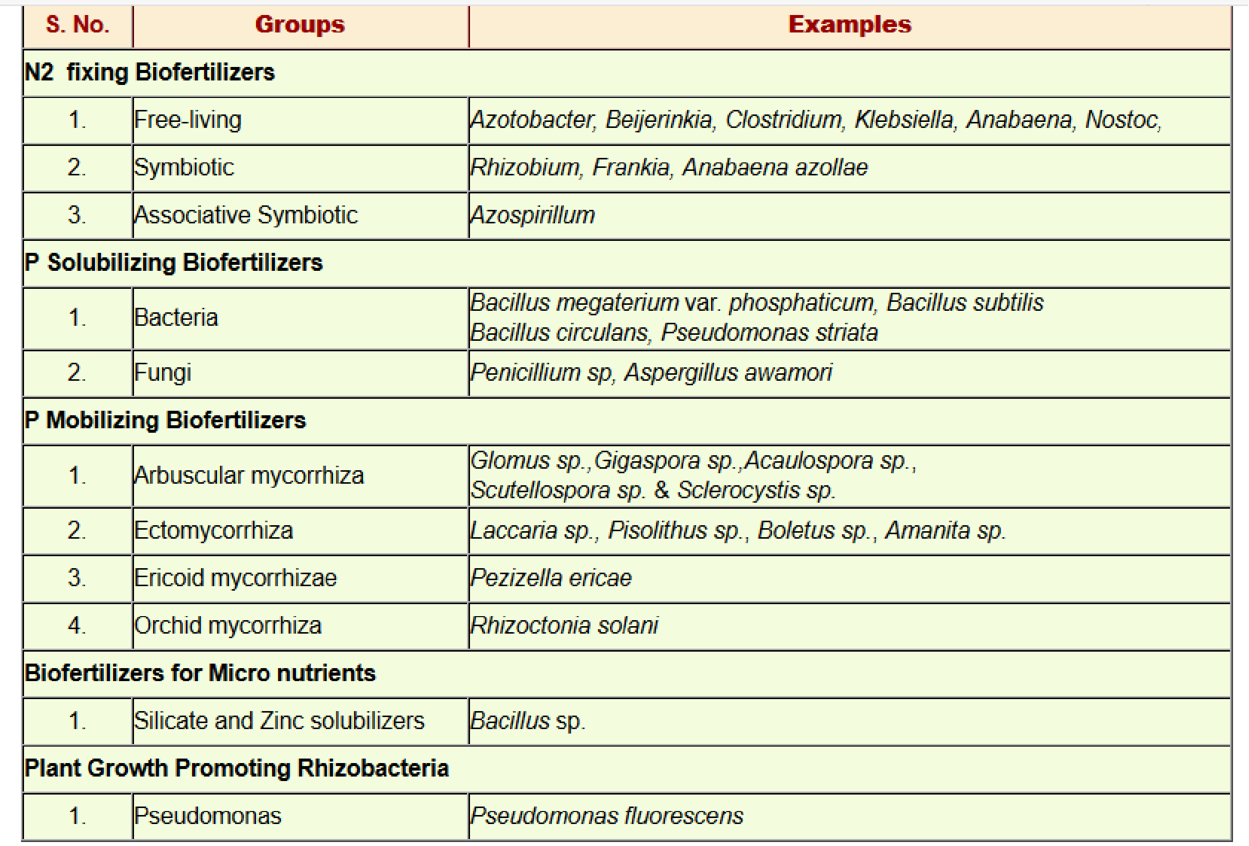
Among the applied fertilizers Phosphorus (P) is one of the major fertilizers because P is essential macronutrients for biological growth and development,(Kramer, 1980) especially in energy transfer. It is present at levels of 400–1200 mg/kg of soil (Fernández C. R. N., 1988). However, the concentration of soluble phosphorus in soil is usually very low, normally at levels of 1 ppm or less than 1ppm (Goldstein A. H., 1994). Although the cell might take up several phosphorus forms but the greatest part is absorbed in the forms of Phosphate (Dobbelaere, 2003)

In general, the soil P is in organic and inorganic phosphates. However, a large proportion is present in insoluble forms, and therefore, not available for plant as nutrition. Organic matter is an important reservoir of immobilized P that accounts for 20–80% of soil P (Richardson A. E., 1994). In addition, the applied P reacts to the soil minerals and the inorganic P occurs in soil mostly in insoluble mineral complexes,(Rodriguez H. T., 2004) some of these appearing after the application of chemical fertilizers. These precipitated forms cannot be absorbed by plants. It has been estimated that in some soil up to 75% of applied phosphate fertilizer may become unavailable to the plant because of mineral phase re-precipitation (Sudhakara, 2002). Hence, phosphate solubilizing bacteria (PSB) are opted as an alternative and have therefore been used to enhance the solubilization of re-precipitated soil phosphate for crop improvement(Young, 1990) due to their ability to convert insoluble phosphates into soluble forms (Viverk, 2001).

1.2.1 Biofertilizer:

Biofertilizer are substances which contain living microorganisms which, when applied to seeds, plant surfaces or soil, colonize the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). Use of bio-fertilizers is one of the important components of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture.(Ali Malboobi, et al., 2009) Several microorganisms and their association with crop plants are being exploited in the production of bio-fertilizers. They can be grouped in different ways based on their nature and function.

Table 1.1 Different Groups of biofertilizers along with their respective examples



S. No.	Groups	Examples
N2 fixing Biofertilizers		
1.	Free-living	*Azotobacter, Beijerinkia, Clostridium, Klebsiella, Anabaena, Nostoc,*
2.	Symbiotic	*Rhizobium, Frankia, Anabaena azollae*
3.	Associative Symbiotic	*Azospirillum*
P Solubilizing Biofertilizers		
1.	Bacteria	*Bacillus megaterium* var. *phosphaticum, Bacillus subtilis* *Bacillus circulans, Pseudomonas striata*
2.	Fungi	*Penicillium sp, Aspergillus awamori*
P Mobilizing Biofertilizers		
1.	Arbuscular mycorrhiza	*Glomus sp.,Gigaspora sp.,Acaulospora sp.,* *Scutellospora sp.* & *Sclerocystis sp.*
2.	Ectomycorrhiza	*Laccaria sp., Pisolithus sp., Boletus sp., Amanita sp.*
3.	Ericoid mycorrhizae	*Pezizella ericae*
4.	Orchid mycorrhiza	*Rhizoctonia solani*
Biofertilizers for Micro nutrients		
1.	Silicate and Zinc solubilizers	*Bacillus* sp.
Plant Growth Promoting Rhizobacteria		
1.	Pseudomonas	*Pseudomonas fluorescens*

(Source: http://agritech.tnau.ac/org_farm/orgfarm_biofertilizers.html)

1.2.2 Role of Phosphate Solubilizing Bacteria as Biofertilizer

Biofertilizers are organisms that enrich the nutrient quality of soil. The main sources of biofertilizers are bacteria, fungi, and cyanobacteria (blue-green algae). Plants have a number of relationships with fungi, bacteria, and algae. After the introduction of chemical fertilizers during the last century, farmers were happy of getting increased yield in agriculture. But slowly chemical fertilizers started displaying their ill-effects which cause serious adverse effects to the human as well as environmental health. The principle behind this strategy is that microbes have various abilities which could be exploited for better farming practices. Some of them help in combat diseases while some have the ability to degrade soil complex compounds into simpler forms which are utilized by plants for their growth. They are extremely beneficial in enriching the soil by producing organic nutrients for the soil. To convert insoluble phosphates to a form accessible to the plants, like orthophosphate, is an important trait for a microorganism for increasing plant yields. Microbes having the ability to dissolve appreciable amount of phosphates is not rare. Some of them are already used as commercial biofertilizers for agricultural improvements (Rodríguez H. a., Phosphate solubilizing bacteria and their role in plant growth promotion, 1999).The use of microbial products has certain advantages over conventional chemicals: They are considered safer than many of the chemicals now in use; they do not accumulate in the food chain; the target organisms seldom develop resistance as is the case when chemical agents are used; and biofertilizing agents are not considered harmful to ecological processes or the environment.

1.2 Current Studies:

Since soil is the manifestation of weathered rocks with supplemented carbon sources mineralization of marginalized land, supplemented with biochar could possibly mimick soil composition. Elements like nitrogen (N), phosphorus (P) and potassium (K) plus magnesium (Mg), are the minerals that plants need in the greatest quantities and as the concentration of these minerals in the soil is rapidly diminishing an NPK-based fertilizer is generally used in very large quantity worldwide to accelerate plant nutrient absorption (https://www.statista.com/statistics/438967/fertilizer-consumption-globally-by-nutrient). Meanwhile biochar is the basis or the integrity of the fertilizer because its porous surface provides enough surface area for the bacteria to flourish and function. Biochar also acts as the storehouse of the accumulated nutrients and as a water reservoir since it can hold water up to 5 times its own weight(Brady, 2008) .This would effectively prevent eutrophication in downstream water system. In addition, biochar would also act as carbon sequestration for long term storage of carbon in the soil.

Intensive research into manifold aspects involving the pyrolysis/biochar platform is underway around the world. From 2005 to 2012, there were 1,038 articles that included the word "biochar" or "bio-char" in the topic that had been indexed in the ISI Web of Science (Verheijen, et al., 2014). Further research is in progress by such diverse institutions around the world as Cornell University, the University of Edinburgh, University of Georgia, the Agricultural Research Organization (ARO) of Israel, Volcani Center, where a network of researchers involved in biochar research (iBRN, Israel Biochar Researchers Network) was established as early as 2009, and the University of Delaware (https://www.ed.ac.uk/geosciences/facilities/biochar) Long-term effect of biochar on soil C sequestration of recent carbon inputs has been examined using soil from arable fields in Belgium with charcoal-enriched black spots dating >150 years ago from historical charcoal production mound kilns. The lower specific mineralization and increased C sequestration of recent C with charcoal are attributed to a combination of physical protection, C saturation of microbial communities and, potentially, slightly higher annual primary production. Overall, this study provides evidence of the capacity of biochar to enhance C sequestration in soils through reduced C turnover on the long term (Maria C. Hernandez-Soriano, 2015). Biochar sequesters carbon (C) in soils because of its prolonged residence time, ranging from several years to millennia. In addition, biochar can promote indirect C-sequestration by increasing crop yield while, potentially, reducing C-mineralization. Laboratory studies have evidenced effects of biochar on C-mineralization using 13C isotope signatures.(Kerre et al, 2016). Fluorescence analysis of the dissolved organic matter from soil amended with biochar revealed that biochar application increased a humic-like fluorescent component, likely associated with biochar-carbon in solution. The combined spectroscopy-microscopy approach revealed the accumulation of aromatic-carbon in discrete spots in the solid-phase of microaggregates and its co-localization with clay minerals for soil amended with raw residue or biochar upon biochar application.

A process developed by University of Florida researchers that removes phosphate from water, also yields methane gas usable as fuel and phosphate-laden carbon suitable for enriching soil (https://eponline.com/articles/2011/05/18/biochar-more-effective-cheaper-at-removing-phosphate-from-water.aspx). Researchers at The University of Auckland also working on utilizing biochar in concrete applications to reduce carbon emissions during concrete production and to improve the strength considerably.(Akhtar, 2018)

1.3 Objectives of the Study:

1.3.1 General Objectives:

To isolate potent *Bacillus spp.* for its study as consortium in biofertilizer for phosphate solubilization.

1.3.2 Specific Objectives

- To isolate the phosphate solubilizing bacteria.
- To perform Gram staining and other biochemical tests for the biochemical identification of the isolated bacteria.
- To extract the genomic DNA and perform PCR for the molecular identification of the bacterial isolates.
- To perform phosphate solubilization test.
- To perform cellulose degradation test.
- To perform pot trial and syntrophic growth.

1.4 Research Hypothesis

1.4.1 Null Hypothesis:

The isolated *Bacillus spp.* will not solubilize the phosphate when used as biofertilizer as in consortium.

1.4.2 Alternative Hypothesis:

The isolated *Bacillus spp.* will solubilize the phosphate when used as biofertilizer as in consortium.

1.5 Rationale of the Study

The study provides an approach about how to tackle with the problem of global warming along with the problems arisen by it; low agricultural yield with even scarce land. The objective of this study is to reduce consumption of chemical phosphate fertilizer and recycling the soil of marginalized land by creating formula for biofertilizer that brings together i)marginalized land soil, ii) useful soil bacteria, and iii) biochar in a well-proportioned and compatible consortium. If this study could be replicated and trials on large scales could be performed; the dependency of chemical fertilizer could be drastically decreased thus contributing to the national and global economy too.

CHAPTER 2

LITERATURE REVIEW

2.1 Phosphorus:

Phosphorus is one of the most important nutrient for the growth of plants and found abundantly in our nature; mostly in soil. It occurs both in organic and in inorganic forms(Corbridge, 1995). The organic form being insoluble and inorganic form being soluble, However,the abundancy of organic form is higher and plants are not able to take these insoluble phosphorus for their growth.(Chen, et al., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities, 2006). The phosphorus cycle is the biogeochemical cycle that describes the movement of phosphorus through the lithosphere, hydrosphere, and biosphere. Phosphorus gradually becomes less available to plants over thousands of years, because it is slowly lost in runoff. Low concentration of phosphorus in soils reduces plant growth, and slows soil microbial growth (B.L, 2003)

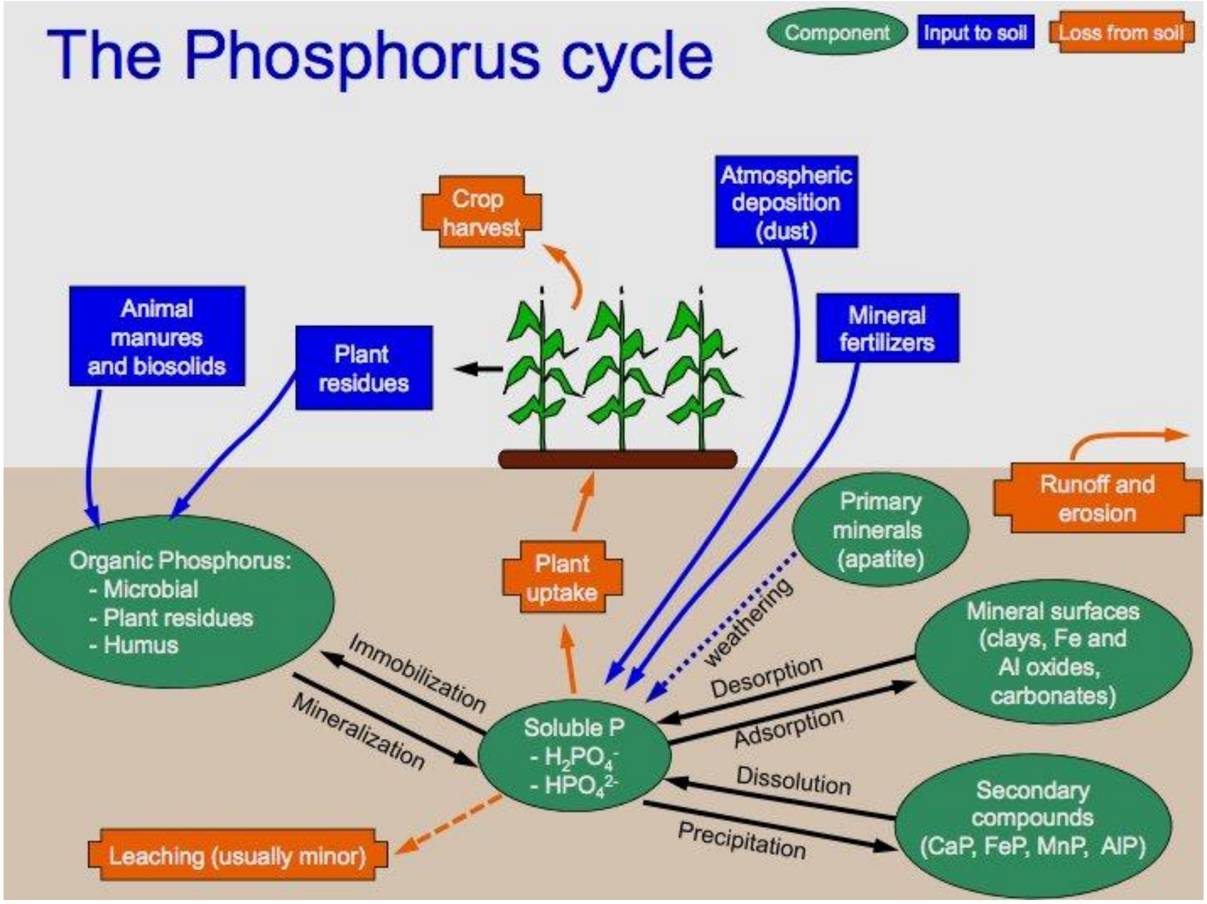


Figure 2.1: The Phosphorus Cycle on land

One of the most abundant organic forms of phosphorous is phytic acid as phytate (salts of phytic acid) and is not readily available to plants because it either forms a complex with cations or adsorbs to various soil components. (Singh & Satyanarayan, 2010). The predominant form of organic phosphorus are phytate (inositol hexa- and penta-phosphates), which constitutes upto 60 % of soil organic phosphorus. This organic form is poorly utilized by plants. Mineral forms of phosphorus are represented in soil by primary minerals, such as apatite, hydroxyapatite, and oxyapatite. They are found as part of the stratum rock and their principal characteristic is their insolubility. In spite of that, they constitute the biggest reservoirs of this element in soil because, under appropriate conditions, they can be solubilized and become available for plants and microorganisms.A large portion of soil and rhizosphere microorganisms is able to utilize phytate as carbon and phosphorus source. Mineral phosphate can be also found associated with the surface of hydrated oxides of Fe, Al, and Mn, which are poorly soluble and assimilable. This is characteristic of ferralitic soils, in which hydration and accumulation of hydrated oxides and hydroxides of Fe takes place, producing an increase of phosphorus fixation capacity (Fernandez, 1988).

