ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA

A

DISSERTATION

Submitted in partial fulfillment of the requirements
for the award of the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

Under the guidance of

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And

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CERTIFICATE

This is to certify that the dissertation entitled **"ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA"** submitted (Registration No: 301105005) by divya Sharma in partial fulfillment of the requirement for the award of degree of Master of Sciences in Microbiology, to Thapar University, Patiala is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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I, hereby declare that the work which is being presented in this dissertation entitled **"ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA"** submitted by the undersigned in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own independent and original research work carried out during the period of six months from Jan 2012 to July 2012, under the supervision of **Dr. M. S. Reddy, Professor & Head**, Department of Biotechnology & Environmental Sciences, Thapar University. The matter embodied in this dissertation has not been submitted in part or full to any other university or institute for the award of any other degree or certificate.

Dated: 17/07/2013

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CONTENT PAGE NO.

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production and biological nitrogen fixation, production of IAA and by HCN production. In the present study PGPRS were isolated from rhizospheric soil and were tested for different plant growth promotion activities. Out of 20 bacterial strains isolated from potato filed, four efficient PGPR strains were selected for further studies. Results of present study showed that these selected strains are efficiently soolubilize the tri calcium phosphate, rock phosphate, aluminium phosphate and ferrous phosphate when suplimented in PKV broth. All the strains are positive for acid, alkaline phosphatase and phytase enzyme production, siderophore production and indole acetic acid production. These selected strains were further tested for their affects on plant growth and soil fertility improvement. When these isolates were used as seed inoculants a significant improvement in plant growth, P uptake and in soil fertility was observed as compared to control soil treatments.

The potentiality of PGPR in agriculture is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements. Growth promoting substances are likely to be produced in large quantities by these rhizosphere microorganisms that influence indirectly on the overall morphology of the plants. Use of PGPR as inoculants increased nutrient availability to plants. The progress, to date in using the rhizosphere bacteria in a variety of applications related to agricultural improvement along with their mechanism of action with special reference to plant growth-promoting traits are discussed in this thesis.

CHAPTER-1

INTRODUCTION

1.1 PHOSPHORUS

Phosphorus (P) is the major essential macronutrients for plants. It helps in plant growth and development. It is applied to the soil in the form of phosphatic fertilizers. However a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizers is immobilized rapidly and becomes unavailable to plants (Shen *et al.* 2005). It is classified as a macronutrient because of relatively large amount of P required by plants. Despite its wide distribution in nature, it is a deficient nutrient in most soils. One of the main roles of P in living organisms is in the transfer of energy (Whitelaw, 2000).

1.2 SIGNIFICANCE OF PHOSPHORUS

Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant. An adequate supply of phosphorus in the early stages of plant growth promotes physiological functions including early root formation and is important for lying down the primordial for reproductive parts of plants (Saber *et al.* 2005).

It is vital to seed formation and its content is higher in seeds than in any other parts of the plants. It helps plant to survive winter rigors and also contribute to disease resistance in some plants. Also known to improve quality of many fruits, vegetables and grain crops. Biological nitrogen fixation depends appreciably on the available form of phosphorus. Phosphorus is an important structural constituent of nucleic acid, phytin and phospholipids (Sagervanshi *et al.* 2012)

1.3 PHOSPHORUS AVAILABILITY IN SOIL

Phosphorus exists in many different forms in soils. Phosphorus normally occurs in nature as part of a phosphate ion, consisting of a phosphorus atom and some number of oxygen atoms, the most abundant form (called orthophosphate) having four oxygens: PO_4^{3-}. Most phosphates are found as salts in ocean sediments or in rocks. A large portion of soluble inorganic phosphorus applied

to agricultural soils as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dadarwal *et al.* 1997).

A second major component of soil P is organic matter, present largely in the forms of inositol phosphate (soil phytate), accounting for up to 50% of the total organic P in the soil is in the form of phosphodiesters including phospholipids and nucleic acids.

Despite so rich, the concentration of soluble P (that is bio-available P) is usually very low in soils due to the phenomenon of chemical fixation of phosphate. These sources are grouped into four general forms:

(1) Plant available inorganic P, and three forms which are not plant available.
(2) Organic P
(3) Adsorbed P
(4) Primary mineral P

The principal characteristic of these primarily minerals (oxyapatite, hydroxyapatite, apatite) is their insolubility (Fig. 1.1).

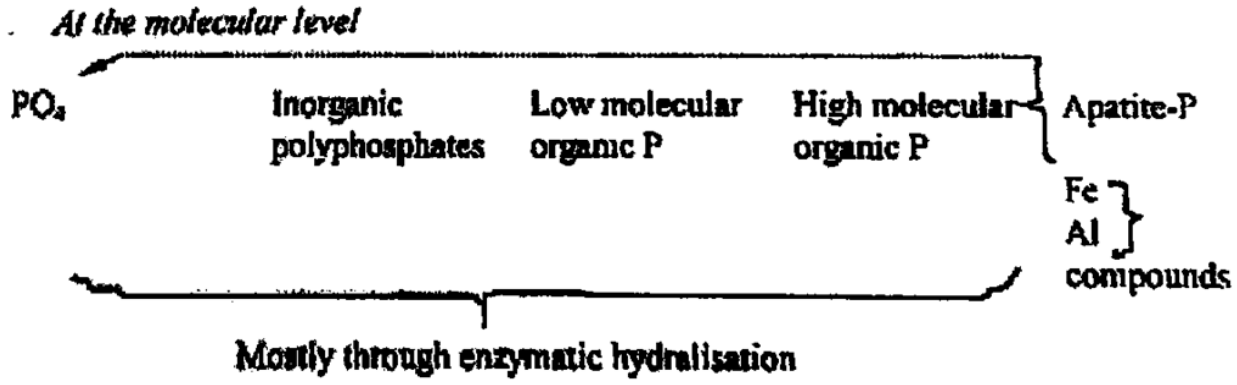

Figure 1.1: Diagram showing relative importance of phosphorus availability at the molecular level (Vollenweider, 1979)

1.4 IMPORTANCE OF PHOSPHORUS IN AGRICULTURE

Phosphorus is often applied to agriculture land to increase crop production. Phosphorus is lost from agricultural land to surface water bodies in sediment bound and dissolved forms.

Microorganisms are involved in a range of processes that effect the transformation of soil P and are thus an integral part of the soil P cycle. In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization (chen *et al.* 2002).

In many soils, although phosphate is available in plenty, application of phosphatic fertilizers is a must to make up for the phosphorus lost due to fixation of soluble phosphate by soil constituents and phosphate run of in P loaded soil (Vikram and Hamzehzarghani, 2008). However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results indicated the amount available to plants is usually a small proportion of this total (Rdresh *et al*. 2004).

1.5 NUTRIENT DEFICIENCY IN PLANTS

Plant requires essential nutrients for normal functioning and growth. A plant's sufficiency range is defined as the range of nutrient necessary to meet the plant's nutritional needs and maximize growth. Nutrient levels outside of a plant's sufficiency range will cause overall crop growth and health to decline due to deficiency. Nutrient deficiency occurs when an essential nutrient is not available in sufficient quantity to meet the requirements of a growing plant (Fig. 1.2). Common nutrient deficiencies are Nitrogen (N) and Phosphorus (P) with some deficiencies of Iron (Fe), Manganese (Mn) and Zinc (Zn).

The three basic tools for diagnosing nutrient deficiencies are:

1. Soil testing
2. Plant analysis
3. Visual observation

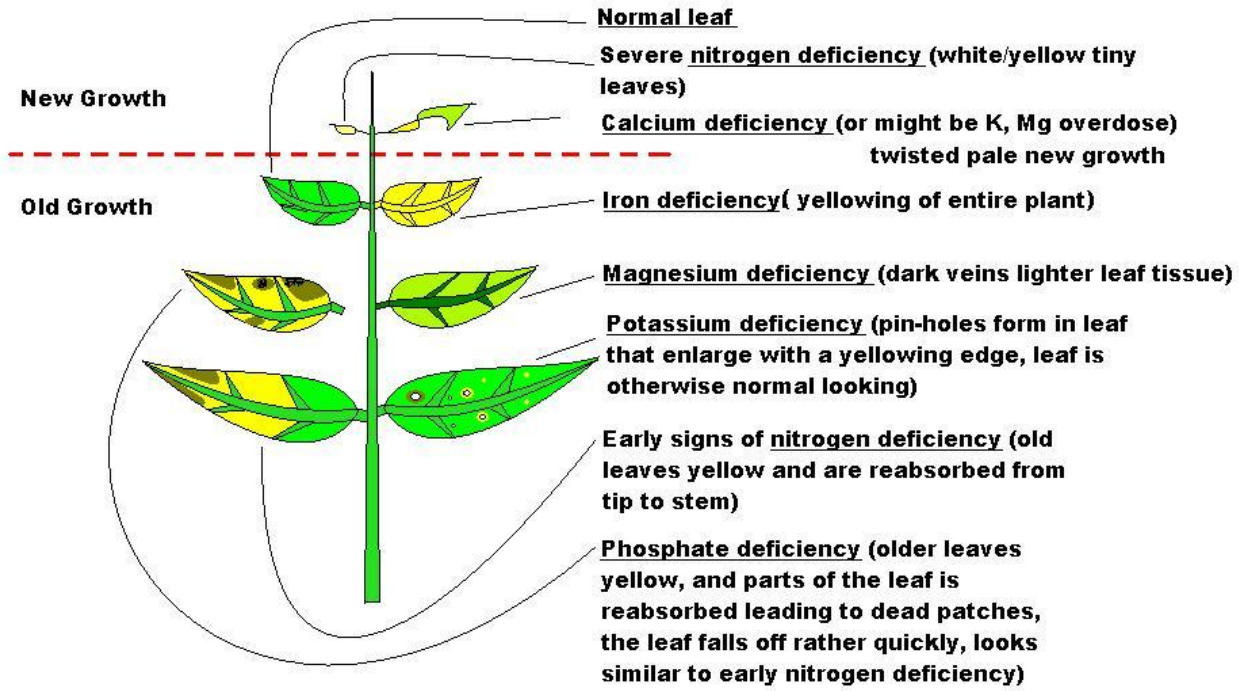

Figure 1.2: Diagram showing nutrient deficiencies in plant.

1.6 EFFECT OF CHEMICAL FERTILIZERS ON PLANTS

Fertilizer is any substance used to add nutrients to the soil to promote soil fertility and increase plant growth. They reduce the food value of plants. The excessive use of chemical fertilizers in plants not only affects the quality of food but also give rise to many diseases like Stomach cancer, goiter, and several vector borne diseases. In infants it is the reason of Blue Baby Syndrome. It also leads to groundwater contamination (Savci, 2012)

As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations (Compant *et al.* 2005). However, increasing use of chemical inputs causes several negative effects, i.e. development of pathogen resistance to the applied agents and their non target environmental impacts (Werger *et al.*1995). The nutrient reservoirs in the soil shrink when crops are removed from the field at harvest. This nutrient export creates a P deficit, necessitating regular P addition to replace the harvested P. This leads to the need of frequent application of chemical phosphate fertilizers, but

its use on a regular basis has become a costly affair and also environmentally undesirable (Reddy *et al*. 2002). Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, and consumer demand for pesticide-free food has led to a search for substitutes for these products. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent (Gerhardson, 2002). Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Welbaum, 2004).

1.7 PLANT GROWTH PROMOTING RHIZOBACTERIA

The rhizosphere is the soil found around the root and under the influence of the root. It is a site with complex interactions between the root and associated microorganisms (Sylvia *et al.* 1998).

The ineffectiveness of PGPR in the field has often been attributed to their inability to colonize plant roots. Recently PGPRs have attracted the attention of agriculturists as soil inoculums to improve plant growth and yield (Fasim *et al*. 2002). Plant growth-promoting rhizobacteria are the rhizospheric bacteria that can enhance plant growth by a wide variety of mechanisms like :

- phosphate solubilization
- siderophore production
- nitrogen fixation
- production of Indole Acetic Acid (IAA)
- Hydrogen Cyanide production

1.7.1. Phosphate Solubilizing Bacteria

Phosphate solubilizing bacteria are group of beneficial bacteria capable of hydrolyzing organic and inorganic phosphorus from insoluble compounds. P solubilization ability of the microorganisms is considered to be one of the most important traits associated with plant phosphate nutrition. It is generally accepted that mechanisms of the mineral phosphate solubilization by the 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 there by converting it into soluble forms. In addition some PSB produce phosphatase like phytase that hydrolyse organic forms of phosphate compound efficiently.

1.7.2. Nitrogen Fixing Bacteria

About 78% of the earth atmosphere is made up of free nitrogen (N_2) produced by biological and chemical processes within the biosphere and not combined with other elements. All plants need nitrogen for their growth. However plants cannot get the nitrogen they need from atmospheric supply. They can use only nitrogen that is available in compound form. Nitrogen occurs in the atmosphere as N_2, a form that is not useable by plants. Nitrogen fixation is the first major mechanism for the enhancement of plant growth by *Azospirillum* (Prasad and Govindarajan, 2001).

The process by which free nitrogen from the air is combined with other elements to form inorganic compounds, such as ammonium ions, which can then be converted by nitrification into nutrients that can be readily absorbed by plants and other organisms for incorporation into more complex organic compounds.

1.7.3. Siderophore production

Siderophores are small, high affinity iron chelating compounds secreted by microorganisms such as bacteria, fungus and grasses. Siderophores are amongst the strongest soluble Fe^{3+} binding agents known. Despite being one of the most abundant elements in the Earth's crust, the bioavailability of iron in many environments such as the soil is limited by the very low solubility of the Fe^{3+} ion. It accumulates in commercial mineral phases such as iron oxides and hydroxides (the mineral that are responsible for red and yellow soil colours) hence cannot be readily utilized by the organisms. Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by the active transport mechanisms.

1.7.4. Production of Indole Acetic Acid (IAA)

PGPRs produce plant hormones both in liquid cultures and natural situation. The major hormones produced are

- Indole acetic acid (IAA) production (Barbieri *et al*. 1986)
- The phytohormones synthesized by PGPRs influenced the root hair development, respiration rate, metabolism and root proliferation which in turn resulted in better mineral uptake of the inoculated plants (Bar and Okon, 1993).

1.7.5. Hydrogen Cyanide production

Hydrogen cyanide (HCN) production is an inorganic compound. It is colorless, extremely poisonous liquid that boils slightly above the room Temperature at 26 °C. Hydrogen cyanide is a linear molecule with a triple bond between Carbon and Nitrogen.

1.8. PGPR AS BIO-INOCULATION

The PGPRs occur in soil, usually their number are not high enough to compete with other microorganisms commonly established in the rhizosphere. Thus the amount of P liberated by them is generally not sufficient for a substantial increase of *in situ* plant growth. Therefore inoculation of plants by a target microorganism at a much higher concentration than the normal found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement (Rodriguez and Fraga 1999). Inoculation of PGPRsin the soil is a promising technique because it can increase P availability (Reyes et al. 2002) and improves the physio-chemical, biochemical and biological properties of soil (Caravaca et al. 2004). The use of PGPR in agriculture can not only compensate for higher cost of manufacturing fertilizers in industries but also mobilizes the fertilizers added to soil.

Objectives

1. Isolation and screening of plant growth promoting rhizobacteria
2. Biochemical and molecular characterization of efficient plant growth promoting rhizobacteria
3. Field evaluation of plant growth promoting rhizobacteria

CHAPTER 2

REVIEW OF LITERATURE

2.1 PHOSPHORUS

Phosphorus is one of the major essential macronutrients for plants, which applied to the soil in the form of phosphatic manure. However a large portion of applied phosphorus is rapidly immobilized being unavailable to plants. In average the content of Phosphorus in soil is about 0.05% however only 0.1% of them are available to plants.

The free phosphatic ion in soil plays a crucial role. The orthophosphatic ion is the only ion which can assimilate in an appreciable amount by plants. Soil microorganisms involve in a wide range of biological processes including the transformation of soil phosphorus. They solubilize soil phosphorus for the growth of plants (Fengling *et al.* 2011).

Phosphorus is one of the most important elements for plant growth and metabolism. It plays key roles in the many plant processes such as energy metabolism, the synthesis of nucleic acid and membranes, photosynthesis, respiration, nitrogen fixation and enzyme regulation (Ragothama, 1999). Adequate phosphorus nutrition enhances many aspects of plant development including flowering, fruiting and root growth. The optimal development of crops demands a high , often costly , input of P fertilizers (Richa *et al.* 2003).

2.2 IMPORTANCE OF PHOSPHORUS IN AGRICULTURE

Phosphorus is an essential element needed in all living organisms and it is also a non-renewable resource. An input of phosphorus is crucial for food production since all plants need an adequate supply of it for successful growth. A shortfall in phosphorus will result in a reduction of crop yield (Tirado and Allsoapp 2012).

Only about 20% of the phosphorus used in agriculture reaches the food we consumed, most of the rest lost in inefficient steps along the phosphorus cycle (Fig. 2.1) (Cordell *et al*. 2011). The increasing scarcity of the phosphorus is of great concern.

In many agricultural systems in which the application of P to the soil is necessary to ensure plant productivity, the recovery of applied P by crop plants in a growing season is very low, because in the soil more than 80% of the P becomes immobile and unavailable for plant uptake because of adsorption, precipitation, or conversion to the organic form (Holford, 1997)

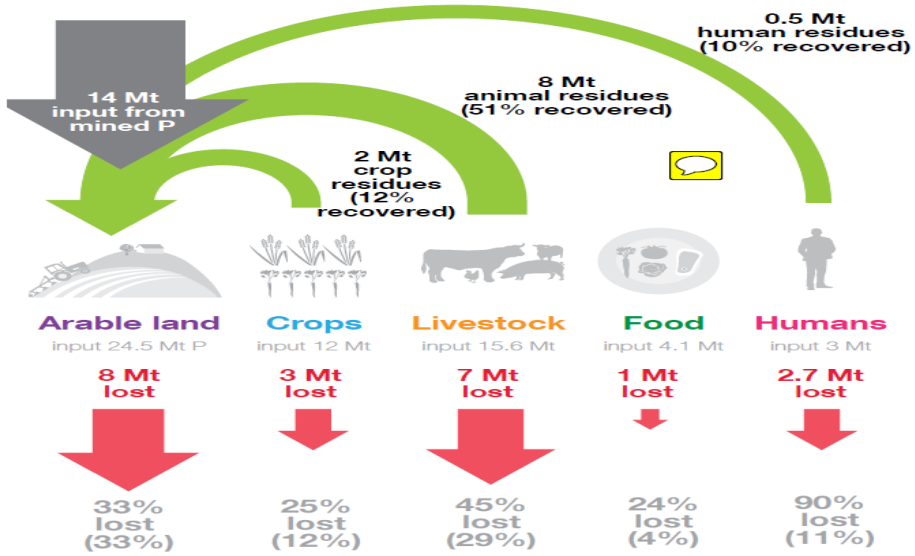

Figure 2.1: Simplified cycle of phosphorus in agriculture based on data from Crdell *et al.* 2009 and cordell *et al.* 2011.

2.3 FORMS OF PHOSPHORUS IN SOIL

Scientists describe phosphorus in soils as existing in four different "pools" on the basis of their accessibility to plants (Syers *et al*. 2008).

- The solution P pool is very small and contains only a fraction of a pound of P per acre. The solution P will usually be in the orthophosphate form, but small amounts of organic P may exist as well. Plants will only take up P in the orthophosphate form. The solution P pool is important because it is the pool from which plants take up P and is the only pool that has any measurable mobility.
- The active P pool is P in the solid phase, which is relatively easily released to the soil solution, the water surrounding soil particles. As plants take up phosphate, the concentration of phosphate in solution is decreased and some phosphate from the active P

pool is released. Because the solution P pool is very small, the active P pool is the main source of available P for crops. The active P pool will contain inorganic phosphate that is attached (or adsorbed) to small particles in the soil, phosphate that reacted with elements such as calcium or aluminum to form somewhat soluble solids, and organic P that is easily mineralized.

- The fixed P pool of phosphate will contain inorganic phosphate compounds that are very insoluble and organic compounds that are resistant to minearlization by microorganisms in the soil. Phosphate in this pool may remain in soils for years without being made available to plants and may have very little impact on the fertility of a soil. The inorganic phosphate compounds in this fixed P pool are more crystalline in their structure and less soluble than those compounds considered being in the active P pool. Some slow conversion between the fixed P pool and active P pool does occur in soils (Lovleen *et al*. 2007).

2.4. PHOSPHORUS CYCLE

The phosphorus cycle in soil is a system which involves soil, land and microorganisms. Major processes includes the uptake of soil phosphorus by plants, recycling (the return of plant and animal residues), biological turnover (mineralization and immobilization) fixation to clay, solubilization (Stevenson, 1986). Phosphorus not supplied through biochemical fixation but, must come from other sources to meet plant requirement. These sources include commercial fertilizers, animal manures, plant residues, wastes and native compounds of phosphorus, both organic and inorganic already present in the soil. The soil 'P' cycle is a dynamic process involving the transformation of 'P' by geochemical and biological process. The next figure shows the general cycle of phosphorus in the soils (portion of organic and inorganic form of phosphorus) into pools based on its availability in plants (Fig. 2.2).

Phosphorus occurs in soils as both organic and inorganic forms. Phosphorus can be found dissolved in the soil solution in very low amounts or associated with soil minerals or organic materials (Fig. 2.3). The relative amounts of each form of phosphorus vary greatly among soils, with the total amount of P in a clayey-textured soil being up to ten times greater than in a sandy soil.

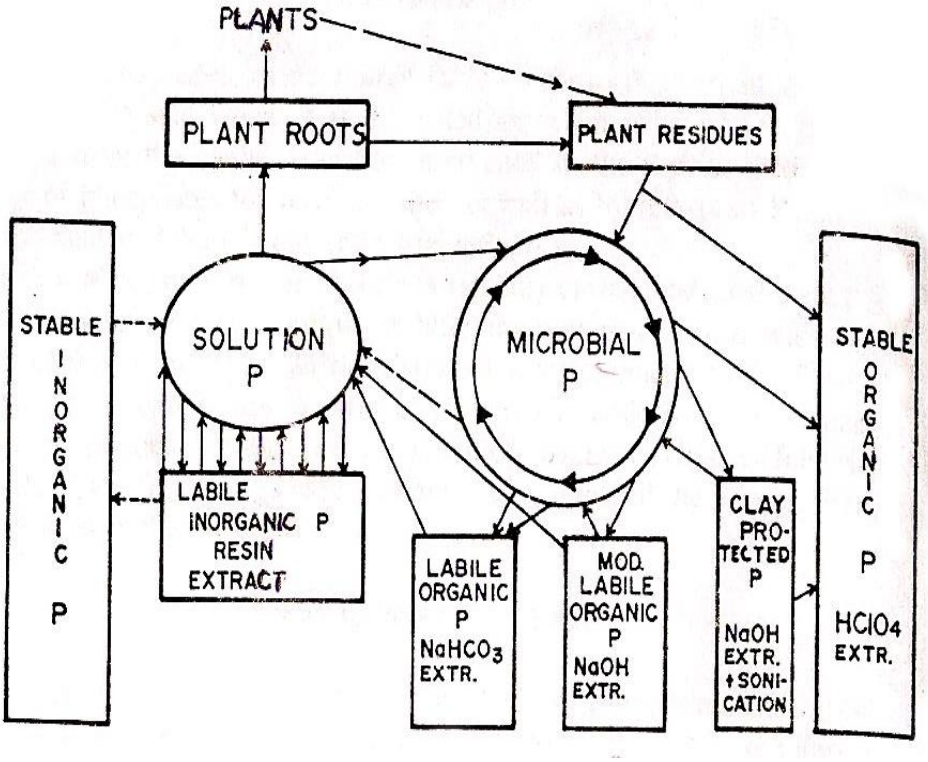

Figure 2.2: Diagram represents phosphorus cycle.

Organic P in soils: A large number of compounds make up the organic P in soils, with the majority being of microbial origin. Organic P is held very tightly and is generally not available for plant uptake until the organic materials are decomposed and the phosphorus released via the mineralization process. Mineralization is carried out by microbes, and as with nitrogen, the rate of P release is affected by factors such as soil moisture, composition of the organic material, oxygen concentration and pH.

The reverse process, immobilization, refers to the tie-up of plant-available P by soil minerals and microbes that use phosphorus for their own nutritional needs. Microbes may compete with plants for P, if the decomposing organic materials are high in carbon and low in nitrogen and phosphorus (i.e., wheat straw). Mineralization and immobilization occur simultaneously in soil. If the P content of the organic material is high enough to fulfill the requirements of the microbial population, then mineralization will be the dominant process.

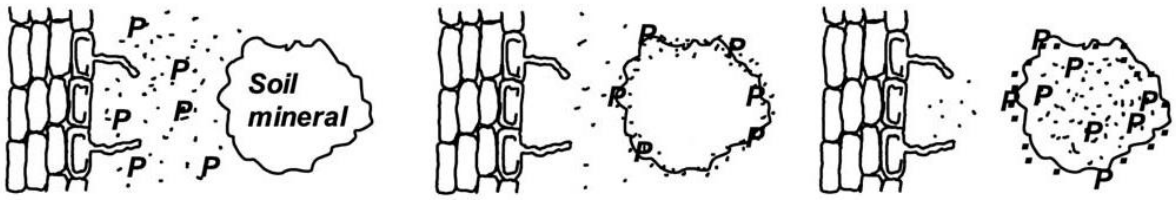

Figure 2.3: Picture shows how phosphorus (phosphates) is tied up by soil minerals. A) A large percentage of the P is available for root uptake immediately after fertilization application. B) P in solution binds rapidly to the surface of soil minerals. Roots may still use this P. C) Eventually, most of the bound P becomes part of the structure of the mineral, with its plant availability being significantly reduced.

Inorganic P in soils: The concentration of inorganic P (orthophosphates) in the soil solution at any given time is very small, amounting to less than 1 lb/A. Phosphorus in the inorganic form occurs mostly as aluminum, iron or calcium compounds. The chemistry of soil P is very complex, with more than 200 possible forms of P minerals being affected by a variety of physical, chemical and biological factors. Soluble P resulting from commercial fertilizer applications or from mineralization reacts with soil constituents to form P compounds of very low solubility (low plant availability). This series of reactions is commonly referred to as sorption or fixation. Iron and aluminum compounds will fix (tie-up) P under acidic conditions (soil pH < 6), while under alkaline conditions (soil pH > 7), phosphorus is preferentially fixed by calcium and magnesium compounds.

Phosphorus availability to plants in most soils is greatest when soil pH is in the range of 6 to 7. Application of liming materials is a common production practice to raise the pH in acidic soils to make P more available. However, lowering the pH of calcareous soils to increase the solubility of P is not an economically viable option, since large amounts of acidifying material are typically needed. Thus, soils with high pH generally have P fertilizer applied every year immediately before planting the crop. Soils that have not received P fertilization for a few years can render much of the P fertilizer applied unavailable. Thus, it is best to maintain proper P fertilization of soils and not to mine those of their P. Otherwise the soil may fix P until the P mineral fractions in the soil have been replenished through proper P fertilization practices (Loveleen *et al.* 2007).

2.5 BENEFITS OF BIOFERTILIZERS OVER CHEMICAL FERTILIZERS

Fertilizers increases efficiency and obtain better quality of product recovery in agricultural activities.It is one of the most important ways. Non-organic fertilizers mainly contain phosphate, nitrate ammonium and potassium salts. However in recent years, fertilizer consumption increased exponentially throughout the world causes serious environmental problems. Fertilization may affect the accumulation of heavy metals in soil and plant system. Plant absorb the fertilizers through the soil, they can enter the food chain. Thus, fertilization leads to water, soil and air pollution (Serpil *et al.* 2012).

Due to harmful effect of chemical fertilizers on crops, researchers have started showing their interest in biofertilizers. Biofertilizers, more commonly known as microbial inoculants, are artificially multiplied cultures of certain soil organisms that can improve soil fertility and crop productivity. Although the beneficial effects of legumes in improving soil fertility was known since ancient times and their role in biological nitrogen fixation was discovered more than a century ago, commercial exploitation of such biological processes is of recent interest and practice.