Some specific growth factors that have been associated with phosphorus are:

- Stimulated root development
- Increased stalk and stem strength
- Improved flower formation and seed production
- More uniform and earlier crop maturity
- Increased nitrogen N-fixing capacity of legumes
- Improvements in crop quality
- Increased resistance to plant diseases
- Supports development throughout entire life cycle

Source (https://www.cropnutrition.com/efu-phosphorus)

2.2 Phosphates:

Phosphates are the naturally occurring form of the element phosphorus, found in many phosphate minerals. There are two components of phosphates in soil: organic and inorganic phosphates. A large proportion is present in insoluble forms, and therefore, not available for plant nutrition. Inorganic phosphate occurs in soil, mostly in insoluble mineral complexes, some of these appearing after the application of chemical fertilizers. These precipitated forms cannot be absorbed by plants. Organic matter, on the other hand, is an important reservoir of immobilized Phosphorus that accounts for 20–80% of soil P. On average, soil contains 400–1200 mg kg−1of total P, of which only 1.00–2.50% is available to plants for uptake (Fernández C. R. N., 1988).Phosphorus is found in soil in both mineral and organic forms. Most of the organic P (20 to 80%) has been found to be inert.

Mineral phosphate in the soil may become unavailable because of:

- Phosphorus fixation
- Adsorption in clay soil
- Precipitation reactions with cations such as Ca-P and Mg-P in alkaline soil or Fe-P and Al-P in acidic soil

As a result, the concentration of mineral phosphate in the soil solution rarely is at levels of 1 ppm or less then 1ppm (Goldstein A. H., 1994)

2.3 Organic Phosphate and its solubilization

A second major component of soil P is organic matter. Organic forms of P may constitute 30–50% of the total phosphorus in most soils, although it may range from as low as 5% to as high as 95% (Paul and Clark, 1988).) Organic P in soil is largely in the form of inositol phosphate (soil phytate). It is synthesized by microorganisms and plants and is the most stable of the organic forms of phosphorus in soil, accounting for up to 50% of the total organic P(Dalal, 1977). Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters. Of the total organic phosphorus in soil, only approximately 1% can be identified as nucleic acids or their derivatives. Various studies have shown that only approximately 1–5 ppm of phospholipids phosphorus occurs in soil, although values as high as 34 ppm have been detected. Large quantities of xenobiotic phosphonates, which are used as pesticides, detergent additives, antibiotics, and flame retardants, are released into the environment. These C-P compounds are generally resistant to chemical hydrolysis and biodegradation, but several reports have documented microbial P release from these sources (Ohtake, 1996).

Organic phosphate solubilization is also called mineralization of organic phosphorus, and it occurs in soil at the expense of plant and animal remains, which contain a large amount of organic phosphorus compounds. The decomposition of organic matter in soil is carried out by the action of numerous saprophytes, which produce the release of radical orthophosphate from the carbon structure of the molecule. The organophosphonates can equally suffer a process of mineralization when they are victims of biodegradation (McGrath J. W. G. B., 1995) . The microbial mineralization of organic phosphorus is strongly influenced by environmental parameters; in fact, moderate alkalinity favors the mineralization of organic phosphorus. The degradability of organic phosphorous compounds depend mainly on the physicochemical and biochemical properties of their molecules, e.g. nucleic acids, phospholipids, and sugar phosphates are easily broken down, but phytic acid, polyphosphates, and phosphonates are decomposed more slowly (McGrath J. W. F. H., 1998)

Phosphorus can be released from organic compounds in soil by three groups of enzymes:

a) **Nonspecific phosphatases**, which perform dephosphorylation of phospho-ester or phosphoanhydride bonds in organic matter.

b) **Phytases,** which specifically cause P release from phytic acid

c)**Phosphonatases and C–P Lyases,** enzymes that perform C–P cleavage in organophosphonates

The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil.

A large portion of soil and rhizosphere microorganisms is able to utilize phytate as carbon and phosphorus source. The ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. The use of phosphate solubilizing bacteria as inoculants increases the phosphorus uptake by plants. Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers. The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphates play a major role in the mineralization of organic phosphorous in soil(Rodríguez & Fraga, Phosphate solubilizing bacteria and their role in plant growth promotion, 1995).

2.4 Inorganic Phosphate and its Mineralization

Several reports have suggested the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein A. H., 1986). In two thirds of all arable soils, the pH is above 7.0, so that most mineral P is in the form of poorly soluble calcium phosphates (CaPs). Microorganisms must assimilate P via membrane transport, so dissolution of CaPs to Pi (H_2PO_4) is considered essential to the global P cycle. Evaluation of samples from soils throughout the world has shown that, in general, the direct oxidation pathway provides the biochemical basis for highly efficacious phosphate solubilization in Gram-negative bacteria via diffusion of the strong organic acids produced in the periplasm into the adjacent environment. Therefore, the quinoprotein glucose dehydrogenase (PQQGDH) may play a key role in the nutritional ecophysiology of soil bacteria. Bacteria may be used for industrial bioprocessing of rock phosphate ore (a substituted fluroapatite) or even for direct inoculation of soils as a 'biofertilizer' analogous to nitrogen-fixing bacteria. Both the agronomic and ecological aspects of the direct oxidation mediated MPS trait (Goldstein A. H., 2003)

Among the bacterial genera with this capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium and Erwinia.*(Goldstein A. H., 1987).

2.5 Mechanism of Phosphate Solubilization

A number of theories have been proposed to explain the mechanism of phosphate solubilization. Important among them are:

- Acid production theory
- Proton and enzyme theory

2.5.1 Acid Production Theory

According to this theory, the process of phosphate solubilization by PSM(Phosphate Solubilizing Microorganisms) is due to the production of organic acids which is accompanied by the acidification of the medium (Puente, 2004). A decrease in the pH of the filtrate from the initial value of 7.0 to a final value of 2.0 was recorded by (Gaur A. C., Phosphate solubilizing microorganisms-An overview, 1999). The analysis of culture filtrates of PSMs has shown the presence of number of organic acids such as malic, glyoxalic, succinic, fumaric, tartaric, alpha keto butyric, oxalic, citric, 2-ketogluconic and gluconic acid (Fasim, 2002) .The amount and type of the organic acid produced varied with the microorganism. The organic acids released in the culture filtrates react with the insoluble phosphate. The amount of soluble phosphate released depends on the strength and type of acid. Aliphatic acids are found to be more effective in P solubilization then phenolic acids and citric acids. Fumaric acid has highest P solubilizing ability. (Idriss, 2002).Tribasic and dibasic acids are also more effective than monobasic acids. In the presence of tribasic acids and dibasic acids, a secondary effect appears due to ability of these acids to form unionized association compounds with calcium thereby removing calcium from the solution and increasing soluble phosphate concentration (Halder A. K., 2005)

Organic acids contribute to the lowering of solution pH as they dissociate in a pH dependent equilibrium, into their respective anion(s) and proton(s). Organic acids buffer solution pH and will continue to dissociate as protons are consumed by the dissolution reaction (Welch, 2002). Similarly, microorganisms often export organic acids as anions (Netik, 1997)

Besides organic acids, inorganic acids such as nitric and sulphuric acids are also produced by the nitrifying bacteria and *Thiobacillu*s during the oxidation of nitrogenous or inorganic compounds of sulphur which react with calcium phosphate and convert them into soluble forms (Gaur and Gaind, 1999).

The most efficient mineral phosphate solubilization (MPS) phenotype in Gram negative bacteria results from extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase to gluconic acid (Gerhardson B. , 2002)The resulting pH change and reduction potential are thought to be responsible for the dissolution of phosphate in the culture medium.

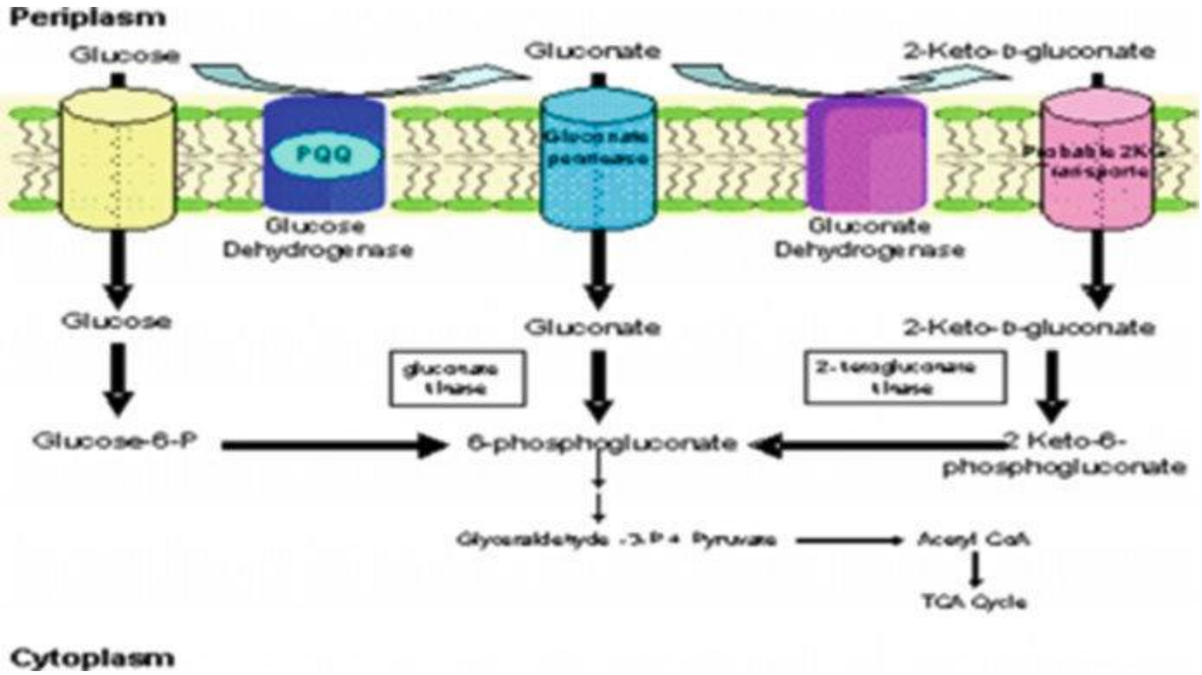


Figure 2.5.1: Production of gluconic acid via the alternative extracellular oxidation pathway of glucose metabolism.

(Source: http://www.ucc.ie/biomerit/simon%20image.gif)

2.5.2 Proton and Enzyme Theory

Esterase type enzymes are known to be involved in liberating phosphorus from organic phosphatic compounds. PSMs (phosphate solubilizing microorganisms) are also known to produce phosphatase enzyme along with acids which cause the solubilization of P in aquatic environment(Alghazali, 2010). (Illmer, 2015) reported that out of the four efficient phosphates solubilizing microbes, *Penicilliumaurathiogriseum, Penicillliumsimplicissimum, Aspergillus niger and Pseudomonas sp,* only*A .niger*could produce orgainic acid. Two most probable explanations for this are:

Solubilization without acid production is due to the release of protons accompanying respiration or ammonium assimilation.

More solubilization occurs with ammonium salts than with nitrate salts as the nitrogen source in the media (Kasahara, 2003).

Besides these two mechanisms the production of chelating substances(Luo, 1993) H_2S mineral acids, siderophores (Bossier et al., 1988) biologically active substances like indole acetic acids, gibberllines and cytokinins (Kucey et al., 1988) are also correlated with

Phosphate solubilization. Chelation involves the formation of two or more coordinate bonds between an anionic or polar molecule and a cation, resulting in a ring structure complex(Whitelaw, 2000). Organic acid anions, with oxygen containing hydroxyl and carboxyl groups, have the ability to form stable complexes with cations such as $Ca^{2+,}$ $Fe^{2+,}$ $Fe^{3+,}$ and $Al^{3+,}$ that are often bound with phosphate in poorly forms (Zehnder, 2001)

Dissolution of phosphate in soil is a very important process for plant growth. Several studies have shown that the phosphate uptake by plants can be markedly increased by either mycorrhizal fungi or inoculation of soil with species capable of solubilizing free phosphate, such as (Uzair, 2006)

2.6 Phosphate –Plant Interaction

Phosphorus is one of one of the major plant nutrient limiting plant growth. It plays a key role in nutrition of plants as it promotes development of deeper roots. The average soil is rich in phosphorus as it contains about 0.05% (w/w) phosphorus (Fernández C. R. N., 1988) but only one tenth of this is available to plants approximately 95–99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants and due to its poor solubility and chemical fixation in the soil (Gaur A. C., 1999)causing a low efficiency of soluble P fertilizers. To increase the availability of phosphorus for plants, large amounts of fertilizer is used on a regular basis. But after application, a large proportion of fertilizer phosphorus is quickly transferred to the insoluble forms. Therefore, very little percentage of the applied phosphorus is used, making continuous application necessary.(Fernández C. R. N., 1988)

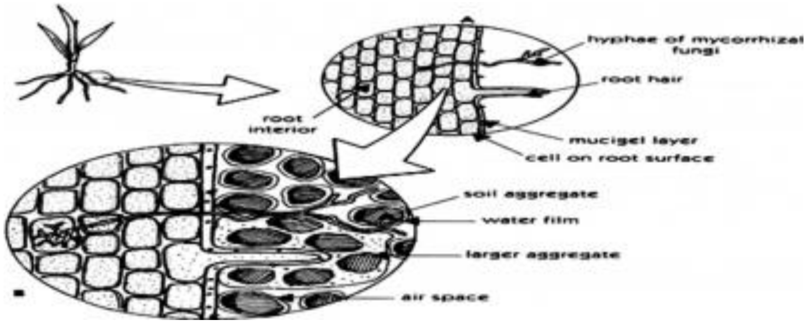


Figure 2.6: Soil and root interactions

(http://www.sare.org/publications/bsbc/fig3_3.jpg)

Soils microorganisms are involved in a range of processes that affect Phosphate transformation and thus influence the subsequent availability of phosphate to plant roots (Richardson A. P., 2001). Free living phosphate solubilizing microorganisms (PSM) are always present in soils. The populations of inorganic Phosphate solubilizing microorganisms are sometimes very low, less than 10^2 CFU g^{-1} of soil as observed in a soil in Northern Spain (Peix, 2001).As observed with other soil microbes the number of PSM is more important in the rhizosphere than in non-rhizosphere soil and the number of phosphate solubilizing bacteria is more important than that of fungi(Ranjan, Plant hormones: Action and Applications, 2003) However, inoculation studies aimed to improving P nutrition in plants involved bacteria and fungi, and is commercially available in Western Canada as the phosphate inoculant Jumpstart . They are sold for wheat, canola, mustard and other legumes and contain bacterial strain of *Penicillium bilaii.* (http://www.philombios.ca/).

2.7 Phosphate fertilizer:

Phosphate fertilizer supplement the deficient amount of phosphate in the soil. It can be commercially produced through chemical processes or by the use of natural raw products.

2.7.1 Rock phosphate (apatite)

Deposits of rock phosphate form naturally in geological formations. It is harvested, ground to powder and added to soil as a source of phosphorus. Rock phosphate still has limited use in domestic cultivation and is refined as an ingredient in other advanced fertilizers. It has phosphate content upto 15-20%

2.7.2 Single Super phosphate

Normal super phosphate is a stronger and more soluble compound than rock phosphate, but it is significantly less potent than concentrated super phosphate or ammonium compounds. The newer concentrated compound contains 46% phosphate, while the normal variety has 20%. Neither of the two supplies nitrogen, a desirable nutrient found in other fertilizers.

2.7.3 Granular triple super phosphate:

Triple super phosphate is made by the action of phosphoric acid on raw rock phosphate (also called appatite). It typically contains 46% phosphate, soluble in neutral ammonium citrate and water. Of this, typically 90% is soluble in water alone. Because the phosphate on TSP is never completely water-soluble, it cannot be used in the manufacture of clear-liquid fertilizers.

2.7.4 DAP (Diammonium phosphate)

Diammonium phosphate fertilizer is a plant food that is rich in the mineral phosphorus. It contains 53-46% of phosphate. Some farmers may choose to blend this type of plant food with other fertilizer forms to meet the specific needs of their crops and soils. It may be applied using a variety of methods before, during, or after crops have been planted.

(http://www.aithercommodities.com/commodities/fertilizers/phosphate-fertilizers)

The first commercial Phosphate mineral fertilizer was single superphosphate (SSP), followed by triple superphosphate (TSP). Both fertilizers are excellent P sources but their use declined when other P fertilizers became available. Artificial phosphate fertilization is necessary because phosphorus is essential to all life organisms, natural phosphorus-bearing compounds are mostly insoluble and inaccessible to plants, and the natural cycle of phosphorus is very slow. Fertilizer is often in the form of superphosphate of lime, a mixture of calcium dihydrogen phosphate ($Ca(H_2PO_4)_2$), and calcium sulfate dihydrate ($CaSO_4{\cdot}2H_2O$) produced reacting sulfuric acid and water with calcium phosphate(N Ahmed, 2009).

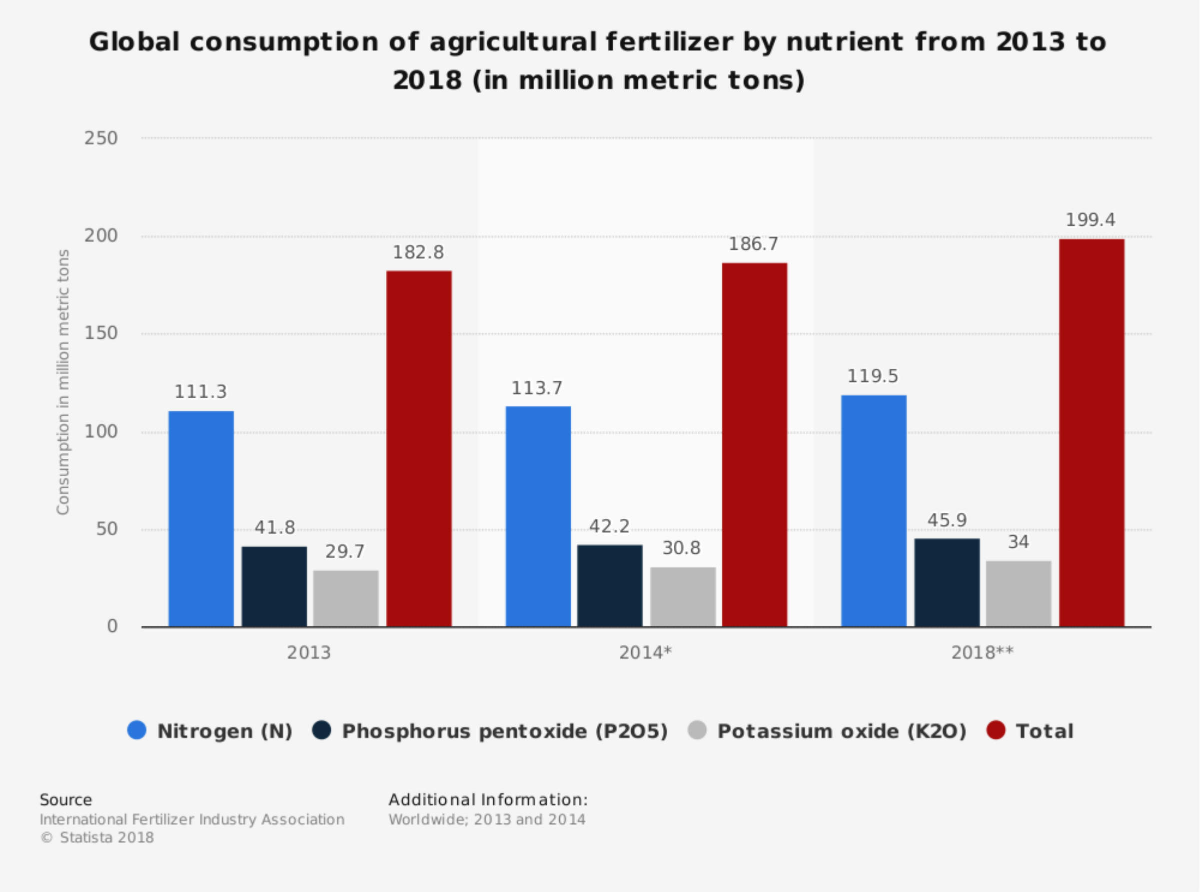


Figure 2.7.1: Global consumption of agricultural fertilizer by nutrient from 2013 to 2108

(Source:https://www.statista.com/statistics/438967/fertilizer-consumption-globally-by-nutrient/)

2.8 Hydroxyapatite

Hydroxyapatite (HA), is a naturally occurring mineral form of calcium apatite with the formula $Ca_5(PO_4)_3(OH)$. It is the hydroxylendmember of the complex apatite group. The OH^-ion can be replaced by fluoride, chloride or carbonate, producing fluorapatite or chlorapatite. It crystallizes in the hexagonalcrystal system. Pure hydroxyapatite powder is white. Naturally occurring apatites can, however, also have brown, yellow, or green colorations, comparable to the discolorations of dental fluorosis. Up to 50% by volume and 70% by weight of human bone is a modified form of hydroxyapatite, known as bone mineral. Carbonated calcium-deficient hydroxyapatite is the main mineral of which dental enamel and dentin are composed.

Hydroxyapatite can be synthesized by chemical process from bones of animals and fishes (Nasser A.M. Barakat, Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods, 2005). This product can be used as a source of phosphate to plants. Since phosphate given from other sources can be overflown by different means, it increases the phosphate content of the soil. Hydroxyapatite is a valuable resource that serves as an important agrochemical fertilizer, although this use is limited due to the relatively limited dissolution. Innovative techniques for production of hydroxyapatite particles that provide slow release of phosphorus may increase the use of this mineral for fertilizer applications. Recently, hydroxyapatite nanoparticles have been fabricated in controlled environments and shown efficacy as a slow-release fertilizer (Liu R, 2014). Another example of novel strategies for increasing the potential use of hydroxyapatite fertilizers was reported by Kottegoda et al(Kottegoda N, 2011)who generated urea-modified hydroxyapatite particles.(U. Shashvatt, 2017)

2.9 Phosphate Solubilizing Bacteria:

Biofertilizers are the substance which contain living microorganisms which, when applied to seeds, plant surfaces, or soil, colonizes the rhizosphere or interior of the plant and promotes the growth by increasing the availability of primary nutrients to host plant(Vessey, 2003). Biofertilizer often referred as Plant Growth Promoting Rhizobia(PGPR) improve the nutrient status of host plant by following ways:

- Aid in biological N2 fixation and phosphorus solubilization.
- Stimulate plant growth through the synthesis of growth-promoting substances.
- It can also provide protection against drought and some soil-borne diseases.
- Intensify the root surface area
- Increase the symbiosis process for the host which is beneficial (Pandey, Trivedi, Kumar, & Palni, 2006)

Phosphate solubilizing bacteria are beneficial bacteria capable of solubilizing inorganic phosphorus from insoluble compounds. The mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids, through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms(Chen, et al., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities, 2006)

PSB have been introduced to the Agricultural community as phosphate Biofertilizer.

The strains of bacteria that have been identified as PSB are:

- *Pantoeaagglomerans (P5),*
- *Microbacteriumlaevaniformans(P7)*
- *Bacillus megaterium*
- *Pseudomonas putida*
- *Bacillus subtilis* (Baas, et al., 2016)

2.10 Role of Phosphate Solubilizing Bacteria as Biofertilizer

Biofertilizers are organisms that enrich the nutrient quality of soil. The main sources of biofertilizers are bacteria, fungi, and cyanobacteria (blue-green algae). Plants have a number of relationships with fungi, bacteria, and algae. After the introduction of chemical fertilizers during the last century, farmers were happy of getting increased yield in agriculture. But slowly chemical fertilizers started displaying their ill-effects such as leaching out, polluting water basins, destroying flora and fauna including friendly organisms, making the crop more susceptible to the attack of diseases, reducing the soil fertility and thus causing irreparable damage to the eco system (Ranjan, Plant hormones: Action and Applications , 2003)

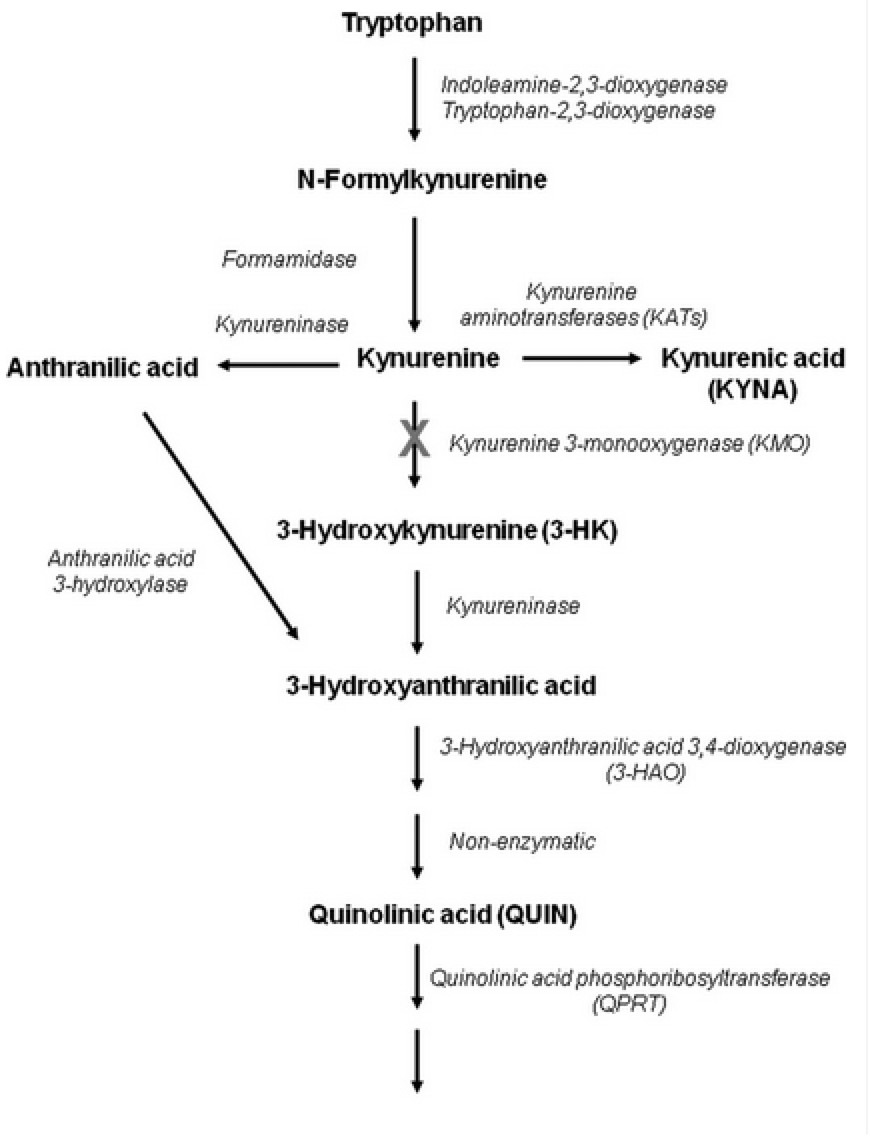
The principle behind this strategy is that microbes have various abilities which could be exploited for better farming practices. Some of them help in combat diseases while some have the ability to degrade soil complex compounds into simpler forms which are utilized by plants for their growth. They are extremely beneficial in enriching the soil by producing organic nutrients for the soil. To convert insoluble phosphates to a form accessible to the plants, like orthophosphate, is an important trait for a PGPB for increasing plant yields ((Halder A. K., 2000). Microbes having the ability to dissolve appreciable amount of phosphates is not rare. Some of them are already used as commercial biofertilizers for agricultural improvement. The use of microbial products has certain advantages over conventional chemicals: they are considered safer than many of the chemicals now in use; they do not accumulate in the food chain; the target organisms seldom develop resistance as is the case when chemical agents are used; and biofertilizing agents are not considered harmful to ecological processes or the environment.

According to Statistics, the worldwide transaction amount of fertilizer is roughly US$40 billion. Out of this, 135 million metric tons of chemical fertilizer is applied each year, with sales volume of about US$30 billion. Although there are no clear application statistics for biofertilizer, however, its sales volume is estimated to be as much as US$3 billion. Commercial biofertilizers claiming to undergo phosphate solubilization using mixed bacterial cultures have been developed. Examples of these are: ‘Phylazonit-M’ (permission at No. 9961, 1992, by the Ministry of Agriculture of Hungary), a product containing *Bacillus megaterium*; *Azotobacter chroococcum*, which allows an increase in N and P supply to the plants; and the product known as ‘KYUSEI EM’ (EM Technologies, Inc.)(Rodríguez & Fraga, Phosphate solubilizing bacteria and their role in plant growth promotion, 1995)

2.11 Bacillus megaterium

Bacillus megaterium is a gram positive, endospore forming, aerobic, rod shaped bacteria. It is considered as one of the largest of all bacteria having size of 4˟1.5µm. It is found in soil and considered a saprophyte(De Vos, 2009). Also recognized as an endophyte and potential agent for biocontrol of plant diseases. It can grow in simple media on more than 62 carbon sources out of 95 tested, including all tri-carboxylic acid cycle intermediates, formate and acetate (BACMAP genome Atlas, n.d.). Industrial applications of enzymes excreted by *B. megaterium* are diverse, starting from amylases used in bread industry to penicillin amidase, which is used for generation of new synthetic antibiotics. It is used for the production of pyruvate, vitamin B12, drugs with fungicidal and antiviral properties.(Vary, 2007) *. B. megaterium* is known to produce poly-γ-glutamic acid and the accumulation of the polymer is greatly increased in a saline (2–10% NaCl) environment, in which the polymer comprises largely of L-glutamate.(Shimizu, 2007)

B. megaterium is vitamin B12 (cobalamin) overproducer and doing this they also overproduce cysteine, riboflavin (vitamin B2), S-adenosylmethionine (SAM) which are critical in reactive oxygen species (ROS) stress management that is critical during augmented photosynthesis and also in developing pathogen resistance. Bacillus megaterium can grow in a media containing L-tryptophan as the sole carbon, nitrogen and energy source.Kyrunenine, anthranilic acid and catechol are the metabolic intermediates suggesting that this organism uses the anthranilic acid pathway for tryptophan degradation(Sadoff, 1975)



NAD^+

Figure 2.11: Anthranilic acid pathway

(Source:https://www.researchgate.net/figure/Major-metabolites-of-the-kynurenine-pathway-of-tryptophan-degradation-KMO-located-in-a_fig1_258280854)

2.12 Bacillus subtilis

Bacillus subtilis are rod-shaped, Gram-positive bacteria that are naturally found in soil and vegetation. They are stress resistant, endospore forming bacteria (Madigan M, 2005) having size of 4-10 micrometers μm in length and 0.25–1.0 μm in diameter(Yu AC, 2014) They also has fungicidal properties and forms preemptive biofilm colonization on the plant rhizome.(Nakano MM, 1998). It also replenishes soil nutrients and they can biodegrade hydrocarbons, stimulates the gene expression of natural defense mechanism in plants and animals.