Biofertilizers have various benefits. Besides accessing nutrients, for current intake as well as residual, different biofertilizers also provide growth-promoting factors to plants and some have been successfully facilitating composting and effective recycling of solids wastes. By controlling soil borne diseases and improving the soil health and soil properties these organisms help not only in saving, but also in effectively utilizing chemical fertilizers and result in higher yield rates.

2.5.1 Type of biofertilizers

Following are the type of biofertilizers:

- **Phosphate solubilizers**
  Bacillus
  Pseudomonas
  Aspergillus
  Penicillium

Trichoderma

- **Nitrogen Fixing bacteria**

  Rhizobium

  Azotobacter

  Azospirillum

  Blue green algae

  Azolla

Table 2.1 Crop varieties to which BFs have been applied (Dixit, 2002)

BFs	Beneficiary Crops.
Rhizobium	Crop specific BFs for legume like groundnut, soybeans, red gram, green gram, black gram, lentil, cowpea, Bengal ran, and fodder legumes
Azospirillium	Cotton , vegetables , mulberry , plantation crop , rice, wheat , barley ,ragi, jowar, mustard, sunflower ,tobacco, fruit, spices, condiment, ornamental flower
Azospirillium	Sugarcane, vegetables, maize, pearl millet, rice, wheat, fodders, oil seed, fruit and flower.
Blue green algae	Rice, banana
Azolla	Rice
PSM	All crops (nonspecific)
VAM fungi	For variety of plant

2.6. PLANT GROWTH PROMOTING BACTERIA

Plant growth promoting rhizobacteria are free living organisms that have beneficial effects on plant. They enhance soil fertility by increasing the amount of available nitrogen and phosphorus and other plant nutrients and synthesize several different phytohormones that can act to enhance various stages of plant growth and also suppress soil borne pathogens by antagonizing them. In recent years, the concept of PGPR mediated plant growth promotion is gaining worldwide importance and acceptance.

2.6.1. Beneficial traits of PGPRs

2.6.1.1. Nitrogen fixation

Among the free living nitrogen fixing bacteria *Azospirillum* is considered to be more efficient with nitrogenase properties comparatively better than the other nitrogen fixers. Nitrogen fixation is the first major mechanism for the enhancement of plant growth by *Azospirillum* (Prasad and Govindarajan, 2001).

- **Nitrogen fixing bacteria**

  Greenhouse and field trials conducted in many places with different crops have revealed that biological nitrogen fixation by root associated *Azospirillum* contribute significant amounts of nitrogen to the plants thereby saving valuable nitrogenous fertilizers. The results also revealed that *Azospirillum* inoculation benefits plant growth and increases yield of crops by improving root development, mineral uptake and plant water relationship (Okon, 1985).

2.6.1.2. Phosphate solubilization

Several heterotrophic and chaemoautotrophic bacteria, fungi and actinomycetes have the capacity to solubilize insoluble mineral phosphates. Many genera of bacteria such as *Achromobacter, Agrobacterium*, *Bacillus*, *Pseudomonas*, *Serratia* and several others have been reported to solubilize varying quantities of phosphorus depending on the efficiency of the strains. The most efficient and dominant solubilizers belong to genera *Bacillus* and *Pseudomonas* (Gaur, 2002), *Aspergillus* (Reddy et al. 2002).

- **phosphate solubilizing bacteria (PSB)**

Phosphate solubilizing microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P soil and make it available to the plants. Phosphate solubilizing microorganisms include largely bacteria, fungi which can grow in media containing tricalcium, iron and aluminum phosphate, hydroxpatite, rock phosphate and similar insoluble phosphate compounds as the sole phosphate source. Such microbes not only assimilate P but a large portion of soluble phosphate is released in quantities in excess of their own requirement. Phosphate solubilizing microorganisms play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers.

The establishment and performance of PSB is however affected severely under stressed condition such as high salt, pH and temperature prevalent in degraded ecosystems represented by alkaline soils with tendency to fix phosphorus. Phosphatase and phytase enzymes secreted by these microorganisms having important role in phosphate solubilization (Achal *et al.* 2005).

Therefore, for agronomic utility, inoculation of plants by target microorganisms at a much higher concentration than those normally found in soil is necessary to take advantage of their beneficial properties for plant yield enhancement (Richa *et al.* 2003).

- **Mechanisms in phosphate solubilization**

Phosphorus Solubilizing Microorganisms are reported to dissolve insoluble phosphates by the production of inorganic or organic acids and/or by decrease the pH (Whitelaw, 2000). Most of the previous reports state that calcium phosphates are dissolved by acidification. Therefore, any microorganism that acidifies its external medium will show some level of phosphorous solubilizing activity (Pradhan *et al.* 2006). It is suggested that the release of H^+ to the outer surface in exchange for action uptake or with the help of H^+ translocation ATPase could constitute alternative ways for solubilization of mineral phosphates.

Production of organic acids and lowering of PH

Phosphorus solubilizing activity is determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe *et al.* 1998). Phosphate solubilization takes place through various microbial processes/mechanisms including organic acid production and proton extrusion (Surange, 1995; Nahas, 1996). Phosphate solubilizing microorganisms convert the insoluble phosphates into soluble forms through the processes of acidification, chelation and exchange reactions (Gerke, 1992). Therefore the applications of P-solubilizing microorganisms (Kucey *et al*. 1989) is a promising approach.

It has been repeatedly shown that low molecular weight organic acids can strongly increase the concentration of phosphorus in solution by mechanisms involving chelation and exchange reactions (Gerke, 1992). Phosphatases are required for the mineralization of organic forms of soil P to release phosphate (Ragothama, 1999). A wide range of phosphatases with differing substrate specificities have been characterized in plant roots (Bosse and Kock, 1998) and soil microorganisms (Richardson, 1994).

Chelation

Chelation involves the formation of two or more coordinate bonds between an anionic and 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. By

complexing with cations on the mineral surface, organic acid anion loose in cation-oxygen bonds of mineral structure and catalyze the release of cations to solution (Welch *et al.,* 2002).

Enzyme production

Phosphate solublizing bacteria produce enzymes like phytase and phosphatases which are beneficial of phosphate solubilization. Phytases, myo-inositol hexakisphosphate phosphohydrolase are classified as the family of histidine acid phosphatases. Phytates account for a large component of the organic P some 20-50% of the total soil organic P yet appear to be only poorly utilized by plants (Richardson *et al*. 2000). Phytase catalyses the de-esterification of phytic acid to myo-inositol and free ortho-phosphoric acid (lovleen *et al*. 2007).

Mineralization of most organic phosphorus compounds that may constitute up to 30-50% of the total phosphorus in most soils is carried out by means of phosphatase enzymes, primarily acid phosphatases. A phosphatase is an enzyme that dephosphorylates its substrate. Alkaline phosphatase is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins. Acid phosphatase is a type of enzyme used to free attached phosphate group from other molecules during digestion (loveleen *et al.* 2007).

Factors effecting solubilization of phosphorus

The suggested possible mechanisms of solubilization are: the production of gluconic acid, of citric acid or the H^+ pump. These mechanisms are influenced by the N, P and C sources. Phosphorus solubilization is a complex phenomenon, which depends on many factors such as the nutritional, physiological and growth conditions of the cultures.

- **Carbon sources**

  Phosphate solubilizers need a carbon source and energy for both the synthesis of new cell material and the oxidation of carbon compounds (Moat and Foster, 1988). Rhizosphere soils present water-soluble C and N compounds mainly as carbohydrates and organic acids and a small portion of amino acid (Ryan *et al*. 2001). It is well known that a considerable number of microorganisms were associated with the plant rhizosphere due to its carbon concentration. Decomposition of plant residues is a mechanism by which carbon sources are recycled in the soil (Ely Nahas, 2003). Organic constituents of plants as cellulose, hemicelluloses and lignin are the most abundant polymers that need be degraded by the microorganisms to provide the carbon sources in the soil (Romano, 2000).

- **Nitrogen Sources**

  The nitrogen source influences the phosphate solubilization. When used as sole source of nitrogen, KNO_3 is less effected as compared to $(NH_4)_2SO_4$. Phosphate solubilizing microorganisms solubilize a good amount of insoluble phosphate in the presence of a nitrogen source. Ammonium sulfate reduces the pH to 3.31 of medium which is beneficial for the phosphate solubilization.

- **pH**

  The optimum pH for maximum solubilization of inorganic phosphate is neutral or slightly acidic in case of bacteria. In general phosphate solubilization was associated to the pH decrease (Fankem *et al.* 2006).

- **Temperatue**

  The optimum temperature for phosphate solubilization is $25^{\circ}C$-$30^{\circ}C$ in case of bacteria and $30^{\circ}C$- $37^{\circ}C$ in case fungi.

2.6.1.3. Mechanisms of biocontrol

In general, competition for nutrients and niche, parasitism and antibiosis through the production of antibiotics, siderophores and other metabolites have been suggested as the major mechanisms of action of antagonistic bacteria and other microorganisms in the biological control of various plant diseases.

- **Siderophores**

Siderophores are low molecular weight iron-binding ligands which can bind to ferric ion and make it available to the producer microorganism (Neliands, 1981). Soil pseudomonads generally produce fluorescent, yellow-green, water soluble siderophores. They can be either pyoverdins or pseudobactins. The production of these siderophores has been linked to the disease suppression potential of certain fluorescent pseudomonads. Fluorescent siderophores that have very strong affinity for ferric ions are secreted during growth under low iron conditions. The resulting ferric siderophore complex is unavailable to the other organisms but the producing strain can utilize this complex via very specific receptor in the outer cell membrane (Hemming, 1986).

Bacteria like *Pseudomonas sp* play vital role in stimulating plant growth and in controlling several plant diseases. They function as a biocontrol agent by depriving the pathogens from iron nutrition, thus resulting in increased yield of crop. Growth and siderophore production by PGPR is attributed to organic acids, sugars, amino acids, minerals, enzymes and several other

components of root exudates. Effects of root exudation on microorganisms is of prime importance as it attracts beneficial organisms and has major influence on the diversity of bacteria in rhizosphere. Any factor influencing either the growth or siderophore production by PGPR would greatly influence the efficacy of that PGPR in plant growth promotion and disease suppression (Mhaske *et al.* 2004).

- **HCN production**

HCN inhibits the electron transport thereby the energy supply to the cell is disrupted leading to the death of the organism. It inhibits proper functioning of enzymes and natural receptors by reversible mechanism of inhibition. It is also known to inhibit the action of cytochrome oxidase (Gebring *et al.* 1993). HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Defago *et al.* 1990).

2.6.1.4. Indole acetic acid production

PGPR can exhibits a variety of characteristics responsible of influencing plant growth. The common traits include production of plant growth regulators (auxin, gibberellins, ethylene etc Indole Acetic Acid is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism by several microorganisms including PGPR (Farah Ahmad, 2004)

Bacteria inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrate exuded from the roots compared with non-rhizospheric soils.

Promotion of root growth is one of the major markers by which beneficial effects of plant growth promoting bacteria is measured. Rapid establishments of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, are advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environments, thus enhancing their chances for survival. Most root-promoting bacteria synthesize IAA, and their effect on plants mimics that of exogenous IAA (Bernard *et al.* 2002).

2.7. INOCULATION EFFECT OF PGPR ON CROP PLANTS

The use of PGPR as inoculants has been found to increase growth, yield and phosphorus uptake by many crop plants. The field and pot trials with PGPR with or without phosphate fertilizers, TCP, MRP, pyrite or hydroxyl apatite showed increase in yield and P uptake from marginal to significant levels (10 – 27%) (Altomare *et al.* 1999). Alagawadi and Gaur (1988) reported

increase in nodulation, nitrogenase activity, dry matter yield, P uptake and grain yield of chickpea plants as well as available P content in soil due to inoculation of *Pseudomonas striata* and *Bacillus polymyxa* as compared to uninoculated control. Increase in grain yield AND p uptake of maize and wheat crop under field condition has also been reported by (Himani and Reddy, 2011).

Kundu *et al.* (2002) studied host specificity of phosphate solubilizing bacteria isolated from different crop rhizospheres and observed greater establishment of the isolates in the rhizosphere of their respective crop plants than other plants. Afzal *et al.* (2005) reported increased yield and P uptake of wheat plants due to inoculation of mixture of *Pseudomonas* and *Bacillus* spp.

CHAPTER 3
MATERIALS AND METHODS

3.1. Collection of soil samples

Soil samples were collected from rhizosphere of potato plants grown in potato field of TIFAC CORE, Thapar university, Patiala. For this purpose the plants were uprooted carefully, shoot portion cut off and roots along with the rhizosphere soil aseptically in small plastic bags / bottles were brought to the laboratory and prior to their processing kept at 4^0C.

3.2. Physiochemical analysis of soil samples

Various physiochemical properties like Electric conductivity (EC), total dissolved solids (TDS), pH, Oragnic Carbon, Total P, Available P and total nitrogen were determined.

3.2.1. Determination of pH

pH of soil was measured potentiometrically in a 1:2 or 1:5 soil water suspension or in a saturated soil plates.

Procedure

1. 25g of air dried soil samples were weighed and taken in a 100 ml beaker.
2. Added 50 ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.
3. Further, it was kept in shaking condition (120 rpm) for 2 hours.
4. Suspension was allowed to settle down for 30 min.
5. Mean while, pH meter was switched on and checked with two buffer solutions of known pH viz. one acidic and other alkaline with the help of standardization knob.
6. The pH of sample was measured by immersing the electrode in supernatant solution and recorded when the reading was stabilized (usually after 30 sec).
7. The electrode was rinsed with distilled water and carefully wiped with filter paper for every sample.

3.2.2 Determination of Electrical conductivity (EC) and Total Dissolved Solids (TDS) of the soil

Electrical Conductivity (EC) and Total Dissolved soils (TDS) in soil sample was measured in a 1:2 soil-water suspension.

Procedure

1. 10g of air dried soil samples were weighed and taken in a 100ml beaker.
2. Added 20ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.
3. Further, it was kept in shaking condition (120 rpm) for 30minutes.
4. The suspension was allowed to settle down.
5. The connections of the analysis kit were switched 'on' and its knob was fixed onto electrical conductivity/ total dissolved solids parameter.
6. Both the parameters were measured by immersing the electrode in supernatant solution and recorded when the readings were stabilized.
7. The electrode was rinsed with distilled water and carefully wiped with tissue paper after use.