It shows competitive inhibition and produce natural antibiotics that suppress pathogenic microorganisms .It has been reported that this Gram-positive soil bacterium is able to utilize nitrate, nitrite and urea in the absence of its preferred nitrogen sources like ammonium ions or glutamine. The metabolism of such compounds is tightly regulated and requires a large energy investment. Recombinant strains pBE2C1 and pBE2C1AB were used in production of polyhydroxyalkanoates (PHA), and malt waste can be used as their carbon source for lower-cost PHA production.(M, 2013). B. *subtilis* is known to have a symbiotic relationship with the *Azotobacter* species. These two work together to lower the levels of insoluble phosphorus within soil. Soils that have a history of applied fertilizer benefit from the symbiotic relationship because of the phosphorus build up. The phosphorus gets trapped within the soil and begins to freeze, because the phosphate ion tightly bonds to the positive cations found in the soil. B. *subtilis* works with this other species by helping to release the phosphate bonds and distribute the phosphate throughout the different plants throughout the entire growing season. Without the addition of B. *subtilis*, the phosphorus can't do its job effectively and further hinders agriculture. The phosphorus can't move around to the plants and help maintain prosperous growth. B. *subtilis* is a good alternative to insoluble phosphate fertilizers.(Dybiec, 2018). Monsanto has isolated a gene from *B. subtilis* that expresses cold shock protein B and spliced it into their drought-tolerant corn hybrid MON 87460, which was approved for sale in the US in November 2011.(Harrigan GG, 2009)

2.13 Carbon Catabolite repression:

Catabolite repression is the regulatory mechanism that allows the cells to choose among several available carbon sources. Usually, it involves regulation at the level of gene expression to prevent transcription of catabolic genes and operons (catabolite repression in a strict sense) as well as regulation at the level of protein activity to prevent uptake or formation of the specific inducers of catabolic operons (inducer exclusion)(Deutscher, 2008). Repression of the *Escherichia coli* lactose operon by glucose was the first example of gene regulation understood at the molecular level, and it is still the paradigm for carbon catabolite repression. However, the molecular mechanisms by which catabolite repression is achieved differ strongly between the different bacteria. In *E. coli*, inducer exclusion and transcription activation of catabolic genes by the complex of the Crp protein with cyclic AMP are crucial(Crasnier, 1996)

2.14 Carbon Catabolite Repression in *Bacillus subtilis:*

In contrast, in *B. subtilis* and most other Gram-positive bacteria, catabolite repression is exerted by the CcpA repressor protein. The Gram-positive bacterium *Bacillus subtilis* uses glucose as the preferred source of carbon and energy. Glucose-mediated catabolite repression is caused by binding of the CcpA transcription factor to the promoter regions of catabolic operons. CcpA binds DNA upon interaction with its cofactors HPr(Ser-P) and Crh(Ser-P).(Fujita, 2009) The formation of the cofactors is catalyzed by the metabolite-activated HPr kinase/phosphorylase. Recently, it has been shown that malate is a second preferred carbon source for *B. subtilis* that also causes catabolite repression. In this work, we addressed the mechanism by which malate causes catabolite repression. (Kleijn, 2010). Genetic analyses revealed that malate-dependent catabolite repression requires CcpA and its cofactors. Moreover, we demonstrate that HPr(Ser-P) is present in malate-grown cells and that CcpA and HPr interact *in vivo* in the presence of glucose or malate but not in the absence of a repressing carbon source. (Rudrappa, 2008). The formation of the cofactor HPr(Ser-P) could be attributed to the concentrations of ATP and fructose 1,6-bisphosphate in cells growing with malate. Both metabolites are available at concentrations that are sufficient to stimulate HPr kinase activity. The adaptation of cells to environmental changes requires dynamic metabolic and regulatory adjustments. The repression strength of target promoters was similar to that observed in steady-state growth conditions, although it took somewhat longer to reach the second steady-state of expression when cells were shifted to malate.

2.15 Biochar

Biochar is the biological residue like charcoal used as a soil amendment. It is a stable solid, porous, low density rich in carbon material and can endure in soil for thousands of years. Like most charcoal, biochar is made from biomass via pyrolysis of organic material like rice husk, wood chips or cattle dung. Biochar is under investigation as an approach to carbon sequestration and improvement of poorly degraded agricultural land(Lean, 2008). Biochar thus has the potential to help mitigate climate change via carbon sequestration(Balal Yousaf, 2016). Independently, biochar can increase soil fertility of acidic soils (low pH soils), slow release of nitrogen (N) and phosphorus (P), increased soil microbial biomass, and improved soil physical properties increase agricultural productivity, and provide protection against some foliar and soil-borne diseases. Studies have reported positive effects from biochar on crop production in degraded and nutrient–poor soils (*www.csiro.au.)* The application of compost andbiochar under FP7 project FERTIPLUS has had positive effects in soil humidity, and crop productivity and quality in different countries (http://sior.ub.edu/jspui/cris/socialimpact/socialimpact00544). Biochar can be designed with specific qualities to target distinct properties of soils.(Novak, 2009). In an Columbian savanna soil, biochar reduced leaching of critical nutrients, created a higher crop uptake of nutrients, and provided greater soil availability of nutrients(Julie, 2009). At 10% levels biochar reduced contaminant levels in plants by up to 80%.

Biochar is a high-carbon, fine-grained residue that today is produced through modern pyrolysis processes; it is the direct thermal decomposition of biomass in the absence of oxygen (preventing combustion), which produces a mixture of solids (the biochar proper), liquid (bio-oil), and gas (syngas) products. The specific yield from the pyrolysis is dependent on process condition, such as temperature, and can be optimized to produce either energy or biochar(Lehmann, Gaunt, & Rondon, 2006). Temperatures of 400–500 °C (673–773 K) produce more char while temperatures above 700 °C (973 K) favor the yield of liquid a nd gas fuel components(Winsley, 2007)

2.16 Features of biochar:

- The burning and natural decomposition of biomass and in particular agricultural waste adds large amounts of CO_2 to the atmosphere. Biochar is a stable way of storing carbon in the ground for centuries, potentially reducing or stalling the growth in atmospheric greenhouse gas levels; at the same time its presence in the earth can improve water quality, increase soil fertility, raise agricultural productivity, and reduce pressure on old-growth forests(Laird, 2008).
- Biochar is a carbon sink useful for long-term storage of carbon in soil
- Biochar increases water retention and nutrient storing capacity of the rhizosphere. Biochar is hygroscopic. Thus it is a desirable soil material in many locations due to its ability to attract and retain water. This is possible because of its porous structure and high surface area. As a result, nutrients, phosphorus, and agrochemicals are retained for the plants benefit. Plants are therefore healthier, and less fertilizer leaches into surface or groundwater.
- Biochar can improve water quality, reduce soil emissions of greenhouse gases, reduce nutrient leaching, reduce soil acidity, and reduce irrigation and fertilizer requirements.
- For plants that require high potash and elevated pH, biochar can be used as a soil amendment to improve yield.(Lehmann J. a., 2003)
- Biochar is the home and storehouse for the inoculant bacteria.
- Biochar reduces the acidity and salinity of soil and also maintains the pH.
- Biochar limits eutrophication by holding nutrients on itself.
- The pyrolysis of forest- or agriculture-derived biomass residue generates a biofuel without competition with crop production.
- Biochar is a pyrolysis byproduct that may be ploughed into soils in crop fields to enhance their fertility and stability, and for medium- to long-term carbon sequestration in these soils. It has meant a remarkable improvement in tropical soils showing positive effects in increasing soil fertility and in improving disease resistance in West European Soils.
- Biochar enhances the natural process: the biosphere captures CO especially through plant production, but only a small portion is stably sequestered for a relatively long time (soil, wood, etc.)(Cornet A., 2009)

CHAPTER 3

METHODOLOGY

3.1 Sample Collection

Samples were collected from Panchase region of Pokhara valley. Alltogether 9 types of samples from different places of Kaski district were collected.

3.2 Isolation of Bacteria

3.2.1 Isolation of Bacillus megaterium

0.5g soil sample was added to 2 ml of acetate base medium (broth) and placed in water bath at 80°C for 10 min. 100 ul of the medium was taken and mixed in soft agar (~ 42°C).The mixture was poured in plates with basal acetate agar medium. The plates were incubated at 30°C for 24-48 hour. After observing the colonies' characteristics the most probable colonies were sub-cultured first on tryptophan media and. The most probable isolates based on colony morphology from tryptophan media were then cultured on phenol media. The bacterial isolates which survived on phenol media were considered as putative *Bacillus megaterium .*

3.2.2 Isolation of *Bacillus subtilis*

0.5 g of each soil sample was taken in a test tube and mixed with 10 ml of sterile water. The samples were serially diluted upto 10^{-2}.Then the tubes were placed in water bath at 80°C for 10 minutes. The samples were inoculated on Nutrient agar medium using the spread plate technique and then to sodium acetate agar plates. All the plates were incubated at 30°C for 24-48 hours. Thus-formed colonies' characteristics were observed and the most probable colonies were sub-cultured first on catechol media. The most probable isolates from catechol media were then cultured on phenol media. The bacterial isolates which survived on phenol media were considered as putative *Bacillus subtilis.*

3.3 Biochemical Identification of bacteria

3.3.1 Gram's staining

Microorganisms were inoculated and smeared on sterile glass slides with an inoculating loop. The smear was allowed to air dry and then heat-fixed quickly. Crystal violet dye was flooded over the smear on each slide and kept for 1 min. The slides were washed with water and then Gram's iodine, a mordant, was flooded over the smear (1 min) after which a decolorizer, ethyl alcohol, was used to wash off the color. Finally, the second dye safranin was flooded over the slides and washed off after 1 min. Smears appearing to be predominantly blue or black were labelled as “Gram-positive,” and indicated the retention of crystal violet dye in a thick peptidoglycan layer of the cell wall. Predominantly pink or red smears were labelled “Gram-negative” because crystal violet was not retained due to a thin layer of peptidoglycan, contained within the outer membrane of the cell wall of gram negative bacteria.

3.3.2 MR-VP test

The two MR-VP medium were first labeled with the name of the microorganism, then the microorganisms were aseptically inoculated in each tube and incubated at 28°C. After 48 hr, 5-6 drops of MR reagent was added to the incubated tubes labeled MR and color change was observed.

For VP, Barritt’s reagent A and B in the ratio of 3:1 were added in the incubated tubes and shaken to provide oxygen. The color change was noticed after 20-30 min of incubation.

3.3.3 Citrate utilization test

Simmon’s citrate agar slants were prepared and labeled with the name of the microorganism. Then the microorganisms were aseptically inoculated on the surface of the medium in a zig-zag way. The tubes were incubated at 28°C for 24-48 hr and the color change in the medium was observed.

3.3.4 Catalase test

A drop of 3% H_2O_2 was placed on opposite ends of clean and grease-free glass slides. Using a sterile wooden stick (toothpick), a portion of the bacterial culture was transferred to the drop of 3% H_2O_2. Effervescence marked by bubbles was examined.

3.3.5 Oxidase test

A small portion of the bacterial culture was taken using a sterile wooden stick and rubbed over the oxidase disc (pre-soaked with 1% tetra methyl-p-phenylenediaminedihydrochloride). Development of purple or blue coloration was examined.

3.3.6 Urease Test

The both medium was inoculated with a loopful of culture of the test organism. The cap of the tube was left loosely and incubated at 35C fo 18-25 hours. Positive test indicate the change of color of the media from yellow to pink-orange.

3.3.7 TSIA (Triple Sugar Iron Agar) test

With a sterilized straight inoculation needle the top of a well-isolated colony was touched. The colony was inoculated in the TSI Agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The cap of the tube was left on loosely and incubated at 35°C in ambient air for 18 to 24 hour.So the expected results of TSI Agar test are:

1. Alkaline slant/no change in butt (K/NC) i.e Red/Red = glucose, lactose and sucrose non-fermenter
2. Alkaline slant/Alkaline butt (K/K) i.e Red/Red = glucose, lactose and sucrose non-fermenter
3. Alkaline slant/acidic butt (K/A); Red/Yellow = glucose fermentation only, gas (+ or -), H2s (+ or -)
4. Acidic slant/acidic butt (A/A); Yellow/Yellow = glucose, lactose and/or sucrose fermenter gas (+ or -), H2s (+ or -).

3.3.8 SIM (Sulphur Indole Motility) Test:

Isolated colonies from an 18-24 hour culture on solid media, SIM Medium was inoculated by stabbing the center of the medium to a depth of 1/2 inch. The medium was incubate the inoculated aerobically at 35ºC for 18-24 hours. After incubation, the tubes were observed for H_2 S production and motility. Once H_2 S and motility reaction have been read and recorded, three drops of Kovacs Reagent was applied to the surface of the medium. Development of a pink to red color was observed for positive result.

Interpretation of results:

A positive H_2S test is denoted by a blackening of the medium along the line of inoculation. A negative H_2S test is denoted by the absence of blackening.

A positive motility test is indicated by a diffuse zone of growth flaring from the line of inoculation.

A negative motility test is indicated by growth confined to the stab line.

A positive test for indole is denoted when a pink to red color band is formed at the top of the medium after addition of Kovacs Reagent. A yellow color denotes a negative indole test after addition of Kovacs Reagent.

3.4 DNA Extraction

Cells were streaked on LB agar plate and incubated overnight at 37°C.The single colony was isolated and inoculated in 2 ml of LB medium and incubated at 37°C for 12 hr at 200 rpm. From the overnight culture 1.5 ml was transferred to sterilized ependroff tubes and centrifuged at 5000 rpm for 5 min at 4°CThe supernatant was discarded by aspiration and the remaining overnight culture (0.5 ml) was added to the same tube containing cell pellet and centrifuged again at 5000 rpm for 5 min at 4°C. Whatever possible amount of the supernatant was removed without disturbing the pellet. The cell pellet was re-suspended in 450 ul of TE buffer by gentle pipetting. The solution was halved and transferred into two freshly sterilized Ependroff tubes by placing 225 ul of the above suspension in each tube. To each tube 180 ul of lysozyme (1mg/ml) was added. Both the tubes were incubated at 37°C for 30 min gently mixing the solution by inverting the tubes every 5 min for proper cell lysis. 45 ul STEP solution was added in both the tubes and incubated in ice for 45 min or until the solution became clear due to cell lysis with gentle inversion in between the ice incubation period. Equal volume of chilled phenol (450 ul) was added and mixed by vortexing. The mixture was centrifuged at 13000 rpm for 10 min. The upper aqueous layer containing DNA was transferred to freshly sterilized Ependroff tubes without carryover of the lower organic phase. Again equal volumes of chilled phenol: chloroform: isoamyl alcohol (25:24:1) was added to the above aqueous solution mixed by vortexing and centrifuged at 13000 rpm for 10 in at 4°C.After collecting the aqueous layer in a fresh tube equal volume of chloroform was added and vortexed.The mixture was then centrifuged at 13000 rpm for 2 min and aqueous phase was collected in a fresh eppendroff tube. To the aqueous solution (450 ul) containing the genomic DNA, 100 ul of 3M chilled sodium acetate pH 5.2 was first added and double that volume 95% ethanol (i.e. 1100 ul) was added and incubated at -20°C for 30 min. The mixture was then centrifuged at 13000 rpm for 20 min at 4°C. The supernatant was poured off and the pellet washed with 250 ul of 70% ethanol without disturbing the pellet. The solution was centrifuged at 13000 rpm for 10 min at 4°C. After draining the supernatant, the remaining ethanol was removed by keeping the tube open at room temperature for 5-10 min. Care was taken so as not to over-dry the DNA pellet. The genomic DNA was re-suspended in 100 ul of autoclaved milliQ water TE buffer pH 8.0 and stored at -20°C until use.

3.5 DNA quantification:

Isolated DNA from each sample was further quantified by an equipment called Nanodrop. The DNA concentration was measured in the unit ng/ul. The device also measured the optical density of the DNA sample at 260 and 280 and their ratio was directly calculated by the device. This ratio gives the purity of the extracted DNA samples. If the value of OD at 260/280 is more than 1.8, the sample is likely to have protein contamination and if the OD is less than 1.8 the sample is likely to have RNA contamination. The results were interpretated accordingly.

3.6 PCR (Polymerase Chain Reaction)

The polymerase chain reaction is a method for making many copies of a specific segment of DNA. It is an exponential process that proceeds as long as the raw materials for sustaining the reaction are available. The PCR can only replicate fairly small pieces of DNA. It uses enzymes to mass replicate a portion of a DNA strand for easier analysis, such as searching for genes of interest. The DNA to be amplified is mixed with deoxyribonucleotides, a thermostable enzyme called Taq polymerase and DNA primers in a vial.