3.3.3 Available phosphorus (P)

Reagents for the estimation of available phosphorus

1. **0.5M $NaHCO_3$ extracting solution –** 84g of sodium bicarbonate was added in distilled water and volume was made upto 2 litre. The pH was adjusted to 8.5 with 1M or 1N NaOH
2. **Reagent A -** 12.0g ammonium molybdate in 250ml distilled water and 0.2908g antimony potassium tartarate in 100ml distilled water was added to 1000ml of 2.5M H_2SO_4 ,mixed thoroughly and volume was made upto 2 litre with distilled water
3. **Reagent B (freshly prepared) –** 1.058 g of ascorbic acid was added in 200ml of reagent A and mixed
4. **Sulphuric acid (2.5M) –** 140 ml of conc. H_2SO_4 was diluted to 1 litre
5. **Stock standard P solution (50 ppm) –** 0.2917 KH_2PO_4 was dissolved in distilled water to a final volume of 1 litre
6. **Working standard P solution(1 ppm) –** 20ml of 50 ppm solution was diluted to 1 litre

Procedure for estimation of available phosphorus (Olsen *et al*., 1954)

1. 2.5g soil was weighed and 50 ml of extracting solution was added to it.
2. Kept on a shaker for 30 minutes and was filtered through whatman filter paper no. 42
3. 10ml aliquot of filterate was transferred to a 100ml beaker

4. 1ml of 2.5M H_2SO_4, 15.5ml distilled water, 8ml reagent B and again 15.5ml of distilled water was added.
5. After 10 minutes, the intensity of the colour was measured at 882 nm against blank
6. Blank was prepared as above without the soil
7. To prepare standard curve, 0, 2, 5,10, 15 and 20 ml of 50 ppm standard stock solution was measured in 50 ml volumetric flask separately and followed the steps as above.
8. The P concentration of these solutions were 0.04, 0.1, 0.2 ,0.3 and 0.4 ppm respectively. After 10 min read the P concentration at 882 nm.

Calculation

Available P in soil (ppm): P in extract (ppm) × 20 (standard soil to solution ratio)

3.3.4 Total phosphorus (P)

Reagents for the estimation of total phosphorus

Vanadomolybdate solution –

1. **Solution A –**25 g ammonium molybdate [$(NH_4)_6\ Mo_7O_{24}.4H_2O$] was dissolved in 300 ml water in a 500 ml beaker
2. **Solution B –**1.25 g ammonium vanadate (NH_4VO_3) was dissolved in 300 ml boiling water, cooled and 250 ml concentrated HNO_3 was added and cooled again. Solution A was added to solution B and was made upto 1000ml in a volumetric flask.
3. **Phosphorus stock standard solution (50 mg/l) –**0.2195 g of dried KH_2PO_4 was dissolved in distilled water and mixed thoroughly. Acidified with 25 ml of 7N H_2SO_4 and made the volume upto 1 litre to get 50 mg/ml P solution. 4 to 5 drops of toluene was added to prevent microbial activity. (KH_2PO_4 was dried to 100°C for 1 hour and cooled

Sample preparation for elemental analysis

For the release of mineral elements from soil and sediments, di acid (HNO_3- $HClO_4$) oxidation of sample was carried out.

HNO_3/ $HCLO_4$ digestion

1. 1 g sample of air dried soil was weighed in digestion tube and added 10 ml concentrated HNO_3 digest on electric heater for 1hr at 145°C in acid proof digestion chamber having fume exhaust system

2. Allowed to cool it and 5 ml $HClO_4$ was added and heated to about 100°C for the first one and then raised the temperature to about 200°C
3. Continued the digestion until the contents become colourless and only white fumes appeared
4. Reduced the acid contents till white matter remains left in the digestion tube
5. After this removed from the heating mental and cooled and 50% diluted HCl was added and filtered through whatman filter paper no. 42
6. 2 or 3 washings with 50% diluted HCl was given and final volume made was 50 ml with diluted 50% HCl

Procedure for the estimation of total phosphorus in soil and plant samples (Kitson and Mellon, 1944)

Ammonium molybdate reacts under acidic conditions to form a heteropoly acid and molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdate acid is formed. The intensity of colour is propotional to phosphorus concentration.

1. 10 ml of acid digests of soil sample was placed in 50ml volumetric flask, 10 ml of the vanadate molybdate reagent was added and diluted to 50 ml
2. Mixed well and read the phosphorus concentration after 10 minutes using spectrophotometer at 420 nm.
3. Blank was prepared by taking 10 ml of distilled water in place of 10 ml of acid digests of soil sample
4. For standard readings, 0, 1, 2, 3, 4 and 5 ml of 100 mg per litre stock phosphorus solution was taken in 50 ml volumetric flask and the colour was developed as mentioned above
5. Calibrated the spectrophotometer with known phosphorus concentration and read the concentration of the sample.

Calculation

P (mg/kg):

$$\frac{Volume\ make\ up\ after\ acid\ digestion}{Weight\ of\ sample(s)\ to\ develop\ colour\ (ml)} \times \frac{50}{volume\ of\ digest\ used} \times P(mg)\ in\ 50\,ml\ solution$$

3.3.5 Organic carbon

Reagents for the estimation of organic carbon

1. **1N potassium dichromate –** 49.04 g was added in distilled water and volume was made upto 1 litre
2. **0.5N ferrous ammonium sulphate –** 198 g was added in distilled water and volume was made upto 1 litre
3. **Diphenyl amine indicator –** 0.5 g of diphenyl amine indicator (DPA) was dissolved in a mixture of 200ml water and 100ml concentrated H_2SO_4

Procedure for estimation of organic carbon and organic matter (Walkley and Black, 1934)

1. 1 g of soil was taken in 500 ml conical flask and 10 ml of 1 N $K_2Cr_2O_7$ was added.
2. The flask was swirled for mixing the soil and reagent.
3. 20 ml of concentrated H_2SO_4 was added and the flask was allowed to stand undisturbed for 30 minutes after which 200ml of distilled water was added
4. 1 ml of diphenylamine indicator was then added
5. Ultimately the contents were titrated with freshly prepared 0.5N ferrous ammonium sulphate till the end point is observed from blue violet to green.
6. Run a blank without soil sample and followed the steps as above

Calculation

Organic carbon (%): $\frac{10(B-T)\times 0.003\times 100}{B\times \text{weight of soil}(g)}$

Where

B is volume of ferrous ammonium sulphate solution for blank titration

T is volume of ferrous ammonium sulphate solution for soil sample

Because organic matter contains 58% carbon, so

Organic matter (%): Organic carbon (%) × 17.24 (van bemmelen factor)

3.3.6 Total nitrogen

Reagents for the estimation of total nitrogen in soil

1. **Concentrated H_2SO_4**
2. **0.02 N H_2SO_4**
3. **Sulphuric salicylic acid –** 1g salicylic acid was mixed with 30 ml sulphuric acid
4. **Sodium thiosulphate**

5. **4% boric acid –** 4g of boric acid was dissolved in 100 ml of distilled water
6. **Mixed indicator –** 0.066 g of methyl red and 0.099 g of bromo cresol green was dissolved in 100 ml of ethyl alcohol
7. **50 % NaOH**
8. **Digestion mixture –** 10 g HgO , 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20)

Procedure for estimation of total nitrogen in soil (Kjeldahl method given by Piper, 1960)

1. 5 g soil was mixed thoroughly with sulphuric salicylic acid and followed by 5 g of sodium thiosulphate. Heating was carried out for 5minutes followed by cooling and addition of 10 g of digestion mixture. The contents were mixed well in a kjeldahl flask
2. The flask was kept in a digestion chamber at 100°C for two hours
3. The colour change was monitored from dark brown to greenish white after which the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15-20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator was added till the end point colour changes from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 until the colour changed from green to pink.

Calculation

$$\textit{Total N (\%):} \quad \frac{(T-B)\times normality\ of\ H_2SO_4 \times 1.4 \times 300}{Weight\ of\ sample(g)}$$

T is titre value for sample

B is for blank

3.4.Isolation and Screening of Plant Growth promoting Rhizobacteria

3.4.1. Isolation of phosphate Solubilizing Bacteria

10.0g of soil samples was suspended in 90ml of sterilized distilled water and 10^{1} dilution was obtained. Serial dilutions were prepared by mixing 1ml of the suspension made into 9ml sterilized water blanks, until the 10^{7} dilution was obtained. From these dilutions 100.0µl was

spread plated on Pikovskaya's Agar plates. These plates were the incubated at 30^{o}C and were observed for 2-7 days. The total bacterial types were counted after 48 hours of incubation. The PSB showing halo zones of clearance were streaked again on PVK agar plate to check for purity and 'P' solubilizing ability. The pure strains forming zone of clearance were maintained by streaking on nutrient agar slants and stored at 4^{o}C.

Composition of Pikovskayas Agar Medium

Ingredients	**gms/liter**
Yeast Extract	0.50
Dextrose	10.0
Tri Calcium Phosphate	5.00
Ammonium Sulphate	0.50
Potassium Chloride	0.20
Magnesium Sulphate	0.10
Manganese Sulphate	0.001
Ferrous Sulphate	0.001
Agar	15.00
Distilled Water	1000 ml
pH	7.0 ± 0.2

All ingredients except Tri-calcium phosphate $Ca_3 (PO_4)_2$ were dissolved in 1000.0ml of distilled water, pH adjusted to 7.0 and autoclaved at 15Psi for 15 minutes. Tri calcium phosphate was autoclaved separately at 15 Psi for 15 minutes. Later these were mixed together and poured in sterilized Petri plates. After solidification of medium, these Petri-plates were incubated at 37^{0}C for overnight to check contamination.

3.4.2. Isolation of nitrogen fixing bacteria

10.0 g of soil samples was suspended in 90 ml of sterilized distilled water and 10^{1} dilution was obtained. Serial dilutions were prepared by mixing 1 ml of the suspension made into 9ml sterilized water blanks, until the 10^{7} dilution was obtained. From these dilutions 100.0µl was spread plated on Jensen Agar plates. These plates were the incubated at 30^{o}C and were observed

for 2-7 days. The total bacterial types were counted after 48 hours of incubation and were maintained by streaking on nutrient agar slants and stored at 4^{o}C.

Composition of Jensen Media

Sucrose	20.00g/l
Dipotassium phosphate	1.000g/l
Magnesium sulphate	0.500g/l
Sodium chloride	0.500g/l
Ferrous sulphate	0.100g/l
Sodium molybdate	0.005g/l
Calcium chloride	2.00g/l
Agar	15.00g/l
Water	1000ml
pH	7.0

3.4.3. Qualitative assay of Phosphate Solubilizing Efficiency and Phosphate Solubilizing Activity of selected isolates

Bacterial strains showed more than 5 mm zone of solubilization were selected for quantitative assay of P solubilization,

3.4.4. Assay for Quantitative 'P' solubilization in liquid medium

All PSB isolates were tested for their ability to release inorganic 'P' form Tri calcium phosphate. The 'P' in solution was determined by using Calorimetric Chlorostannous reduced molybdo phosphoric acid blue method (Jackson, 1973).

3.4.4.1. Preparation of Chloromolybdic Acid

15.0 g ammonium Molybdate was dissolved in about 400.0ml of distilled water. Filtered and then 400.0ml of 10N HCl was added slowly with rapid stirring. Volume was made to 1000.0ml with distilled water and stored in amber glass bottle.

3.4.4.2. Preparation of Chlorostannous acid

Stock Solution

$SnCl_2$ Crystals	10.0g
Conc. HCl	25.0ml

$SnCl_2$ crystals were dissolved in conc. HCl and solution was kept in glass under airtight stopper.

Working Solution

Fresh working solution was prepared by adding 1.0 ml of the above solution to 132.0 ml of distilled water.

3.4.4.3. Assay procedure for quantitative 'P' solubilization in liquid medium

The PSB were grown in 50 ml NB for 24 hours at 30^{o}C in incubator shaker. 1ml of each PSB was aseptically transferred to 50.0 ml PVK broth contained in 150 ml conical flask. The flasks were incubated at 30^{o}C for 3-7 days incubator shaken at 120 rpm. After 3 days of incubation, 5.0 ml culture was withdrawn from each flask. Cultures were centrifuged at 10,000 rpm for 30min. The supernatant was diluted to 100.0ml with autoclaved distilled water. Then 5.0ml aliquot of each dilution was transferred to 50.0ml volumetric flask. This was followed by addition of 10.0ml chloromolybdic acid, which was added along the sides of the flask. The contents of the flasks were diluted to 40.0 ml with distilled water. Then 5 drops of chlorostannous acid was added. After mixing, the volume was made up to 50.0ml with distilled water. The blue colour intensity of the solution was measured in a spectrophotometer at O.D. 660nm. The soluble 'P' was estimated from standard curve of KH_2PO_4 (0-2 ppm) drawn against O.D. 600 nm.

3.5. Enzyme activities

3.5.1. Acid phosphatase activity

Reagents for estimation of acid phophatase activity

1. **p– nitrophenyl phosphate solution (0.115 M) –** 4.268 g of p- nitrophenyl disodium salt hexahydrate was dissolved in 100 ml of modified universal buffer(MUB) pH 5

2. **NaOH (0.5N)** – 20 g of NaOH was dissolved in distilled water and the volume was made up to 1 litre
3. **p-nitrophenol –** 1mg per ml solution in modified universal buffer(pH 5)
4. **5X modified universal buffer (pH 5) (Skujins *et al*., 1962)**

Tris(hydroxyl methyl) amino methane	12.10g
Maleic acid	11.60g
Citric acid	14.00g
Boric acid	06.28g
NaOH	488 ml

Volume was made upto 1000ml with distilled water

Procedure for the estimation of acid phosphatise activity (Tabatabai and Bremner, 1969)

Phosphatase activity was indicated as the amount of p-nitrophenol released in the filterate from the p- nitrophenyl phosphate substrate per ml of supernatent.

The p- nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.

1. 1 ml supernatant was taken in the test tube
2. 4 ml of modified universal buffer (pH 5) and 1 ml of filter sterlized 0.115M p- nitrophenyl phosphate solution was added to the flask
3. The tubes were swirled for few seconds to mix the contents
4. The tubes were stoppered and incubated at 37°C for 1hour in dark
5. 4 ml of 0.5M NaOH was added to stop the reaction
6. The intensity of the yellow colour formed was measured at 410 nm
7. To perform control the above procedure was followed without the sample and 1 ml p-nitrophenyl phosphate was added after 0.5N NaOH

3.5.2. Alkaline phosphatise activity

Reagents for the estimation of alkaline phosphatise activity

1. **p–nitrophenyl phosphate solution (0.115 M)** –4.268 g of p-nitrophenyl disodium salt hexahydrate was dissolved in 100 ml of modified universal buffer (MUB) pH 9
2. **NaOH (0.5N)** – 20 g of NaOH was dissolved in 100 ml distilled water

3. **p-nitrophenol solution (1 mg/ml)–** in modified universal buffer (pH 9)

   5X modified universal buffer (pH 9) **(Skujins *et al*. 1962)**

Tris(hydroxyl methyl) amino methane	12.10g
Maleic acid	11.60g
Citric acid	14.00g
Boric acid	06.28g
NaOH	488ml

Procedure for the estimation of alkaline phophatase activity (Tabatabai and Bremner, 1969)

Phosphatase activity was indicated as the amount of p-nitrophenol released inthe filterate from the p- nitrophenyl phosphate substrate perml of substrate. The p- nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0,10,20,30,40 and 50µg of p-nitrophenol.

1. 1 ml supernatant was taken in the test tube.
2. 4 ml of modified universal buffer (pH 9) and 1 ml of filter sterlized 0.115 M p- nitrophenyl phosphate solution was added to the tube.
3. Tubes were swirled for few seconds to mix the contents
4. The tubes were stoppered and incubated at 37°C for 1hour in dark
5. 4 ml of 0.5M NaOH was added to stop the reaction
6. The intensity of the yellow colour formed was measured at 410 nm
7. To perform control followed the above procedure without the soil sample and the addition of 1 ml p- nitrophenyl phosphate was made after 0.5N NaOH
8. Phosphate activity was indicated as amount of p-nitrophenol released in the filterate from p-nitrophenyl phosphate substrate per ml of sample.
9. Data was recorded as microgram p-nitrophenol per ml.
10. Phosphatase activity was calculated in the unit of µM PNP/ml/hour.

Phytase activity (Kim and Lei 2005)

Reagents

1. Citrate buffer (0.2M*)*, pH 5.5

2. Sodium phytate 1% (9 mM) in 0.2 M citrate buffer, pH 5.5

3. Trichloroacetic acid (TCA) 15% (w/v) (room temperature)

4. Color reagent (prepared fresh daily): mixed three volumes of 1 M sulfuric acid and one volume of 2.5% ammonium molybdate, one volume of 10% ascorbic acid (w/v) and mixed well.

5. KH2PO4 solution 9 mM was prepared. Then made a series of dilutions: 1:100, 1:200, 1:400, 1:800 and 1:1600 (corresponding to 90, 45, 22.5, 11.25, and 5.625 μM phosphate, respectively).

Procedure

1. Three aliquots (0.2 ml each) of samples were taken in 10 ml test tubes and incubated the test tubes in 37 °C water bath for 5 min.

2. 0.2 ml of 1% (w/v) sodium phytate in the citrate buffer was added to start the enzymatic hydrolysis of phytate. Incubated for 15 min at 37 °C.

3. The reaction was stopped by adding 0.4 ml of 15% TCA (room temperature).

4. Centrifuged the mixture at 3,000 rpm for 10 min and transferred the supernatant fraction to a new tube.

5. Mixed 0.2 ml of supernatant fraction with 1.8 ml of MiliQ water (Millipore).

6. Added 2.0 ml of fresh color reagent to each tube and mixed well. Incubated the mixture at 50 °C for 15 min and took the tubes to room temperature.

7. Read the absorbance of each sample solution at 820 nm, using water as the blank and the series diluted potassium phosphate solutions as standards.

Calculated the phytase activity per ml of sample. Enzyme activity was expressed as micromoles of inorganic P released per hour per milliliter of culture material from sodium phytate at 37 °C.

3.6. Indole acetic acid production

Reagents for the estimation of indole acetic acid (IAA) activity

1. **0.5M $FeCl_3$** – dissolved 810 mg $FeCl_3$ in 10 ml distilled water
2. **35% $HClO_4$-**50 ml $HClO_4$ (70%) M was mixed with 50 ml of distilled water
3. **Tryptophan**- 0.1 % w/v
4. **Salper's reagent –** mixed 1 ml of 0.5M $FeCl_3$ with 50ml of 35% v/v $HClO_4$ and the total volume was made upto 51ml. this reagent was freshly prepared.

Procedure for estimation of IAA activity with and without tryptophan (Gordon and Paleg, 1957)

1. 1% of the bacterial inoculum was inoculated in 5ml nutrient broth both with and without tryptophan (0.1%) in test tubes for 12 – 14 hrs
2. 2ml of the culture was taken and centrifuged at 10,000 rpm for15 min and supernatant was filtered through whatman filter paper
3. 1 ml of supernatant of each isolate was taken in separate test tubes and 2 ml salper's reagent was added dropwise but rapidly with continous mixing in each tube.
4. The samples were placed in dark for 30 minutes.
5. Development of pink colour was assayed with spectrophotometer at 535 nm

3.7. HCN production activity

HCN production was tested by the method of Bakker and Schippers(1987).

Reagents

1. **Nutrient Agar:** 28.0 g/l
2. **Glycine:** 4.4 g/l
3. **2% sodium carbonate:** 2g sodium carbonate in 100ml
4. **0.5% Picric Acid:** 0.5g Pecric acid in 100ml

Procedure of HCN production activity

1. Bacterial culture was inoculated on petridish containing Nutrient Agar supplemented with Glycine
2. A whatman filter paper soaked in 2% w/v sodium carbonate in0.5%(w/v) picric acid solution was plate dinside the lid of a petriplate3.
3. The plate was then related with parafilm and incubated at30^0C for 2days.4.
4. A change in the filter paper colour from yellow to reddish brown was considered to be an indicator of HCN production.

3.8. Biochemical characterization of selected isolates

Biochemical characteristics of selected bacterial isolates will be studied by performing:

- Gram Staining
- Methyl Red test(MR test)
- Urease Test
- Catalse Test
- Carbohydrate fermentation test
- Citrate utilization test
- Indole production test
- Cellulose test

Procedure of biochemical tests

3.8.1. Gram Staining

1. Bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times**.**
2. The slides were flooded with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute.
3. The slide were washed with water and decolorized with 95% ethyl alcohol with the help of dropper until no violet colour was visible from drain off solution.
4. The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water.
5. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter.

6. Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

3.8.2. Indole production test

1. Prepared 1% tryptone broth by dissolving 10g of peptone in one litre of distilled water. Sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Inoculated one tube with the test organism, second with *E.coli* and other one uninoculated as control.
3. The tubes were then incubated at 37^0C for 48 hours.
4. Added 1 ml of kovac's reagent to each tube and shake them after intervals for 10-15 minutes
5. Allowed the tubes to stand to permit the reagent to come to the top.
6. Development of cherry red colour in the top layer of the tube indicates a positive test while the absence of this colour indicates negative test.

3.8.3. Methyl-Red and Voges-Proskauer (MRVP) tests

1. Prepared MRVP broth (pH 6.9) tubes and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Inoculated one tube with the test organism, second with *E.coli* and other one uninoculated as control.
3. The tubes were then incubated at 37^0C for 48 hours.
4. For methyl red test, added 5 drops of methyl red indicator to the each set of the tube.
5. For Voges-proskauer test added 12 drops of V-P reagent 1 and 2-3 drops of V-P reagent 2.
6. Shook the test tubes gently for 30 seconds with caps off to expose the media to oxygen.
7. Allowed the VP reaction to complete for 15 minutes.
8. The formation of red color in methyl red test indicates positive result while turning of methyl red to yellow is a negative test.
9. The development of crimson to ruby pink colour is positive while no change in colour indicates a negative result for VP test.

3.8.4. Carbohydrate fermentation tets

1. Prepration of fermentation medium with different carbohydrate such as glucose, sucrose,mannitol,fructose and lactose

2. Label each of specified fermentation tubes of media with the name of the organisms to be inoculated.
3. Inoculate the different sugar fermentation broth with each bacterium and keep one uninoculated tube of each fermentation broth as a comparative control.
4. Incubate all the inoculate and uninoculatesd tubes at 35^0C for 24-48hrs.

3.8.5. Citrate utilization test

1. Prepared Simmon's citrate agar slants (pH 6.9) in tubes and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Inoculated one tube with the test organism, second with *E. aerogens* and other one uninoculated as control.
3. The tubes were then incubated at 37^0C for 48 hours.
4. Change in the medium from green to blue is positive result while no change in colour is negative result.

3.8.6. Urease test

1. Prepare urea agar medium and urea broth
2. Inoculate a loopful organisms in both agar medium and broth.
3. Incubate for 24-48hrs at 37^0C
4. A pink colour show the positive result.

3.8.7. Catalase test

1. Take 2-3ml of hydrogen peroxide in test tube
2. Take a colony of test organisms and immerse it into hydrogen peroxide solution.
3. Formation of bubbles indicate oxygen production.

3.8.8. To check Antibiotic resitance activity

Procedure for Ab resistivity

1. Adjust turbidity according to 0.5 Macfarland (add 450µl of overnight grown culture) with saline.
2. Take 100µl and spread on MHA plate
3. Make well with the help of cork bore.
4. Fill these wells with 450µl resistant antibiotic
5. Incubate it at37^0C for 24hrs

3.9. Evaluation of efficient strains under field conditions

A field experiment was conducted in the Thapar university, Patiala to check the effect of selected effeicient strains on plants growth and soil fertility. Maize Kohinoor variety was selected for the field study. The isolates used in this field study were selected based on their efficiency of nitrogen fixation/P-solubilization and/or production of growth promoting substances under *in vitro* conditions. Bio inoculants were applied in the soil as seed inoculations.

Table 3.1: Different field treatments

S. No.	Treatments
Treatment 1	Soil
Treatment 2	Soil + PSB-3
Treatment 3	Soil + PSB-4
Treatment 4	Soil + DN-3
Treatment 5	Soil + DN-13

3.9.1 Seed inoculation

Reagents for preparation of slurry

1. **10% sugar solution** – dissolved 3 g of sugar in 30 ml of sterile distilled water
2. **40% gum arabic-** dissolved 12 g of gum arabic in the above solution and mixed well

Methodology for preparation of inoculum

1. Inoculated 1000 µl of bacterial inoculum in 500 ml broth culture
2. Kept in 37°C at 130 rpm for 3-5 days
3. Centrifuged at 8000 rpm for 5 minutes
4. Prepared bacterial inoculums in steri distilled water
5. Repeat the last two steps thrice

Methodology for preparation of slurry

1. Added 10g sugar in 100 ml sterile distilled water and heat the solution
2. After heating , added 40 g of gum arabic in hot solution
3. Mixed well and made a slurry

4. Cool at room temperature
5. And add the inoculum to slurry and mixed well

Seed treatment

Reagents for the seed treatment

1. Maize mature seeds harvested from field-grown plants.
2. 80% Ethanol (~300 ml for ~200 seeds).
3. 50% bleach solution: mix 450 ml of commercial bleach (5.25% hypochlorite) with 450 ml of Millipore water containing 2 drops of the surfactant Tween-20. Use ~900 ml for ~200 seeds.
4. 15% bleach solution: mix 15 ml of commercial bleach (5.25% hypochlorite) with 85 ml of Millipore water containing 1 drop of the surfactant Tween-20. Use ~100 ml for ~200dissected embryos.
5. Millipore water (autoclaved).

Seed treatment protocol

1. Place 50 seeds in a 250 ml beaker along with a stir bar.
2. Add ~ 75 ml of 80% ethanol, cover with the aluminum foil and place the beaker on a stir plate. Stir at medium speed for 3 minutes.
3. Take the beaker to the flow bench and decant the ethanol into a liquid-waste container.
4. Add ~ 75 ml of 50% bleach solution, cover with the aluminum foil and stir for 15 minutes on medium speed.
5. In the flow bench, decant the bleach into the liquid-waste container.
6. Sterilize the seeds a second time by repeating Steps 4 and 5.
7. Rinse the seeds 5 times with sterile Millipore water (~ 75 ml each time).
8. After the last rinse, keep seeds in ~50 ml sterile water (just enough to cover the seeds – do not overfill the container), cover with aluminum foil and leave the beaker inside the flow bench for 24 hours.

Seed inoculation

Seed inoculation of PGPR is done by mixing the PGPR culture i.e inoculum with slurry to whish treated seeds are added with the result a uniform coat of PGPR culture around is formed the seed. The inoculated seeds were dried in shade and then sown in the field.

Field experiment was conducted in Thapar university Patiala on maize crop in which selected isolates were amended in soil as seed inoculation. Five field plots measuring 2x2 m^2 each were Prepared. The plots with different treatments were arranged in a randomized complete block design with three replicates per treatment. Maize was sown in first week of March. Size of inocum on each seed was 2×10^5 to 2.5×10^5. During the field study irrigation was done once before maize was sown to have adequate soil water storage for seedling establishment. The plants were irrigated regularly. No fertilizer was added. The crop was harvested in first week of July and studied for various parameters such as groth parameters and P content in the plant parts. From each plot, ten randomly selected plants were uprooted and checked for shoot height. The plants were oven dried at 65 °C for 24 hours and measured for shoot weight and root weight and total P (Kitson and Mellon 1944). The soil was analyzed for its pH, EC, organic carbon (Walkley and Black 1934), total P (Kitson and Mellon 1944), available P (Olsen et al. 1954), total N content and phosphatase (Tabatabai and Bremner 1969) and phytase activity (Kim and Lie 2005). One week after before harvesting, the viability of inoculated bacteria in the soil was determined by isolating PSB bacteria using serial dilution technique.

3.9.2 Enzyme activities of soil

3.9.2.1 Acid phosphatase activity

Reagents for estimation of acid phophatase activity

5. **p– nitrophenyl phosphate solution (0.115 M) –** 4.268 g of p- nitrophenyl disodium salt hexahydrate was dissolved in 100 ml of modified universal buffer(MUB) pH 5
6. **NaOH (0.5N)** – 20 g of NaOH was dissolved in distilled water and the volume was made up to 1 litre
7. **p-nitrophenol –** 1mg per ml solution in modified universal buffer(pH 5)
8. **5X modified universal buffer (pH 5) (Skujins *et al*., 1962)**

Tris(hydroxyl methyl) amino methane	12.10g
Maleic acid	11.60g
Citric acid	14.00g
Boric acid	06.28g
NaOH	488 ml

Volume was made upto 1000ml with distilled water

Procedure for the estimation of acid phosphatise activity (Tabatabai and Bremner, 1969)

Phosphatase activity was indicated as the amount of p-nitrophenol released in thefilterate from the p- nitrophenyl phosphate substrate per gram of soil.