The first step is denaturation i.e. heating the mixture to cause melting of the DNA. This breaks the hydrogen bonds between the two strands, forming single strands that are susceptible to copying. The next step is called annealing. The primers, which are custom-made, short DNA strands, are designed specifically to bond to sites at the beginning and end of the segment to be copied. The Taq polymerase synthesizes the complementary strands of DNA using the primers as the starting point. The final step in the cycle is called extension, where the temperature is raised again to separate the DNA strands and then lowered sufficiently to allow the primers to attach. Taq polymerase then synthesizes new strands of DNA and the cycle is repeated until sufficient DNA is produced. (Detail PCR works in Appendix 3)

3.7 Phosphate Solubilization test:

For this test, the bacterial strains were cultured on media containing Hydroxyapatite (HA) as the sole source of phosphate. The absorbance of the media was taken before the inoculation of the bacterial culture and on 7th, 14th and 21st days of incubation at 827nm.

Procedure:

40 ml of sample was pipetted into 50 ml graduated flask. To this, 8 ml of the mixed reagent was added and diluted to volume 50ml. Color was allowed to develop for 24 hours at room temperature (20°C). The optical density of the solution was measured at 827nm.

3.8 Cellulose Degradation test:

For this test, the bacterial strains were cultured on media containing different types of cellulose as the sole source of carbon. The culture was incubated at 28-30°C for 7 days. After incubation, the culture was subjected to Molisch's test for the detection of carbohydrates because it was assumed that the bacteria could degrade cellulose polymers present in the media to carbohydrates.

3.9 Syntrophic growth and Pot trial:

Syntrophic growth is a phenomenon in which one species lives off the products of another species. In this association, the growth of one partner is improved or depends on the nutrients, growth factors or substrate provided by the other partner. In this experiment, the syntrophic relation is observed between different types of bacteria where each of them have individual and specific function in enhancing to soil fertility. The artificial soil made from char and crusher dust provide appropriate habitat for the sustainence of the microbial life.From the biochemical identification, the most potent bacterial strains from our study and of our fellow thesis students were selected to be cultured in Nutrient broth. The species included *Bacillus subtilis*, *B. megaterium*, *B. mucilaginosus*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Geobacter*, *Shewanella*, *Frateuria*. These mixture of bacteria were mixed with crusher dust, biochar and limestone dust in the ratio of 2:1:1. This mixture was used as artificial soil for the growth of bonsai plants.

The ratio of bacterial strains used in the study is as follows:

Table 3.9: List and concentration of bacteria used in the study

Name of the bacteria	Concentration
a) *Azotobactervinelandii*	*x*
b) Psedomonasfluorescens	*x*
c) Lactobacillus lactis	*x*
d) Geobactersulfurredens	*x*
e) Bacillus subtilis	2x
f) Bacillus megaterium	*2x*
g) Shewanellaoneidensis	*2x*
h) Frauteriaaurentina	*4x*
i) Azospirillum spp.	*4x*
j) Bacillus mucilaginosus	*4x*

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and Identification of *Bacillus megaterium:*

Isolation of *Bacillus megaterium* was performed for its potential to solubilize insoluble phosphate(Chen, et al., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities, 2006); mainly its ability to pyruvate, vitamin B12 and mild acids such as poly-γ-glutamic acid.(Shimizu, 2007). The soil sample 0.5gm that was collected from different parts of Nepal was supplemented with 10 ml distilled water and mixed properly. Then, the solution was subjected to heat shock to kill non-spore forming vegetative cells as pasteurization is employed for such inhibition. (Fellows, 2017). Then, 100ul bacterial solution was added to 10 ml of warm top agar medium with sodium acetate as sole reduced carbon source and poured in media containing same composition as top agar medium. Sodium acetate was chosen as sole reduced carbon source as acetate is known to induce stress in bacteria (Leone S., 2015) and is fatal to those which cannot utilize it. Thus, the growth of spore formers and acetate utilizing bacteria would only grow in the medium and *Bacillus megaterium* is known to utilize acetate acetate (BACMAP genome Atlas, n.d.) and is spore forming. (De Vos, 2009).

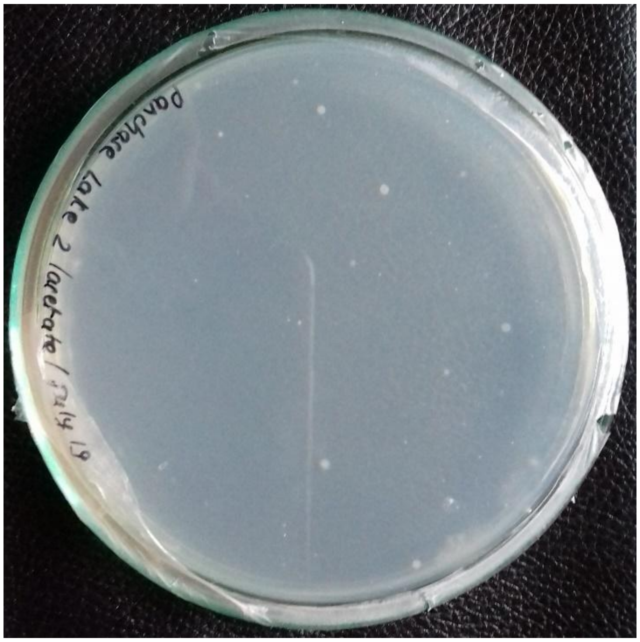


Fig 4.1.1: Culture of bacteria on sodium acetate agar medium

The fast growing colony was assumed to contain *Bacillus megaterium* in the sodium acetate medium (Fig 4.1.1). Then the isolates were further cultured in tryptophan based media where the DL-tryptophan was used as sole reduced carbon source as *Bacillus megaterium* is known to grow in L-tryptophan.(Sadoff, 1975). The isolates that survived in tryptophan media (Fig4.1.2) was further cultured in phenol as *Bacillus megaterium* is known to utilize this as sole reduced carbon source.(Hassan A. N, 2015) . The isolates that survived in this medium (Fig 4.1.3) were presumed as putative *Bacillus megaterium* isolates.

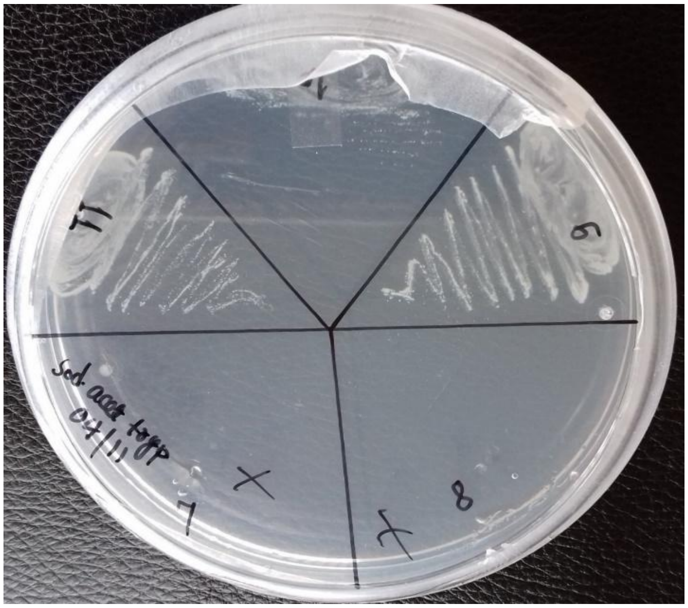


Fig 4.1.2: Culture of bacteria from sodium acetate agar media on tryptophan media

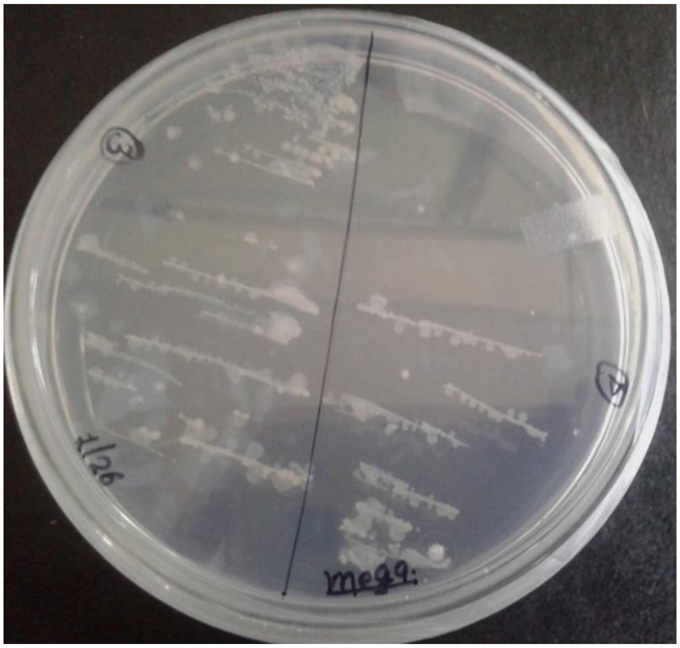


Fig: 4.1.3 Culture of bacteria from tryptophan media on phenol media

The concentration 0.05mM is maintained upon several hit and trials and this was the concentration in which the bacteria grew optimally. Further, the bacterial samples were subjected to various biochemical and molecular analysis.

4.1.1 Gram Staining and Biochemical tests:

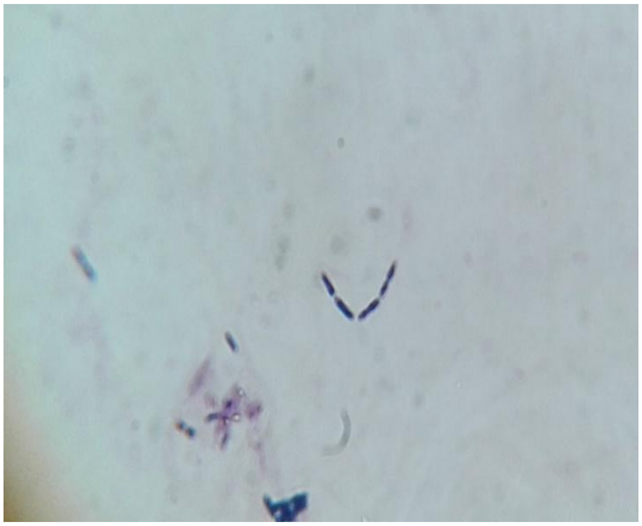


Fig 4.1.1.1: Gram positive rod bacteria

Gram staining is a technique used to differentiate bacterial species into two large groups: Gram-positive and Gram-negative; based on the chemical and physical properties of their cell walls(T.J., 2001). Since Bacillus species are Gram positive, all the Gram negative Bacterial samples were discarded and only Gram positive samples were further subjected to biochemical tests.

The putative bacterial isolates were further characterized by biochemical assay to screen respective bacteria as bacterial physiology differs from species to species. These differences in carbohydrate metabolism, fat metabolism, production of certain enzymes and acids, ability to utilize a particular compound etc. helps identifying by the biochemical tests. Thus all together 9 biochemical tests were performed and the isolates having the same biochemical tests to the preferred bacteria were further subjected to molecular tests.

Table 4.1.1.1: Results of Biochemical tests of putative *Bacillus megaterium*

SN	Tests	1	2	5	9	11	13	14	18	20	21	23
1.	Gram staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2.	Citrate	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve
3.	MR	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve
4.	VP	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
5.	TSIA	Y/Y	Y/Y	Y/Y	Y/Y	R/Y	R/R	Y/Y	R/Y	Y/Y	Y/Y	Y/Y
6.	Urease	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7.	Catalase	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve
8.	Sulphur	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve
9.	Indole	-ve	-ve	-ve	-ve	_ve	-ve	-ve	-ve	+ve	-ve	-ve
10.	Motility	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve

Table 4.1.1.2: Results of biochemical tests of putative *Bacillus megaterium*

S.N.	Tests	25	27	28	31	32	35
1.	Gram stain	+ve	+ve	+ve	+ve	+ve	+ve
2.	Citrate	-ve	-ve	+ve	-ve	-ve	-ve
3.	MR	+ve	+ve	-ve	+ve	+ve	+ve
4.	VP	-ve	-ve	-ve	-ve	-ve	+ve
5.	TSIA	Y/Y	Y/Y	R/Y	R/Y	Y/Y	R/R
6.	Urease	-ve	-ve	-ve	-ve	-ve	-ve
7.	Catalase	+ve	-ve	+ve	+ve	+ve	-ve
8.	Sulphur	+ve	+ve	-ve	+ve	+ve	+ve
9.	Indole	-ve	-ve	-ve	-ve	-ve	+ve
10.	Motility	+ve	+ve	+ve	-ve	+ve	-ve

Out of 40 samples isolated, 23 bacteria were Gram negative so they were discarded because we needed only Gram positive bacteria. The tables above are the biochemical tests performed on remaining 17 bacteria. Only 11 bacteria from the biochemical test were found to be likely as *Bacillus megaterium* by studying their biochemical reactions.

4.1.2 Genomic DNA Extraction and quantification *Bacillus megaterium:*

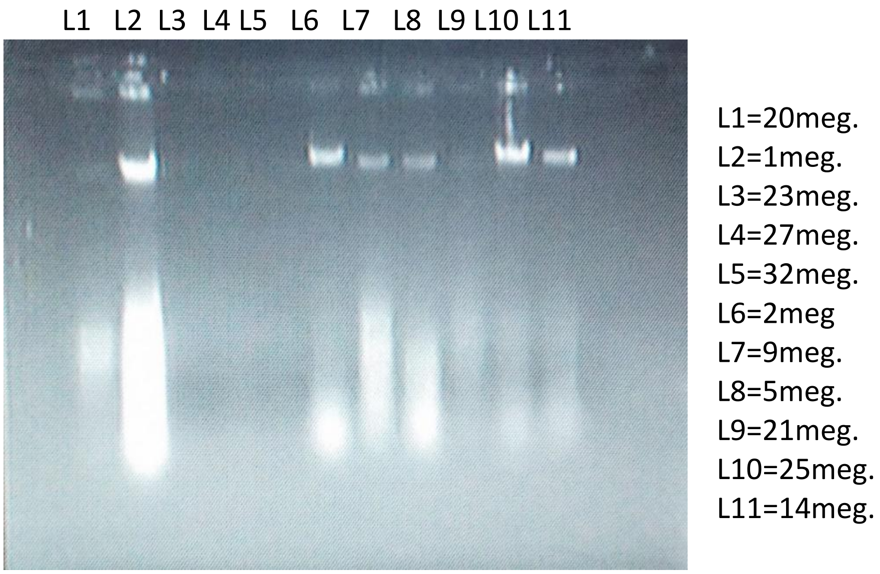


Fig: 4.1.1.2 Genomic DNA of putative *B. megaterium* on 0.8% Agarose gel electrophoresis

Genomic DNA extraction was done on 11 bacterial samples from which 6 samples showed good quality DNA bands. These 6 samples were further quantified by Nanodrop and amplified by PCR by using 16s universal primers.

Table 4.1.2: DNA concentration of isolated putative *Bacillus megaterium*

Sample	Nucleic acid concentration(ng/µl)	OD$_{260/280}$ ratio
1meg	256.12	1.38
2meg	235.39	1.75
9meg	178.53	1.72
5meg	175.05	1.68
25meg	209.86	2.01
14meg	193.68	1.89

The concentration of DNA of the samples were determined by Nanodrop. The highest DNA concentrations were found of the samples 1meg, 2meg, 25megsub and 14meg whose concentrations were 256.12, 235.31, 209.86 and 193.68 respectively. Most samples like 1meg, 2meg, 5meg, showed RNA contamination in their respective lanes at the bottom.

Their ratio of Optical density 260/280 were less than 1.8 which also proves point. RNA contamination might had occured because the enzyme RNase was not used. Protein contamination was observed in the samples 25meg, 14meg, 1meg. The ratio of Optical density 260/280 of samples 25meg and 14meg were more than 1.8 DNA. The reason behind protein contamination might be due to the fact that proteinase K, the enzyme used for protein denaturation might had not worked properly or there might be some handling error during the experiments. The extracted DNA samples were diluted and used for Polymerase Chain Reaction (PCR).