The p- nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.

8. 1 g of air dried soil was weighed and transferred to flask
9. 4 ml of modified universal buffer (pH 5) and 1 ml of filter sterlized 0.115M p- nitrophenyl phosphate solution was added to the flask
10. The flask was swirled for few seconds to mix the contents
11. The flasks were stoppered and incubated at 37°C for 1hour in dark
12. 4 ml of 0.5M NaOH was added to stop the reaction
13. Mixture was swirled and filtered through whatman filter paper no.2
14. Filterate was transferred to the glass cuvettes
15. The intensity of the yellow colour formed was measured at 410 nm
16. To perform control the above procedure was followed without the soil sample and 1 ml p-nitrophenyl phosphate was added after 0.5N NaOH

Calculation :

Phosphatase activity (µM PNP/g /hour): $\frac{Concentration\ of\ PNP\ (\mu M)}{2 \times Weight\ of\ sample\ (g)}$

3.9.2.2 Phytase activity in soil (Kim and Lie method 2005)

Extraction

1. Weighed 5g of sample into a 250 ml shaking bottle and 10 ml of sodium bicarbonate (0.5M) extractant was added.
2. Shook for 30 minutes and centrifuged for 5 minutes at 12000 rpm.
3. Took supernatant in aliquots of 0.2 ml and determined the phytase activity by method given by Kim and Lie (2005) as described previously.

3.10. Statistical analysis

Three replicates were used for each experiment. The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey‘s test at $P < 0.05$. In the field experiment the plots with different treatments were arranged in a randomized complete block

design with three replicates per treatment. Data was analyzed by ANOVA and the means were compared using Tukey‘s test at $P < 0.05$. All the analysis was performed by using GraphPad Prism 5.0 software.

CHAPTER 4

RESULTS AND DISCUSSION

The nutrient status of cultivated soil is major contributing factor for optimal yield of seasonal crops. Irrigated cultivated soils with reference to exogenous applications of commercial/synthetic fertilizers have long term toxic effects on microbial flora and fauna. Leaching of these additives have tendency to magnify the residual load of synthetic fertilizer and soil-water table. The long term prophylactic measures include bio-organic management practices to maintain the productivity status of cultivated soils.

4.1. Physiochemical properties of soil

For the isolation of PGPRs soil samples were collected from rhizospheric soil of five potato plants grown in potato field of TIFAC, CORE, Thapar university, Patiala. Prior to the processing for PGPR isolation, soil samples were tested for their physiochemical properties like pH, TDS, EC, Organic carbon, total phosphorus, available phosphorus, total nitrogen were determined (Table 4.1).

Table 4.1: physiochemical properties of soil samples

pH	TDS ($mScm^{-1}$)	E.C. ($mScm^{-1}$)	Organic carbon (%)	Available P (mg kg^{-1})	Total P (mg kg^{-1})	Total nitrogen (%)
8.15±0.04	0.14±0.04	0.16±0.02	0.33±0.10	0.15±0.008	274.9±8.4	0.15±0.12

Values are mean ± SD (n=3).

4.2. Isolation of Phosphate Solubilizing Bacteria

Significance of major nutrients (N, P, and K) is interrelated to permeation of soil base; soil bound solubilized inorganic/organic sample and permeable nutrients which can be accomplished by judicious monitoring of macronutrients. Phosphate-solubilizing bacteria have

the specific contribution in rhizospheric soil bio-aggregates to coordinate with various other macro and micro nutrients.

Use of Pikovskayas medium for isolating Phosphate Solubilizing Bacteria (PSB) was a simple way to detect PSB through the formation of halo (zone) on agar plates containing Tri calcium phosphate as a sole 'P' source. Glucose was used as the preferred organic source for the isolation of PSB from the rhizosphere.

To isolate the Phosphate Solubilizing Bacteria, five soil samples were collected from rhizosphere of potato plants. From the rhizospheric soil bacteria selected for quantitative analysis. A total of 12 bacterial isolates showed halo zone on PKV agar plates (Pikovskayas, 1948) and selected for further study (Fig. 4.1, 4.2 and 4.3).

4.3. Isolation of nitrogen fixing bacteria

Nitrogen fixing bacteria were isolated by serial dilution method on Jensen agar plates. A total of 8 colonies were showed positive growth on Jensen agar plates (Jensen, 1942). On the basis of morphology 5 colonies were of similar morphology and 3 colonies were of again similar morphology so total 2 colonies of different morphology were selected as nitrogen fixing bacteria.

Only the phosphate in a soluble ionic form (Pi) is effective as a mineral nutrient (Ae, 1991; Kucey, 1989). Several attempts to overcome the 'P' deficiency problems including the application of chemical 'P' fertilizers have been very effective due to high refixing ability of phosphate in the soil (Goldstein *et al.,* 1993). Rhizospheric bacteria are known to play a very significant role in plant growth promotion by different mechanisms, one of them being the ability to solubilize mineral phosphate in rhizosphere, thus make it available for plant uptake (Gyaneshwar *et al.,* 2002).

Root exudates are known to serve as a substantial source of reduced carbon compounds which are released in rhizosphere. Microbes in rhizosphere utilize root exudates as their major nutrient source and this forms the basis of rhizosphere colonization. Maximum exudation occurs near the root tip so large numbers of bacteria occur near the growing root area. Thus, the

rhizospheric zone for the isolation of Mineral Phosphate Solubilizing (MPS) bacteria is very effective (Patel *et al.*, 2008).

4.4. Qualitative screening of isolated Phosphate Solubilizing Bacteria

Screening of bacterial isolates for phosphate solubilization revealed variations among different groups of organisms. Table 4.2 shows the selection of efficient Phosphate Solubilizing Bacterial isolates on qualitative basis. Similar criteria of selection of efficient PSB were followed by Oswal and Bhide, 1992.

Fig. 4.1

Fig. 4.2

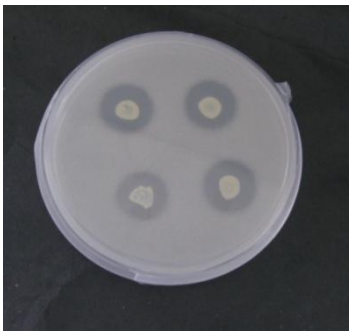

Fig. 4.3

Fig. 4.1 Control (Pikovskayas Agar Medium

Fig. 4.2 & 4.3 Point inoculation of the selected 'P'-Solubilizers, for isolation of effective zone form

Table 4.2 Selection of efficient Phosphate Solubilizing Bacterial isolates obtained from rhizosphere of selected plants

Crop (Dec-March)	No. of soil samples	Total No. of 'P' Solubilizing Bacteria	Total No. of nitrogen fixing bacteria	No. of more efficient bacteria (<5mm zone of solubilization)
Potato	1	12	8	20

4.5. Phosphate Solubilization in liquid medium

After confirming the Phosphate Solubilizing Activity on solid medium, the phosphorus solubilization in liquid medium (PVK Broth) was confirmed. Different investigators have used various media for studying phosphate solubilization in liquid medium. Pradhan and Sukla, (2006) found a suitable medium formulation as an ideal one for new isolates. Considering amount of glucose used in medium and corresponding efficacy of 'P' solubilization, PVK medium proved to be most effective without compromising the solubilization.

Data presented in Table 4.3 and 4.4 summarizes the amount of 'P' (µg/ml) solubilized in PVK liquid medium containing Tri calcium phosphate, rock phosphate, aluminium phosphate and ferrous phosphate (equivalent to 100 mg P_2O_5/100 ml). All bacteria tested were found to be solubilizers of Tri calcium phosphate, rock phosphate, aluminium phosphate and ferrous phosphate (equivalent to 100 mg P_2O_5/100 ml) in PKV broth. The 'P' content released into the medium from Tri calcium phosphate, rock phosphate, aluminium phosphate and ferrous phosphate (equivalent to 100 mg P_2O_5/100 ml) were given in the Table 4.3 and 4.4. Results showed that isolates PSB-3, PSB4, DN-3 and DN-13 showed maximum P solubilization in liquid medium. It was evident that in the medium with Tri calcium phosphate, the values of dissolved phosphate obtained with all the isolates were convincingly showing that the tested isolates have effectively converted the inorganic, insoluble phosphate into soluble form and were selected for further studies.

Table 4.3 Phosphate Solubilizing Ability of selected Bacterial isolates at day 7th of incubation in PKV broth supplemented with tri calcium phosphate and rock phosphate (100 mg P_2O_5/100 ml)

Bacterial isolates	Tri calcium phosohate		Rock phosphate	
	pH	'P'-released in medium (µg. P. ml^{-1})	pH	'P'-released in medium (µg. P. ml^{-1})
PSB-1	5.73 ± 1.3ab	135±3g	6.82±0.04a	99±2e
PSB-3	**4.13 ± 0.9abc**	**396±13a**	**3.94±0.04i**	**295±7a**
PSB-4	**5.32 ± 1.9abc**	**365±4b**	**3.96±0.12i**	**298±7a**
PSB-5	4.75 ± 0.2abc	325±4c	53.86±0.18abcde	141±7c
PSB-6	5.91±1.2ab	337±5c	4.67±0.11fghi	118±3d
PSB-11	4.55±0.1abc	329±3c	5.65±1.02bcdef	118±3d
PSB-12	4.67±0.54abc	268±3d	4.60±0.37fghi	69±13fg
PSB-13	5.19±1.3abc	275±5c	5.00±0.55fghi	89±6e
PSB-15	5.45±0.1abc	144±5g	5.11±0.48cdefgh	85±4i
PSB-17	4.50±0.4abc	209±5	5.27±0.23cdefg	99±3eef
PSB-18	6.13±0.3a	78±3f	5.96±0.22abc	24±4i
PSB-19	5.35±0.4abc	135±6g	4.90±0.42defghi	22±3i
DN-1	6.06±0.5a	115±4h	4.80±0.29efghi	50±7h
DN-2	4.46±0.1abc	227±3e	4.64±0.41fghi	128±5cd
DN-3	**3.70±0.1bc**	**386±4a**	**4.17±0.07hi**	**265±8b**
DN-5	6.16±0.8a	135±4g	5.93±0.30abcd	26±5i
DN-6	6.24±0.8a	144±5g	5.48±0.09bcdefg	85±4ef
DN-9	5.80±0.2ab	65±3i	5.30±0.25cdefg	59±4gh
DN-13	**3.30±0.3c**	**339±3c**	**4.70±0.37ghi**	**289±3a**
DN-15	4.67±0.02abc	336±10c	6.44±0.09ab	17±4i
LSD (p<0.05)	1.255	8.815	0.563	9.091

Values are Mean ± SD (n =3)Means sharing a common letter within the column are not significantly different at P<0.05

Table 4.4 Phosphate Solubilizing Ability of selected Bacterial isolates at day 7th of incubation in PKV broth supplemented with aluminium phosphate and ferrous phosphate (100 mg P_2O_5/100 ml)

Bacterial isolates	aluminium phosohate		ferrous phosphate	
	pH	'P'-released in medium (µg. P. ml^{-1})	pH	'P'-released in medium (µg. P. ml^{-1})
PSB-1	5.62±0.2b	59±2d	6.53±0.02a	39±2de
PSB-3	**4.16±0.1d**	**159±6ab**	**5.73±0.09bcd**	**131±3b**
PSB-4	**4.00±0.1d**	**166±3ab**	**5.34±0.58de**	**158±7a**
PSB-5	4.90±0.3c	119±5c	5.60±0.07cd	104±9c
PSB-6	6.47±0.3a	48±4de	6.75±0.20a	35±4def
PSB-11	6.09±0.1ab	40±7e	6.50±0.24a	43±2d
PSB-12	6.02±0.1ab	46±4de	6.21±0.14abc	37±2de
PSB-13	6.22±0.1ab	43±4de	6.82±0.04a	23±3f
PSB-15	6.13±0.2ab	48±5de	6.65±0.21a	35±4def
PSB-17	6.44±0.2a	43±5de	6.54±0.06a	28±3fe
PSB-18	6.45±0.1a	48±4de	6.50±0.14a	38±4de
PSB-19	6.55±0.43a	45±4de	6.64±0.10a	34±3def
DN-1	6.21±0.1ab	43±5de	6.67±0.40a	39±3de
DN-2	6.16±0.2ab	46±8de	6.68±0.28a	34±4def
DN-3	**4.35±0.1cd**	**170±8a**	**4.92±0.29e**	**129±3b**
DN-5	6.08±0.1ab	16±4f	6.31±0.06ab	41±2d
DN-6	5.93±0.2ab	48±5de	6.66±0.15a	38±4de
DN-9	6.25±0.1ab	50±3de	6.43±0.17a	38±4de
DN-13	**4.64±0.5cd**	**148±9b**	**5.06±0.12de**	**159±6a**
DN-15	6.19±0.1ab	50±10de	6.56±0.22a	34±3def
LSD (p<0.05)	0.351	9.303	0.365	6.642

Values are Mean ± SD (n =3)Means sharing a common letter within the column are not significantly different at P<0.05

Results showed that with increased in P solubilization pH was significantly decresed form its initial value 7.0 to 3.94 in all the different P sources (Tri calcium phosphate, rock phosphate, aluminium phosphate and ferrous phosphate (equivalent to 100 mg P_2O_5/100 ml).) suplimented in PKV broth.

Phosphorus solubilizing bacteria are reported to dissolve insoluble phosphates by production of inorganic or organic acids and/or by the decrease of the pH (Whitelaw, 2000). Most of the previous reports stated that calcium phosphates are dissolved by acidification. Therefore any microorganism that acidifies its external medium will show some level of Phosphate Solubilizing Activity (Pradhan and Sukla, 2006). It is well known that Phosphate Solubilizing Bacteria in soil solubilize insoluble phosphates mainly by secreating acids into the medium (Dave and Patel, 2003; Chung et al, 2005).