4.2 Isolation and Identification of *Bacillus subtilis:*

Isolation of *Bacillus subtilis* was done because of its capability to solubilize insoluble phosphate(Chen, et al., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities, 2006) and ability to utilize nitrate, nitrite and urea in the absence of its preferred nitrogen sources like ammonium ions or glutamine(an Dijl JM, 2013)The soil sample 0.5gm that was collected from Panchase of Nepal was supplemented with 10 ml distilled water and mixed properly. Then, the solution was subjected to heat shock to kill non-spore forming vegetative cells as pasteurization is employed for such inhibition. (Fellows, 2017). The bacterial solution was cultured in Nutrient agar medium for faster bacterial growth. The bacterial colonies from NA media were then transferred to medium with sodium acetate as sole reduced carbon source. Sodium acetate was chosen as sole reduced carbon source as higher concentration of acetate is known to inhibit cellular growth and induce stress in bacteria(Leone S., 2015) and is fatal to those which cannot utilize it. Thus, the growth of spore formers and acetate utilizing bacteria would only grow in the medium and *Bacillus subtilis* is known to utilize acetate (BACMAP genome Atlas, n.d.)and is spore forming(Tan S. I., 2013)

Fig: 4.2.1 Culture of bacteria from NA plate to sodium acetate agar medium

The bacterial colonies which survived in the acetate agar were further transferred into catechol media as *Bacillus subtilis* is known to utilize catechol as carbon source (Crawford R L, 1997) (figure 4.7). The bacterial isolates that survived in the catechol media were further cultured in phenol media because *B. subtilis* can utilize it in smaller concentrations.(Hassan A. N, 2015) . Higher concentration causes protein denaturation. The bacterial isolated that survived in phenol media too were assumed as putative *Bacillus subtilis*.

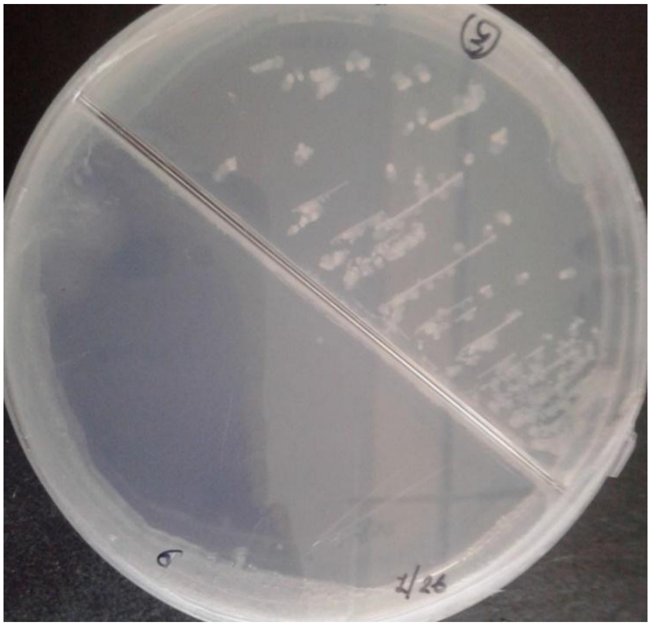


Fig 4.2.2: Culture of bacteria from sodium acetate agar plate on catechol media

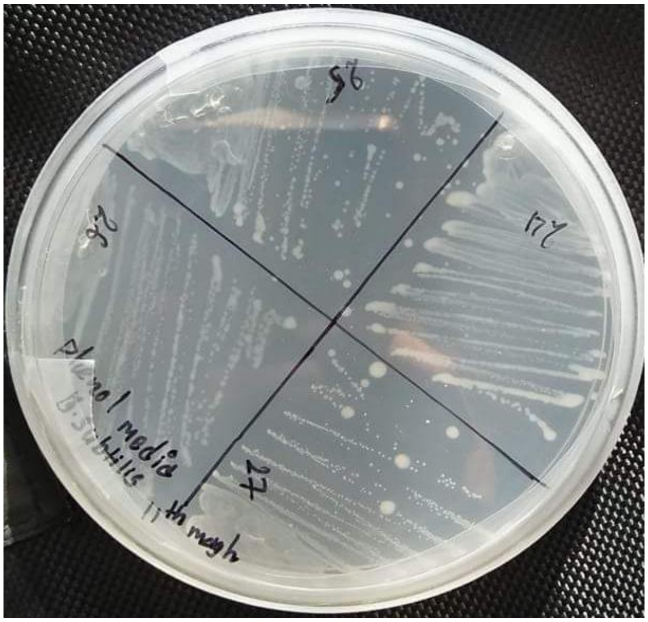


Fig 4.2.3 Culture of bacteria from catechol media on phenol media

Isolation of both the phosphate solubilizing bacteria were done on the basis of carbon catabolite repression. Phenol, Catechol and tryptophan which are known to be growth inhibitors were degraded by the bacteria which is suggested by their capability to grow in media containing these compounds. Phenols are generally produced during the breakdown of cellulose and lignocellulosic compounds. Since compost includes various cellulosic compounds which are remained and not degraded. Thus, it can be inferred that these bacteria can be used in the maturation of organic compost.

4.2.1 Gram staining and Biochemical tests:

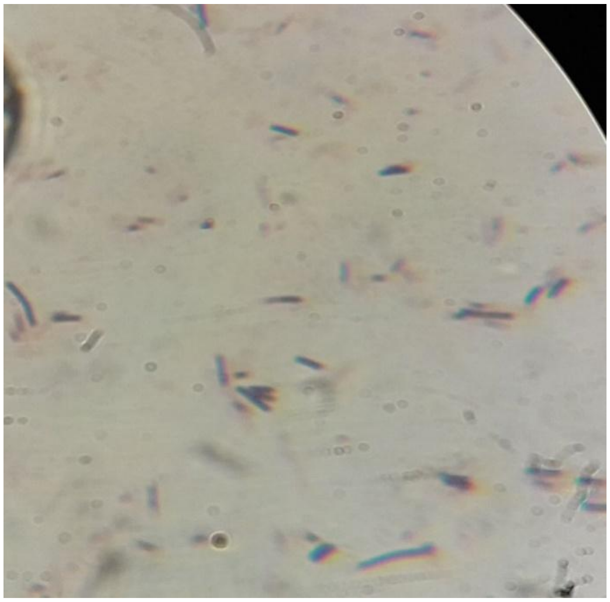


Fig 4.2.1.1: Gram positive rod bacteria

Table 4.2.1.1: Results of Biochemical tests of putative *Bacillus subtilis*

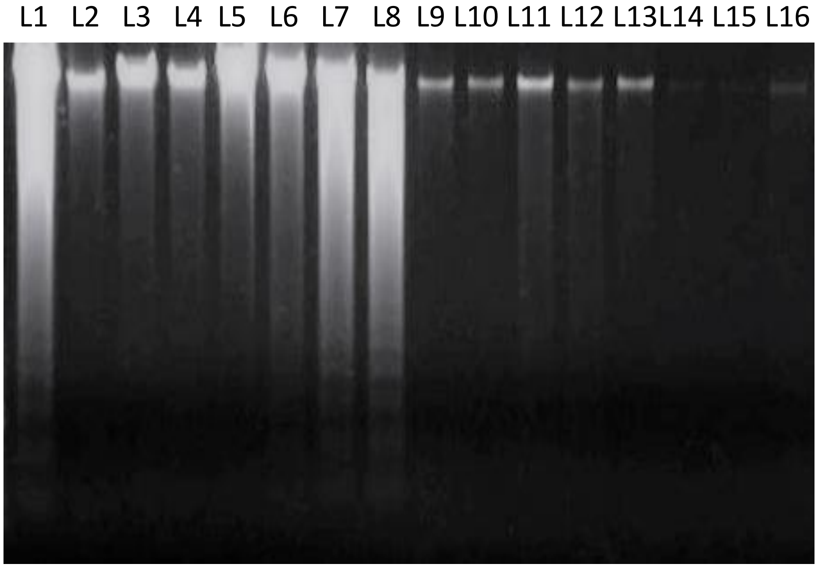
S. N	Tests	1	2	3	4	8	9	11	12	13	15	20	25	26	28	31
1.	Gram staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2.	Citrate	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
3.	MR	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
4.	VP	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
5.	TSIA	Y/Y	Y/Y	Y/Y	Y/Y	R/Y	Y/Y	R/Y	R/R	Y/Y	Y/Y	Y/Y	R/Y	Y/Y	Y/Y	R/R
6.	Urease	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve
7.	Catalase	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
8.	Sulphur	+ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve
9.	Indole	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10.	Motility	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve

Table 4.2.1.2: Results of Biochemical tests of putative *Bacillus subtilis*

S.N	Tests	16	23	29	33	34	35	39	41	43	46
1.	Gram stain	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2.	Citrate	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
3.	MR	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve
4.	VP	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve
5.	TSIA	R/Y	Y/Y	Y/Y	R/Y	Y/Y	R/R	Y/Y	Y/Y	Y/Y	Y/Y
6.	Urease	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve
7.	Catalase	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve
8.	Sulphur	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
9.	Indole	+ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
10.	Motility	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve

Out of 50 samples isolated, 24 bacteria were Gram negative so they were discarded because we needed only Gram positive bacteria. The tables above are the biochemical tests performed on remaining 26 bacteria. Only 16 bacteria from the biochemical test were found to be likely as *Bacillus subtilis* by studying their biochemical reactions.

4.2.2 Genomic DNA isolation of *Bacillus subtilis:*



L1= 26sub.
L2= 3sub.
L3= 20sub.
L4= 2sub.
L5= 28sub.
L6= 41sub.
L7= 34sub.
L8= 29sub.
L9= 1sub.
L10=13sub.
L11=11sub.
L12=9sub.
L13=4sub.
L14= 43sub.
L15= 39sub.
L16= 46sub.

Fig 4.2.2.1: Genomic DNA on 0.8% agarose gel electrophoresis

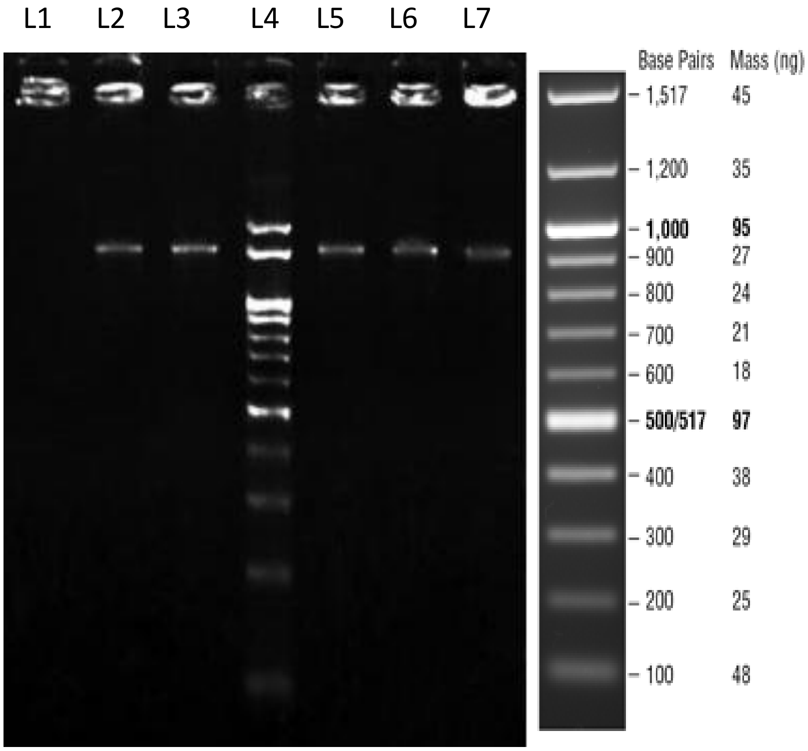
Genomic DNA extraction was done on 16 bacterial samples from which 11 samples showed good quality DNA result.

Table 4.2.2: DNA concentration of isolated putative *Bacillus subtilis*

Sample	Nucleic Acid concentration(ng/ul)	$OD_{260/280}$ ratio
3sub	286.05	1.64
20sub	359.68	1.68
2sub	310.59	1.65
28sub	579.63	1.60
41sub	383.92	1.58
1sub	153.82	1.76
13sub	161.00	1.75
11sub	184.95	1.62
9sub	132.59	1.69
4sub	141.39	1.78
46sub	78.53	1.78

The concentration of DNA of the samples were determined by Nanodrop. The highest DNA concentrations were found of the samples 20sub, 2sub, 28sub and 41sub whose concentrations were 359.68, 310.59, 579.63 and 383.92 respectively. Most samples like 26sub, 29sub, 34sub, showed RNA contamination in their respective lanes at the bottom. Their ratio of Optical density 260/280 were less than 1.8 which also proves point. RNA contamination might had occured because the enzyme RNase was not used. Almost no protein contamination was observed in the DNA bands. The extracted DNA samples were diluted and used for Polymerase Chain Reaction (PCR).

4.3. Polymerase Chain reaction (PCR)



L1= 9meg.
L2= 2meg.
L3= 5meg.
L4= DNA ladder of size 100bp
L5= 3sub.
L6= 11sub.
L7= 13sub.

Fig 4.3: PCR product on 1% agarose gel electrophoresis with 100 bp DNA ladder

PCR was performed using 16s primers on 6 samples and the result was positive for all except one sample 9meg. A ladder of size 100base pairs was used and the amplified DNA had the size of 1100 base pairs. The size of PCR product of bacterial DNA is between 1100-1500 base pairs. This experiment concludes that the samples isolated were bacterial samples for sure. The gene specific amplification was also done but results were negative. So, new primers are needed to be designed and PCR is needed to be done using these bacterial samples.

4.4 Phosphate solubilization test

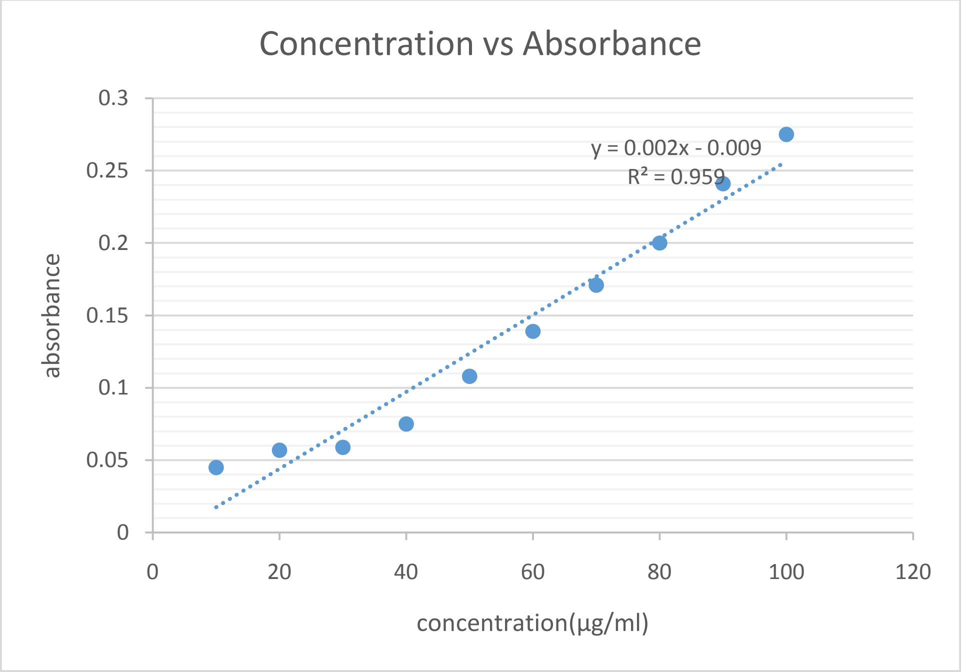


Figure 4.4.1: Standard curve of phosphate using Potassium dihydrogen phosphate

Table 4.4.1: Concentration of phosphate calculated in the media before the culture of *Bacillus subtilis*

S.N.	Sample	Absorbance	Concentration(µg/ml)
1)	20sub	0.0523	39.261
2)	2sub	0.0719	41.374
3)	13sub	0.0552	30.721
4)	9sub	0.0401	27.007
5)	4sub	0.0580	31.012
6)	3sub	0.0471	28.905
7)	1sub	0.0822	42.726
8)	11sub	0.0703	40.573