Isolates showed maximum P solubilization activity might have used the same mechanism to solubilize the insoluble form of phosphate into soluble form. Similar method was also used by Achal *et al.,* (2007) to analyze the soluble content of phosphate in culture filtrate of *Aspergillus tubingensis* and by Himani and Reddy, (2011) to analyze the soluble content of phosphate in culture supernatant of *Bacillus sp.*

4.6 Enzyme production

4.6.1 Acid and alkaline Phosphatase and phytase production by selected isolates

These solubilizers of inorganic phosphate were further screened for Phosphatase and phytase enzyme production in culture supernatant of four selected strains inoculated in PKV broth supplemented with Tri calcium phosphate, rock phosphate, aluminium phosphate and ferrous phosphate (equivalent to 100 mg P_2O_5/100 ml). Data presented in Table 4.5, 4.6 and 4.7 showed that all the selected isolates were significantly produced the phytase, acid and alkaline phosphatase enzymes in PKV broth supplemented with different insoluble P sources. The results were also presented graphically (Fig. 4.4, 4.5 and 4.6).

Similar, results were obtained by De Souza and Chandramohan, (2002) to screen the Phosphatase production by solubilizers of inorganic phosphate. Phosphatases are enzymes of wide specificity which cleave phosphate ester bonds and this plays an important role in

hydrolysis of insoluble polyphosphates and organic phosphates (Yadav et. al., 2004). In all the treatments, the phytase activity was observed to be more than phosphatase activity. This may be due to the higher extracellular phytase enzyme activity of selected bacterial isolates as compared to extracellular phosphatase enzyme activity similar to which was reported by Aseri et al. (2009) that microbes executes extracellular phytase activity many times more than extracellular phosphatase activity.

Table 4.5. Phytase production by selected isolate

Treatments	**Phytase Activity (µ mol. P. $ml^{-1}hrs^{-1}$)**			
	PSB-3	**PSB-4**	**DN-3**	**DN-13**
RRP	252±4.7b	262±2.0b	251±4.5b	247±6.1b
TCP	518±6.0a	564±7.5a	472±9.2a	550±10.9a
$AlPo_4$	172±9.0c	156±4.1c	170±3.5c	137±5c
$FeFo_4$	124±3d	141±8.14c	153±3c	152±4.5c
LSD (p<0.05)	11.53	11.31	10.68	13.41

Values are mean ± SD (n=3).

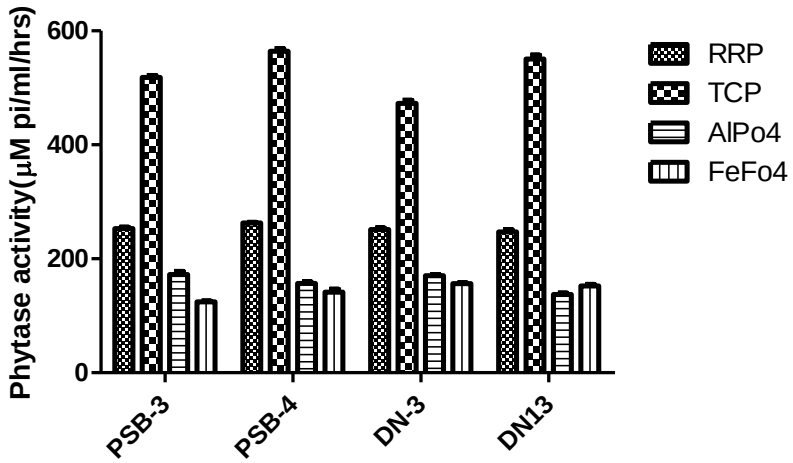

Fig. 4.4 Phytase activity of all the selected strains in different P sources supplemented in PKV broth

Table 4.6. Acid phosphatase production by selected isolates

Treatments	Acid Phosphatase Activity (µ mol. P. $ml^{-1}hrs^{-1}$)			
	PSB-3	**PSB-4**	**DN-3**	**DN-13**
RRP	78±2a	54±5b	59±2ab	73±2a
TCP	59±2b	82±2a	63±2a	54±2b
$AlPo_4$	63±9b	57±2b	56±2b	56±2b
$FeFo_4$	57±4b	60±3b	61±3ab	55±3b
LSD (p<0.05)	10.09	5.57	3.99	3.52

Values are mean ± SD (n=3).

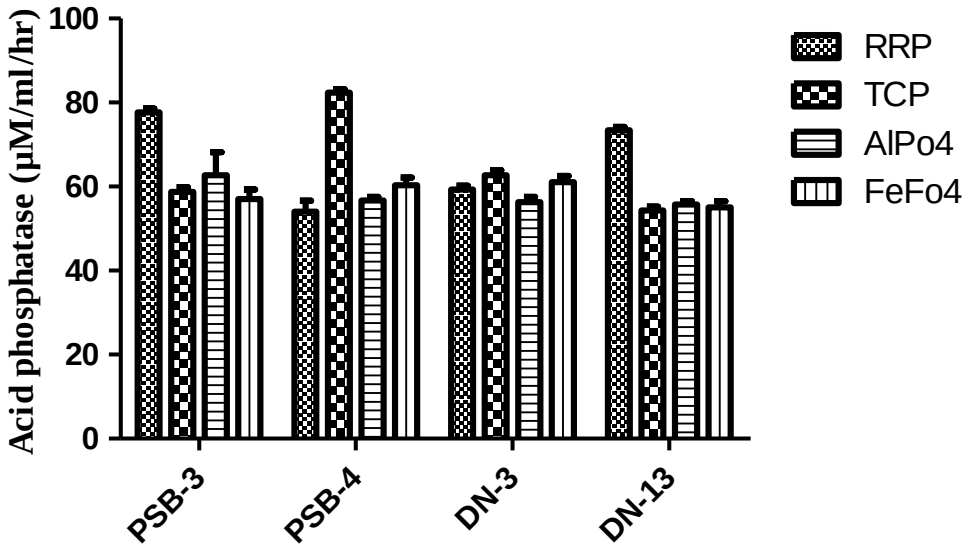

Fig. 4.5. Acid phosphataes activity of all the selected strains in different Psources suplimented in PKV broth

Table 4.7. Alakline phosphatase production by selected isolates

Treatments	Alkaline Phosphatase Activity (µ mol. P. $ml^{-1}hrs^{-1}$)			
	PSB-3	**PSB-4**	**DN-3**	**DN-13**
RRP	86±1.14a	65.44±2.4c	65.70±1.44c	88.31±0.7a
TCP	66.4±3.7a	88.5±2.5a	72.26±1.10b	78.42±1.06bc
$AlPo_4$	68.2±1.09a	74.55±2.02b	64.01±2.3c	81.4±1.2b
$FeFo_4$	65.8±3.33a	84.28±0.03a	85.08±3.8a	74.96±1.5c
LSD (p<0.05)	35.03	3.92	4.25	2.37

Values are mean ± SD (n=3).

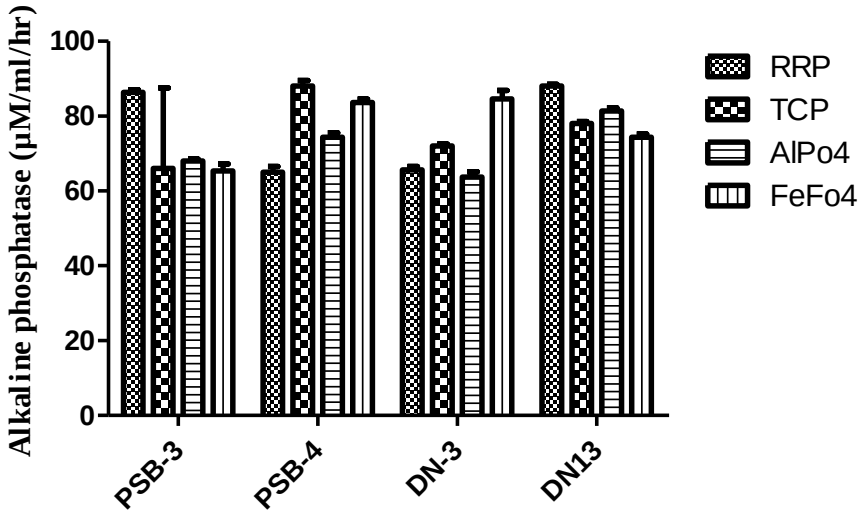

Fig. 4.6. Alkaline phosphataes activity of all the selected strains in different Psources suplimented in PKV broth

4.8. Siderophore Production Ability

Bacterial isolates were further studied for Siderophore production by using Chromo Azurol 'S' Agar medium**.** All the four isolates showed development of orange halo against dark blue background ater 48 hrs of incubation suggests Siderophore production (Fig. 4.7 and 4.8).

Data presented in Table 4.8 showed the diameter of the colony (C) that of the halo zone (Z) and the ratio Z/C help in evaluate the Siderophore Production Activity of given bacterial isolates on agar plates containing Chromo Azurol 'S' (CAS) dye as an indicator. Maximum Siderophore Production Activity was found in PSB-3(1.50) followed by DN-3 (1.44), PSB-4(1.41) and DN-13 (1.37) (Table 4.6).

Siderophore is an iron chelating compound secreated by microorganisms (bacteria, fungi), many plants (Neilands, 1995). Iron Fe^{3+} ions have very low stability at neutral pH and therefore cannot be utilized by organisms. Siderophores dissolve these ions by chelation as soluble Fe^{3+} complexes that can be taken up by active transport mechanism. Siderophore producing microorganisms deprive pathogens of iron required for their growth and pathogenesis (Alexandra., 1995).

Chemical compounds produced by microorganisms in the rhizosphere increase the availability and uptake of certain essential minerals, such as iron. Plants such as Oats are able to assimilate iron via these siderophores. It has been demonstrated that plant are able to use the hydroxamate type siderophores ferrichrome, rodotorulic acid and ferrioxamine B; the catechol-type siderophores, agrobactin; and the mixed ligand catechol-hydroxamate-hydroxy acid siderophores biosynthesized by saprophytic-root colonizing bacteria. All of these compounds are produced by rhizospheric bacterial strains, which have simple nutritional requirements, and are found in natural soils, foliage, fresh water, sediments, and seawater (Carrillo-Castaneda *et al.*, 2002).

Siderophores have applications in medicine for iron and aluminium overload therapy and antibiotics for better targeting (Olmo *et al.,* 2003).

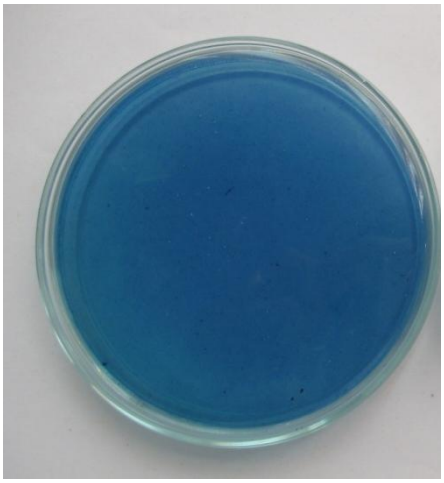

Fig. 4.7

Fig. 4.8

Fig. 4.7 Control (Chromo Azurol ‘S’ Agar plate)

Fig.4.8 Point inoculation of ‘P’-Solubilizer forming yellow coloured zone surrounding the colony (Siderophore production)

Table 4.8 Siderophore Producing Ability of selected efficient Phosphate Solubilizing Bacterial isolates

Bacterial isolates	Dia. of zone (Z) in mm	Dia. of colony (C) in mm	Siderophore Production Activity (Z/C)
PSB-3	21±0.02a	14±0.1a	1.5±0.01a
PSB-4	17±0.05b	12±0.3a	1.41±0.04a
DN-3	13±0.02c	9±0.1b	1.44±0.01a
DN-13	11±0.01c	8±0.2b	1.37±0.01a
LSD (p<0.05)	1.88	1.88	0.126

Values are mean ± SD (n=3).

4.8 Indole acetic acid production

The results of present study clearly showed that all the selected isolates used in this study had the ability to produce IAA and consequently, considered as IAA producing rhizobacteria (Table 4.9). Production of IAA, even without addition of precursor indicated that bacteria were actively involved in the synthesis of IAA in pure culture.Production of IAA by *Pseudomonas fluorescens* RAF15 was observed only in presence of L- tryptophan (Park et al., 2009) Contrary to this we observed that both the isolates were able to produce IAA without tryptophan also This shows that these strains probably synthesize IAA through tryptophan pathways. There is increasing evidence that phosphobacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing available phosphorus. Further evaluation of the isolates exhibiting multiple plant growth promoting traits on soil plant system is need to uncover their efficacy as effective PGPR.

Table 4.9 Indole acetic acid production by selected efficient Bacterial isolates with and without tryptophane

Isolates	With tryptophan (µg/ml)	Without tryptophan (µg/ml)
PSB-3	14.6±0.99b	12.6±0.3a
PSB-4	17.6±0.3a	4.5±0.4c
DN-3	17.5±0.4a	3.6±0.5c
DN-13	16.2±0.5ab	6.5±0.6b
LSD (p<0.05)	1.21	0.99

Values are mean ± SD (n=3).

4.10 Morphological and Biochemical Characterization of selected efficient Phosphate Solubilizing Bacterial isolates

Four selected efficient Bacterial isolates were characterized on the basis of morphological and biochemical tests Out of these four efficient bacterial isolates, three isolates PSB-3, PSB-4, DN-13 are gram negative and DN-3 is found to be Gram's positive. All the results of biochemical characterization were presented in table 4.10 (Fig. 9.9 to 9.11). Table 4.11 showed the antibiotic profile of selected isolates (Fig. 9.12).

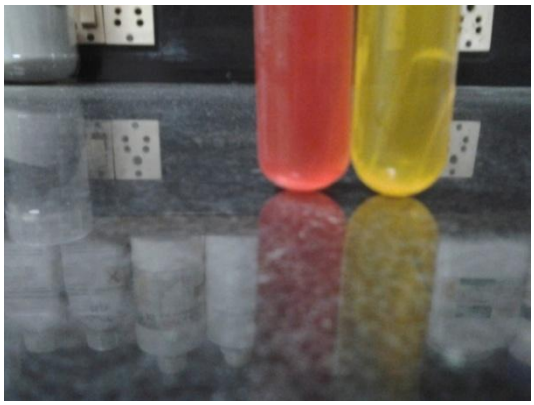

Figure 4.9 Shows the positive and negative result of MR test. Red colour shows positive and yellow color shows negative result.