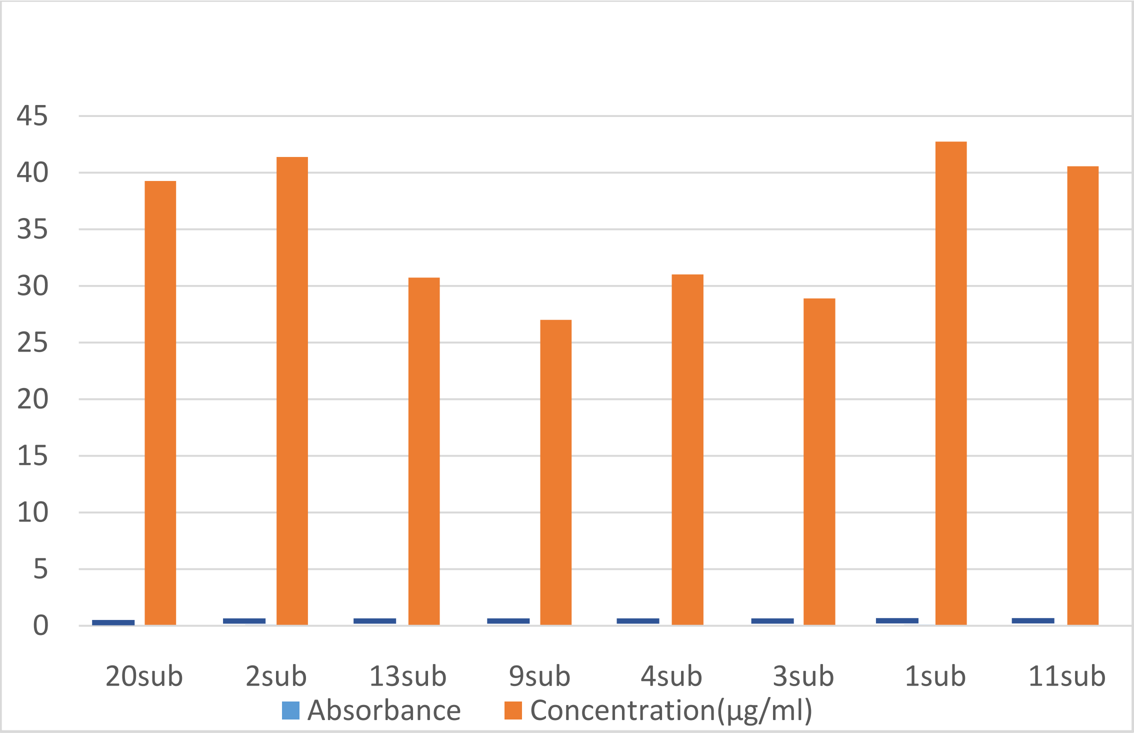


Figure 4.4.2: Absorbance vs. concentration of phosphate in the media before the culture of putative *Bacillus subtilis*

Table 4.4.2: Concentration of phosphate calculated in the media using *Bacillus subtilis* as sample

S.N.	Sample	Absorbance	Concentration (µg/ml)
1)	20sub.	0.0906	44.723
2)	2sub.	0.0825	42.272
3)	13sub.	0.0192	-
4)	9sub.	0.0627	32.312
5)	4sub.	0.0968	46.606
6)	3sub.	0.0978	46.905
7)	1sub.	0.0944	45.877
8)	11sub.	0.0215	-

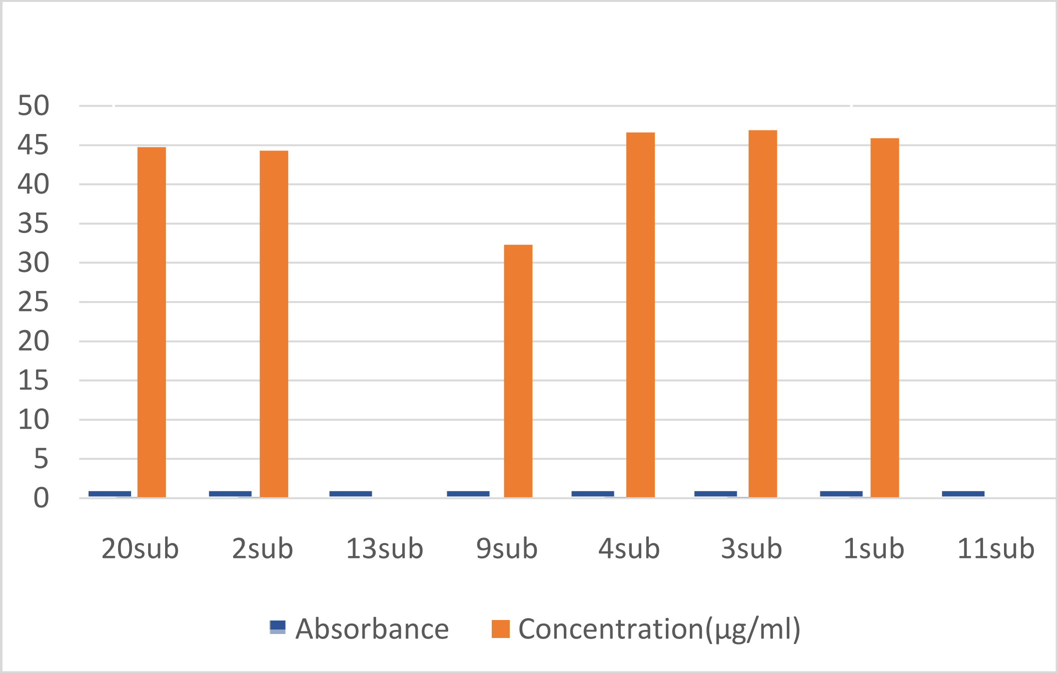


Figure 4.4.3: Absorbance vs. concentration of phosphate in the media after the culture of *Bacillus subtilis*

Table 4.4.3: Concentration of phosphate calculated in the media before the culture of *Bacillus megaterium*:

S.N.	Sample	Absorbance	Concentration (µg/ml)
1)	5meg	0.0381	10.439
2)	1meg	0.0259	-
3)	2meg	0.0461	17.820
4)	9meg	0.0419	17.105
5)	21meg	0.0318	10.117

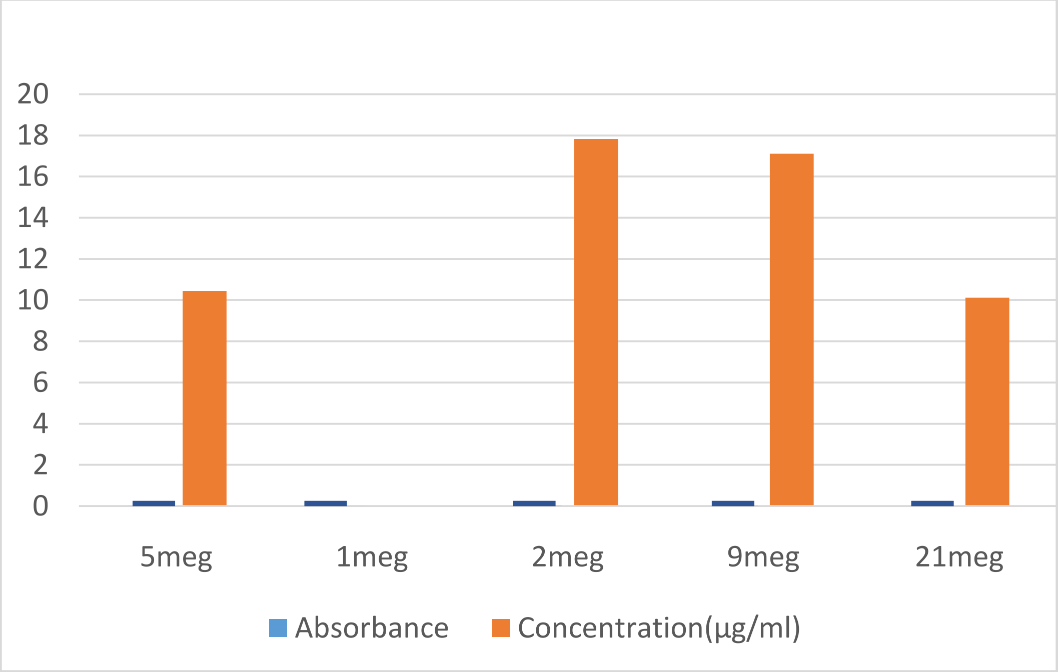


Figure 4.4.4: Absorbance vs concentration of phosphate in the media before the culture of *Bacillus megaterium*

Table 4.4.4: Concentration of phosphate calculated in the media using *Bacillus megaterium* as sample

S.N.	Sample	Absorbance	Concentration (µg/ml)
1)	5meg.	0.0567	19.750
2)	1meg.	0.0708	37.375
3)	2meg.	0.0532	16.833
4)	9meg.	0.0957	46.272
5)	21meg.	0.0480	12.560

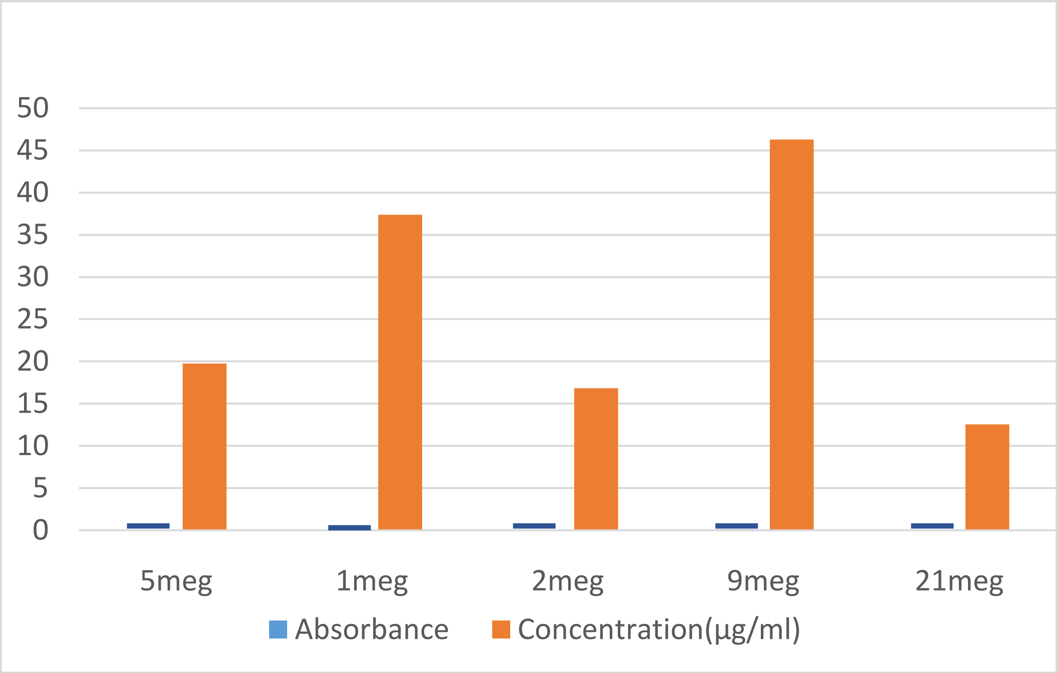


Figure 4.4.5: Absorbance vs. concentration of phosphate in the media after the culture of *Bacillus megaterium*

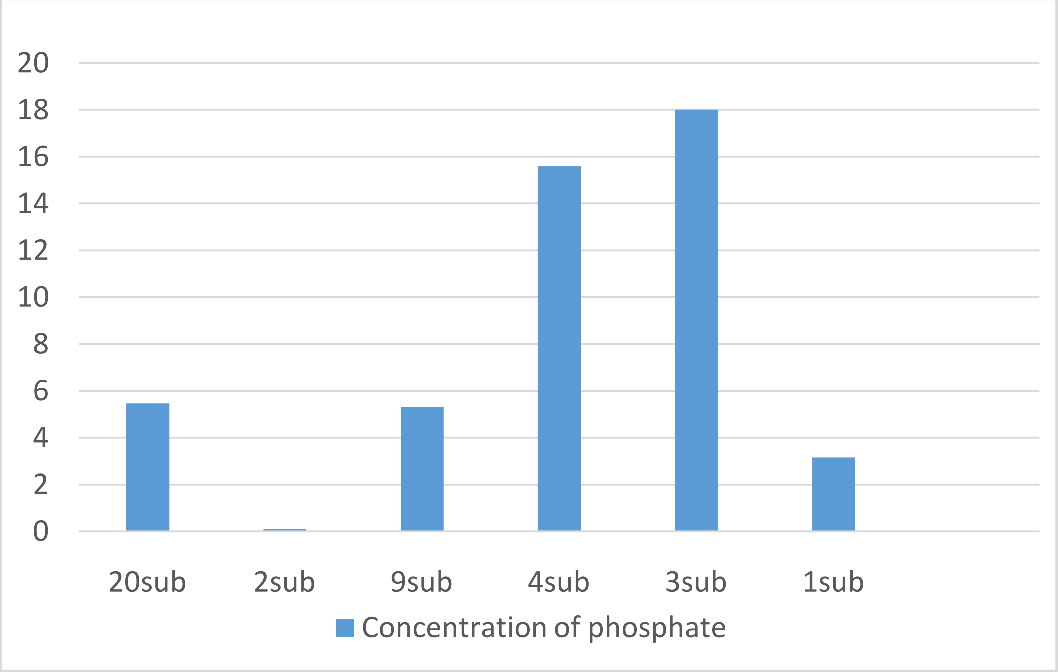


Figure 4.4.6: Final concentration of phosphate using putative *Bacillus subtilis*

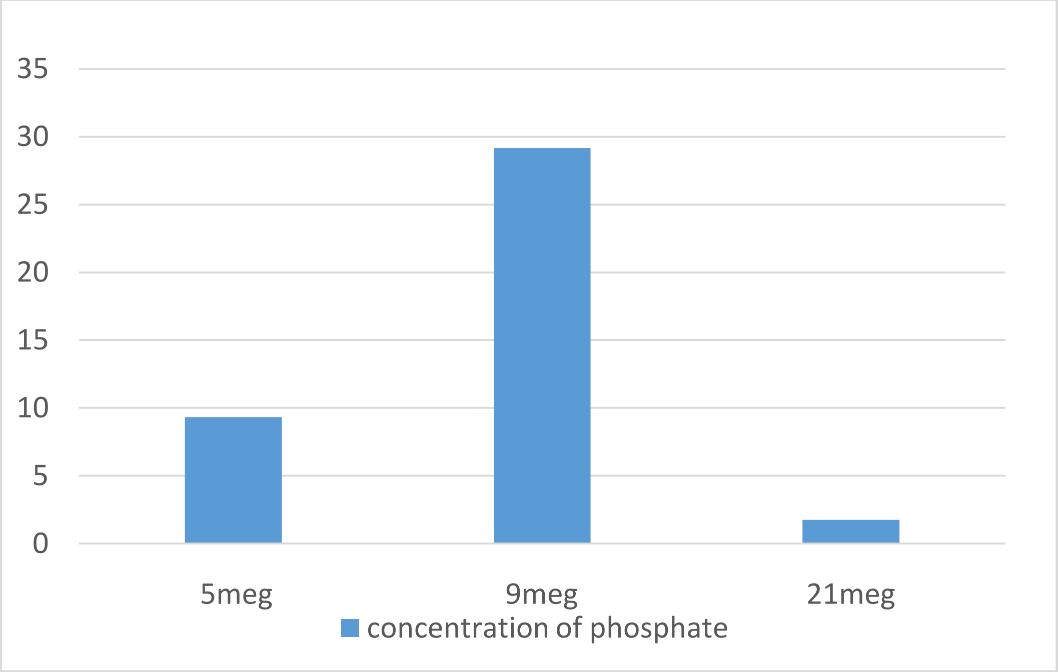


Figure 4.4.7: Final concentration of phosphate using putative *Bacillus megaterium*

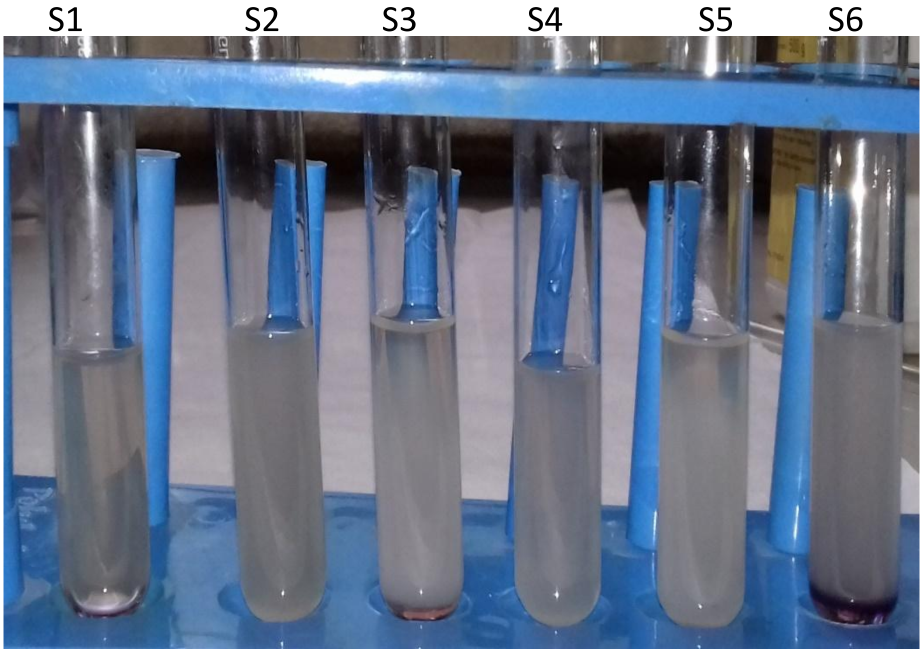
This experiment was done to find whether our isolated bacteria can solubilize the insoluble source of phosphate or not. Hydroxyapatite was used as the insoluble form of phosphate. The soluble mineral form of phosphate was substituted in the sodium acetate media by hydroxyapatite by amount of moles equal to soluble phosphate. The concentration of phosphate in the media was calculated before the culture of the bacteria. Then the bacteria were allowed to grow in the media and the amount of phosphate in the concentration of µg/ml was calculated. The reading was taken in 7th, 14th and 21st days. However, from the reading of 21st day, the concentration of phosphate was found very negligible. So the reading of 21st day was discarded. The 7th and 14th days readings showed very similar concentrations therefore an average of both the readings was taken and the concentration was calculated by using a standard curve of phosphate. The concentration of both before and after the culture of bacteria in the respective species were calculated and the results were found to be significant.

In the case of *Bacillus subtilis*, the average concentration of phosphate in the media before the culture of bacteria was found to be 35.20ug/ml. Similarly, the average concentration of phosphate after the culture of bacteria was found to be 43.12ug/ml. The samples 4sub, 3sub and 1sub showed the highest concentrations of phosphate in the media.

Similarly, in the case of *Bacillus megaterium*, the average concentration of phosphate in the media before the culture of bacteria was found to be 13.88ug/ml. Similarly, the average concentration of phosphate after the culture of bacteria was found to be 26.56ug/ml. The samples 9meg, 1meg and 5meg showed highest concentrations of phosphate in the media.

The final average concentration of soluble phosphate in the media solubilized by *Bacillus subtilis* was found to be 7.93ug/ml and that by *Bacillus megaterium* was found to be 13.40ug/ml. Comparatively, the putative *Bacillus megaterium* showed better results in the solubilization of insoluble phosphate in hydroxyapatite based media in comparison to *Bacillus subtilis*.

4.5 Cellulose degradation test:



S1= Raw wheatstraw
S2= Gelucel
S3= PBAT 60
S4= PBAT 20
S5= PBAT 40
S6= MCC wheatstraw

Figure 4.5.1: Samples of cellulose degradation test

Figure 4.5.2: MCC WS sample showing positive Molisch's test indicated by purple colored ring

In this experiment, the isolated bacteria was used in the study to solubilize polymers of cellulose. The same media was used in this study too where moles of glucose was substituted by moles of cellulose. The bacteria were inoculated in the media and left for incubation for 7 days. After that, the solution of the media was subjected to Molisch's test for the presence of carbohydrates. 3 out of 6 samples showed positive results. Since Molisch's test can detect the presence of carbohydrates but not glucose only. So the formation of glucose in the media is not justified but the presence of carbohydrates in the media is justified.

4.6. Pot Trail and Syntrophic Growth:

This experiment was a study that integrated biochar can be used both as a soil ammendment as well as artificial soil. Biochar as waste from Ruslan Vodka factory, Jawalakhel Group of Industries were obtained. This biochar was integrated with 10 different types of bacteria each having individual function in strengthening the quality of soil. This biochar was used as bio-fertilizer as well as artificial soil. 6 different types of bonsai plants were used in the study. In 4 of them the biochar was used as artificial soil and in others as fertilizer. A study of 6 months showed that the plants survived healthily. No other artificial fertilizer was used. The problems like yellowing of leaves also disappeared but reappeared after several months which maybe due to lack of micronutrients or lack of controlled environmental parameters. Similar kind of study was done by students of SANN College where they used integrated biochar as artificial soil for the growth of rice seedling and *Paulownia tomentosa* seeds with better results compared to MS growth media. From the biochemical identification, the most potent bacterial strains from our study and of our fellow thesis students were selected to be cultured in Nutrient Broth. The species included *Bacillus subtilis*, *B. megaterium*, *B. mucilaginosus*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Geobacter*, *Shewanella*, *Frateuria*. and *Lactobacillus* spp. These mixture of bacteria were mixed with crusher dust and biochar in certain ratio of and mixed properly. This mixture was used as artificial soil for the growth of bonsai plants.

Three basic components of soil; i.e. rock, microorganisms and sand were substituted by crusher dust, isolated microorganisms and char respectively. Limestone dust was added further into the mixture for maintaining alkalinity. The three components crusher dust, char and limestone dust were mixed in the ratio of 2:1:1. Along with these, 10 different types of bacterial strains were added in the mixture. Bonsai plants which were healthy were grown in the mixture as artificial soil. Water was given on a regular basis and the plants showed good growth in this growth medium than the previous one. All the plants tested survived for more than six months without additional supplementation of chemical fertilizer or any other organic fertililzer but only irrigating with the water. Some of the bonsai plants used in the study as sample as shown in the pictures below.

Table 4.6.1: List and concentrations of bacteria used in the study along with their functions

S.N.	Name of the bacteria	Concentration	Functions
1.	*Azotobacter vinelandii*	x	N2 fixation
2.	*Psedomonas fluorescens*	x	make insoluble nutrients more readily available to the plants
3.	*Lactobacillus lactis*	x	Slow release of nutrients to rhizosphere to be absorbed by plants over time
4.	*Geobacter sulfurredens*	x	Reduction of sulphur
5.	*Bacillus subtilis*	2x	Phosphate solubilization
6.	*Bacillus megaterium*	2x	Phosphate solubilization
7.	*Shewanella oneidensis*	2x	Reduction of metal ions
8.	*Frauteria aurentina*	4x	Potassium solubilization
9.	*Azospirillum spp.*	4x	N2 fixation
10.	*Bacillus mucilaginosus*	4x	P, K and Si solubilization

Figure 4.6.1: Plants where the mixture was used as artificial soil

Figure 4.6.2: Plants where the mixture was used as biofertilizer and sprinkled over the surface of the soil

CHAPTER 5

SUMMARY

Due to global warming and pollution, the quality and quantity of every natural resource on earth is constantly degrading and depleting. Be it water; be it air or be it land. The chemical revolution has brought a temporary solution to the challenges like agricultural yield increase but there is a bigger side to it to face for us. The extensive use of chemical fertilizers have highly affected the quality of our soil. It has lead to several acres of productive agricultural areas to turn into barren land. The quality of soil is attributed to the presence of macronutrients like Nitrogen, Phosphorus and Potassium and micronutrients like Iron, Zinc etc. Along with these; the presence of soil microorganisms like bacteria and fungi which often form symbiotic association with the plant rhizosphere enhance the quality of the soil. Other bacteria have the ability to convert the insoluble form of minerals into soluble form; contributing to soil fertility. Thus, it was studied that bacteria could fix the insoluble phosphate into soluble form and hypothesized that similar experiments could be done by isolating the bacteria from soil.

Hence phosphorus solubilizing bacteria *Bacillus*spps. were isolated from the soil sample of Pokhara Valley. Earlier, the students of SANN International College had done similar kinds of work and we were able to replicate their along and took the work a step further by studying the phosphate solubilizationcapabilty and pot trial using crusher dust and residual char.

Soil samples from rom nine differentplaces around Panchase area; Pokhara valley having different altitudes were collected. Initially 40 isolates of putative *Bacillus megaterium* and 50 isolates of *Bacillus subtilis* were isolated in medium containing sodium acetate as reduced carbon source. Further, according to the carbon catabolite repression, the isolated bacteria were further selected on the basis on their ability to grow on their preferred carbon sources like L- tryptophan, catechol, phenol. The putative *Bacillus megaterium* isolates survived in both sodium acetate, L-tryptophan and phenol and the putative *Bacillus subtilis* isolates survived both in sodium acetate, catechol and phenol.

Further, the selection of the most probable Bacillus strain was done on the basis of biochemical tests. Series of various biochemical tests were done and the biochemical reactions similar to the respective bacterial strain was only further subjected to molecular characterization. The genomic DNA of 11 putative *Bacillus megaterium* and 16 putative *Bacillus subtilis* strains was extracted out of which 6 and 11 samples showed proper DNA bands of each strains respectively. Thus, Genomic DNA of each isolates of putative *Bacillus spp.* were extracted and PCR amplification of 16s rRNA genomic DNA was done to confirm whether it is bacteria using universal primers. Once the isolates were confirmed to be bacteria they were further subjected to PCR using genus specific 16srRNA primers. The primers wasdesigned by the students of SANN college where the primer was designed in such a way that it only amplifies the sequence relevant to *Bacillus* spp. only. But, on several repeated experiment no specific band was observed upon gel documentation. Further, the primer also didnt work on the bacterial strain which which was already confirmed to be of

Bacillus species which implied that due to appropriate storage conditions or mishandling the primer solution might have been degraded. But still, this could be a new screening protocol for the isolation of *Bacillus spp.* from mix soil culture which is fast and reliable.

Moreover, the isolates obtained from the culture as putative *Bacillus megaterium* and *Bacillus subtilis* were subjected for the ability for the solubilization of phosphate in the media. The average concentration of phosphate calculated in the media by putative *Bacillus megaterium* was 13.40µg/ml and that by putative *Bacillus subtilis* was 7.93µg/ml.

Since, this work was an attempt to develop a technology where the concept of artificial soil can be modified into simple one and made available for the common people who do not have the complex understanding of science. For this, three basic components of soil; i.e rock, microorganisms and sand were exchanged by crusher dust, isolated microorganisms and char respectively. Limestone dust was added further into the mixture for maintaining alkanility. The three components crusher dust, char and limestone dust were mixed in the ratio of 2:1:1. Along with these, 9 different types of bacterial strains were added in the mixture. Bonsai plants which were healthy were grown in the mixture as artificial soil. Water was given on a regular basis and the plants showed good growth in this growth medium than the previous one. All the plants tested survived for more than six months without additional supplementation of chemical fertilizer or any other organic fertililzer but only irrigating with the water.

CHAPTER 6

CONCLUSION

This research is an attempt to develop a technology as a prototype which could potentially help substitute the use of chemical phosphate fertilizers and introduce the concept of artificial in a simpler and understandable manner. *Bacillus spp*. have already been used as a bio-fertilizer in phosphate solubilization as reported in several literatures and similar kind of works have also been done. To tackle with environmental pollution, global warming, various health hazards; new technologies should always been developed to minimize their effects. Scarcity of food and rapidly depleting sources of fossil fuel have need are the matters of serious concern and need to be addressed very soon. If not the world would face a serious damage in the form of unrest and chaos which will ruin everything we have ever worked for. To save the most intelligent form of life i.e humans, the most primitive forms i.e bacteria contribute a lot and we have benefitted from them since a very long period of time. These bacteria apart from their natural habitat were isolated by growing them in their preferred carbon source. These bacteria were used to solubilize insoluble mineral phosphate in artificial media and its concentration was estimated. The cellulose degradation capability of the isolated bacteria were also studied. These bacteria were also used in the artificial soil used as pot trial which showed satisfactory results.

RECOMMENDATION

- The isolated bacteria isolates should be confirmed by sequencing.
- The source of insoluble phosphate used in the study which is hydroxyapatite could be replaced by some other lower cost, phosphate rich source.
- Pot trial could be done on a large scale and controlled parameters so that the effects of the artificial soil could be studied in more detailed manner.

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APPENDICES

Appendix 1

Composition and preparation of different culture Media

1.1 Nutrient agar media (100ml, pH 6.8)

Beef extract	0.3g
Peptone	0.5g
Sodium chloride	0.5g
Agar	1.5g

1.2 Sodium acetate agar medium (1000 ml, pH 7.0):

Sodium acetate	10 g
$(NH_4)_2SO_4$	1 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.5 g
$CaSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
Agar	12 g

1.3 Tryptophan media (1000 ml, pH 7.0):

DL Tryptophan	10.917 g
$(NH_4)_2SO_4$	1 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.5 g
$CaSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
Agar	12 g

1.4 Catechol media (100 ml):

Catechol	0.556g
$(NH_4)_2SO_4$	1 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.5 g
$CaSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
Agar	12 g

1.5 Phenol media (100 ml):

○ Phenol	0.471g
○ $(NH_4)_2SO_4$	1 g
○ K_2HPO_4	0.8 g
○ KH_2PO_4	0.2 g
○ $MgSO_4.7H_2O$	0.5 g
○ $CaSO_4.7H_2O$	0.05 g
○ $FeSO_4.7H_2O$	0.01 g
○ Agar	12 g

APPENDIX 2

Preparation of working solutions and buffers used in the study

2.1. Tris-Cl (1 M, pH 7.5-8)

Tris-Cl buffer was prepared by adding 12.11 g of Tris base in 80 ml of MilliQ water and pH was adjusted to 7.5-8.0 by adding concentrated HCl. The final volume was maintained to 100 ml. The solution was sterilized by autoclaving and stored at 4C.

2.2. EDTA (0.5 M, pH 8.0)

18.61 g of disodium EDTA 2H2O was added to 80 ml of MilliQ water, stirred vigorously on magnetic stirrer and pH was adjusted to 8.0 with NaOH. The final volume was made 100 ml and sterilized by autoclaving and stored at 4C.

2.3. TE 1 solution

50mMTris-Cl (pH7.5)

50 mM EDTA (pH 8.0)

2.4. STEP solution

50mMTris-Cl (pH 7.5)
0.2mMEDTA (pH 8.0)
0.5% SDS
1 mg/ml proteinase K

2.5. Lysozyme (1mg/ml)

1 mg of lysozyme was dissolved in 10 mMTris-Cl (pH 8.0) with final volume of 1 ml. The solution was prepared immediately before use.

2.6. Liquid Phenol

Crystalline phenol was removed from the -20°C freezer and thawed in 60-65°C.Desired volume of phenol was added in appropriately sized bottles.Equal volume of 10x TE was added to the phenol and vigorously shaken. The layers were allowed to separate.The upper aqueous top layer was aspirated off.The process was repeated with a second equal volume of 10x TE.An equal volume of 1x TE was added to the phenol. The subsequent steps were repeated with a second equal volume of 1x TE.A small layer of 1x TE was left above the phenol after final aspiration.The final pH of the TE was made 8.0 using a pH strip.

2.7. 10 X TAE Buffer

Tris base	4.84 g
Glacial acetic acid	1.142 ml
0.5 M EDTA (pH 8.0)	2 ml
MilliQ water	upto 100 ml

The working solution (0.5 X) of TAE buffer was prepared by diluting 17.5 ml of 10 X TAE stock solutions with MilliQ water to make the final volume upto 350 ml.

2.8. Ethidium Bromide (10 mg/ml)

100 mg of ethidium bromide was weighed and dissolved in 10 ml of MilliQ water. The solution was protected from sun light by wrapping with aluminum foil and stored at room temperature

2.9. Standard solution of phosphate

0.1757g potassium dihydogen phosphate was dissolved in distilled water. The solution was diluted making the final volume 1 litre. The solution was stored in dark glass bottle for further use.

2.10. 5N H2SO4 solution

70litres of concentrated H2SO4 was diluted to 500ml.

2.11. 5% Ammonium molybdate solution

20 gm. of ammonium molybdate was dissolved in distilled water and diluted upto 500ml.

2.12. Ascorbic Acid (0.1M) solution

A solution of 1.76 gm. ascorbic acid was dissolved in 100 ml. distilled water.

2.13. Mixed Reagent

125ml. 5N H2SO4 and 37.5 ml. of 4% ammonium molybdate was mixed properly. To this solution, 75ml. of ascorbic acid solution was added and diluted to make the volume 250 ml. This solution should be prepared an hour before use.

APPENDIX 3

3.1. Primers used for gene amplification

Forward primer: 27F (5’-AGAGTTTGATCCTGGCTCAG-3’)

Reverse Primer: 1392R (5’-GGTTACCTTGTTACGACTT-3’)

3.2. Volume of reagents used in PCR:

DNA template - 1µl

Master Mix - 7µl

Forward primer - 1µl

Reverse primer - 1µl

Table: PCR program for gene amplification:

S.N.	**Steps**	**Temperature(˚C)**	**Time**
1.	Enzyme Activation	95	2 mins.
2.	Denaturation	95	30 secs.
3.	Annealing	52	30 secs.
4.	Extension	72	2.5 mins.
5.	Final extension	72	5 mins.
6.	Hold	4	∞