Table 4.10 Morphological and Biochemical Characterization of selected efficient Bacterial isolates

Biochemical tests	PSB-3	PSB-4	DN-3	DN-13
Morphology	Irregular,raise, ntire,brown colour	Circular,corve x,entire,cream y,white colour appearance	Circular ,entire ,sticky, white colour colony	Irregular, raised ,undulated,brown
Gram staining	- ive, rod shaped	- ive, coccus	+ive, Bacillus	-ive, Bacillus
Methyl Red test	-	-	-	+
Citrate utilization test	+	+	+	+
Carbohydrate fermentation (acid production)				
Sucrose	+	-	+	+
Mannitol	+	-	+	+
Glucose	+	+	-	-
Lactose	-	-	+	-
Fructose	+	-	+	+
Carbohydrate fermentation (Gas production)				
Sucrose	-	-	-	-
Mannitol	-	-	-	-
Glucose	-	-	-	-
Lactose	-	-	-	-
Fructose	-	-	-	-
Ureas	-	-	-	-
Vogesproskauer test (V-P)	-	+	+	+
Cellulose test	+ive, (2.5mm dia.)	-	+ive, (2mm dia.)	+ive, (2mm dia)
HCN production	-	-	-	-

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Figure 4.10 Shows results of carbohydrate fermentation. Red clour shows positive and yellow color shows negative results.

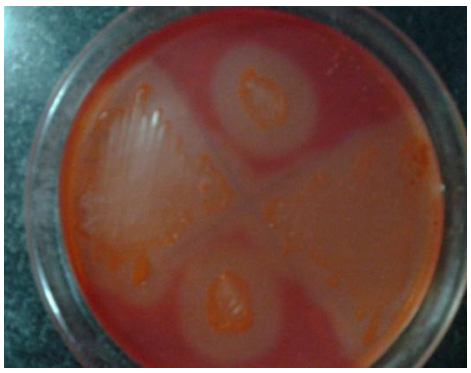

Figure 4.11 Show the zone formation that indicates positive result in cellulase activity.

4.11. Antibiotic resistance activity

Isolates	Ampicillin	Tetracyclin	Penicillin	Vancomycin	Chloroamphenicol	Carbenicillin	Streptomycin
PSB-3	+	-	+	-	-	+	-
PSB-4	-	-	-	-	-	-	-
DN-3	+	-	-	-	-	+	-
DN-13	+	+	-	+	-	-	-

+ive: resistant; -ive: sesitive

a. b.

Figure 4.12 showing bacterial antibiotic resistant activity.

4.10. Field study

the results of present study clearly showed that selected isolates effectively enhance the plant growth total P uptake (Table13) and soil fertility (table 14 and 15) in all the treatments as compared to conrol soil treatment. Many studies in relation to crop improvement by PGPR were carried out either in pot cultures or field conditions (Whitelaw *et al*. 1997; Saber *et al*. 2009 and Omar, 1998). Microbial populations are key components of soil plant entity where they are associated in a framework of interactions affecting plant development (Vassilev *et al*., 2006). In the present study significant increase in plant growth was recorded with the inoculation of selected PGPR strains. The microorganisms involved in plant growth promotion can enhance plant growth by increasing the availability of plant growth promoting substances and other trace elements. There was significant increase in the height of maize plants in all the treatments compared to control. Similarly increase in shoot and root dry weight was significantly increased in all the treatments in comparison to that of control. PGPR may effectively increase the surface area of roots (Bashan *et al*., 2004) and the root weight (Bertrand *et al*., 2001). Similar findings were reported by Puente *et al*. (2004). This shows higher nutrient uptake by inoculated roots significantly improved seedling growth. Kucey *et al*., (1989) reported that besides providing P, phosphate solubilization microorganisms produces considerable amounts of N and plant growth promoting substances in the rhizosphere. Kundu and Gaur (1984) also reported, increased P uptake and plant growth in various crops inoculated with PSMs. PSB are also reported to produce metabolites such as phytoharmones which aid in plant growth (Kloepper *et al*., 1989).

Thus the inoculation of these bacterial isolates considerably increased P uptake and enhanced plant growth as compared to uninoculated soil. These results could be attributed to the ability of these microorganisms to solubilize phosphorus already present in the soil.

Figure 4.13 Maize experiment under field conditions. Maize crop at different growth stages.

Slight reduction in pH was noticed after final harvest. Son *et al*., (2003) reported that, phosphate solubilisation was mainly due to the acidification of the culture by bacteria; however high level of phosphate solubilisation may not be achievable in soil because most soils have a great pH buffering capacity. Moreover the final pH did not reduce to strongly acidic levels. It is known that the production of organic acids by soil microorganisms and commensurate pH decrease is the major mechanism of phosphate solubilisation (Whitelaw *et al*., 1999).

Table 4.12. physiochemical properties of experimental field soil

pH	TDS ($mScm^{-1}$)	E.C. ($mScm^{-1}$)	Organic carbon (%)	Available P (mg kg^{-1})	Total P (mg kg^{-1})	Total nitrogen (%)
8.48±0.09	0.07±0.01	0.12±0.04	0.25±0.15	0.18±0.002	253±7	0.025±0.14

Values are mean ± SD (n=3).

Table 4.13. Effect of different treatments on plants growth parameters and P uptake of maize plants grown in experimental field of Thapar university, Patiala, Punjab

Treatments	Shoot height (cm)	Shoot dry wt. (g)	Root dry wt. (g)	Total P (mg kg^{-1}) in tissue	
				Shoot	Root
Soil	117±2b	18±7b	7.49±0.3b	115±3b	103±11b
Soil+PSB-3	145±5a	39±5a	9.77±0.2a	141±6a	134±8a
Soil+PSB-4	138±8a	36±6ab	9.62±0.3a	136±13a	121±9ab
Soil+DN-3	128±5ab	37±12ab	9.65±0.5a	125±3ab	119±3ab
Soil+DN-13	129±11ab	31±6ab	9.72±0.4a	138±3a	121±10ab
LSD (p<0.05)	12.5	14	0.665	13.10	15.96

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05

Table 4.14. Effect of different treatments on rhizosphere soil characteristics of maize plants grown in experimental field of Thapar university, Patiala, Punjab

Treatments	pH	Organic carbon (%)	E.C. ($mScm^{-1}$)
Soil	8.57 ± 0.03a	0.26±0.01b	0.14 ±0.05a
Soil + PSB-3	8.20±0.08b	0.35±0.05a	0.15±0.15a
Soil + PSB-4	7.87±0.05c	0.34±0.02ab	0.13±0.02a
Soil + DN-3	7.58±0.05d	0.33±0.03ab	0.15±0.05a
Soil + DN-13	8.28±0.04b	0.35±0.04a	0.14±0.03a
LSD (p<0.05)	0.10	0.057	0.041

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05

Table 4.15. Effect of different treatments on rhizosphere soil characteristics of maize plants grown in experimental field of Thapar university, Patiala, Punjab

Treatments	Total nitrogen (%)	Available P ($mg\ kg^{-1}$)	Total P ($mg\ kg^{-1}$)	Acid phosphatase ($\mu M\ g^{-1}\ hr^{-1}$)	Phytase activity ($\mu M\ g^{-1}\ hr^{-1}$)
Soil	0.015±0.003a	1.67±0.12b	242±16a	254±14b	3167±9b
Soil + PSB-3	0.016±0.002a	2.78±0.17a	228±20a	294±5a	6161±16a
Soil + PSB-4	0.012± 0.002a	2.59±0.10a	234±5a	278±14ab	6151±35a
Soil + DN-3	0.019±0.002a	2.65±0.13a	247±7a	280±5ab	6153±6a
Soil + DN-13	0..014±0.001a	2.54±0.30a	237±20a	282±11ab	6149±12a
LSD (p<0.05)	0.005	0.335	28	19	34

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05

The available P increased in the soil having bacterial inoculums as compared to control (Table 4.15). P solubilising microorganisms can solubilise and mineralize P from organic and inorganic pools of total soil P and may be used as inoculants to increase P availability to plants (Kucey *et al*., 1989; Richardson, 2001; Illmer *et al*., 1995; Whitelaw *et al*., 1999). Phosphate solubilising bacteria are slowly emerging as important organisms used to improve soil health as *in vitro* studies have demonstrated that they reduce P deficiency in soil (Yosef *et al.*, 1999). Thus the P content was more in bio inoculated treatments. Previous studies involving plants inoculated with PSMs showed growth enhancements and increased P contents but large variations were found (Kucey *et al*., 1989).

The organic carbon level was significantly increased in all the treatments in comparison to the initial values. Our results are in agreement with Himani and Reddy (2011) who reported an increase in soil organic carbon level in bio-inoculated and RP fertilized soil as compared to control soil. Little decrease in soil pH was observed in all the treatments compared to control soil. Inorganic P is solubilized by the action of organic and inorganic acids secreted by P-solubilizing bacteria in which hydroxyl and carboxyl groups of acids chelate cations and decreases the pH in basic soils and increases the concentration of phosphorous in soil (Stevenson 2005).

Soil enzymes have been suggested as potential indicators of soil quality because of their relationship to soil biology, ease of measurement, and rapid response to changes in soil management (Dick et al. 1996). Inorganic P is released from organic matter by hydrolysis of C-O-P ester bonds by phosphatases, which are therefore important in the P nutrition. Activities of enzymes such acid phosphatase and phytase in all the treatment soils were higher than the control soil. In all the treatments, the phytase activity was observed to be more than phosphatase activity. This may be due to the higher extracellular phytase enzyme activity of selected bacterial isolates as compared to extracellular phosphatase enzyme activity similar to which was reported by Aseri et al. (2009) that microbes executes extracellular phytase activity many times more than extracellular phosphatase activity. This statement is in agreement with our findings which showed that in laboratory conditions extracellular phytase activity was more pronounced than extracellular phosphatase activity by selected isolates. Higher enzyme activities in soils indicated the potential of soil to affect the biochemical transformations necessary for the maintenance of soil fertility (Rao et al. 1990).All the results together suggested that the

treatments of maize crops with PGPR improved the crop growth, yield and soil field experimental conditions.

After haevesting the viability of inoculated P solubilizing bacteria were tested and it was found that there was significant increase in the viability of bacteria which was 3.7×10^7 to 2.4×10^7 in all the inoculation treatments compared to control soil. In control soils where inoculums was not added, it was found no halozones were present around the grown colonies which suggested that phosphate solubilizing bacteria were absent in the control soils.

SALIENT FINDINGS

- In the present study plant growth promoting rhizobacteria were isolated from rhizospheric soil of potato plant grown in potato field of TIFAC, CORE Thapar university, Patiala.
- A total of 12 bacterial isolates were first selected on the basis of their phosphate solubilization activity and 8 were selected on the basis of their growth on Jensen medium.
- Out of these 20 isolates four isolates were selected for their further studies. Out of these four two isolates were P solubilizers and two were nitrogen fixing bacteria.
- These four isolates were showed significant P solubilization in different P sources (tri calcium phosphate, rock phosphate, aluminium phosphate and ferrous phosphate) suppplimented in PKV broth with reduction in PH of culture medium.
- All the isolates were positive for indol acetic acid production and siderophore production
- These isolates were further tested for their plant growth promotion effects in field on maize crop.
- Selected isolated as a seed bio-inoculant showed a significant improvement in plant growth and soil fertility in compared to control soil.
- So we can conclude from the present study that these selected isolates can be used as bio-inoculant. That may be cost effective and ecofriendly in nature compared to chemical fertilizers.

CHAPTER 5

SUMMARY

The rhizosphere is the volume of soil surrounding and under the influence of plant roots, where rhizoplane comprises of plant root surfaces and strongly adhering soil particles. There are many species of bacteria which are found in soil reported to promote plant growth by producing growth regulators, inducing root exudation and enhancing the availability of nutrients to plant, besides controlling soil borne plant pathogen. The means by which PGPR enhance the nutrient status of host plants can be categorized into five areas: (1) biological nitrogen fixation, (2) increasing the availability of nutrients in the rhizosphere, (3) inducing root surface area, (4) enhancing other beneficial symbiosis of the host, and (5) combination of modes of action. The roots of leguminous plants are colonized by numerous rhizospheric microorganisms and which cause definite influence on the survival and nodulation ability of seed inoculated rhizobia. Rhizospheric microorganisms may not only influence the inoculated rhizobia adversely through saprophytic competition, but also help them in survival through synergism resulting in an increase in their nodulation ability and N2 fixing efficiency.

In the present study we have isolates the PGPER from rhizospheric soil of potato field. Atotal of 20 bacterial isolates were selected based on their growthon Pikovskayas agar plates and Jensen agar plates.

Isolates were quantitatively analysed for their P solubilization activity in different isoluble form of P (tri calcium phosphate, rockphosphate, aluminium phosphate and ferrous phosphate). Out of 20 bacterial isolates 4 staains were showed maximum P solubilization were selected for further study

Four selected isolates significantly released the soluble P in the PKV broth with decreased in pH of the medium. All the isolates were positive for acid, alkaline phosphatses and phytase enzyme production.

Bacterial isolates were further tested for their plant growth promotion activities. All the selected isolates were positive for siderophore production. But none of the isolates showed HCN production

Selected isolates were tested for their indole acetic acid production with and without tryptohane and were showed positive results. The production of IAA was more pronounced in tryptophane supplemented medium compared to non suplimented medium. But in the absence of of tryptophane isolates were produced IAA But the amount was less as compared to tryptohane suplimented medium

A Field experiment was conducted to check their effects as PGPR in field condition. All the strain showed a stimulatory effect on plant growth and total P uptake. Soil physiochemical properties were also significantly improved in inoculation treatments as compared to control soil. Soil organic carbon, available P, enzyme activities and Phosphate solubilizing bacterial population was significantly improved in all the treatment compared to control soil.

It was found that these phosphate solubilizing strains along can substitute the chemical fertilizer, might be used to reduce the alkalinity of soil by neutralization phenomenon through organic acid exudation and can survive in the soil system to retain the phosphate solubilizing potential for long time.

CHAPTER 6

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CHAPTER 6

APPENDIX

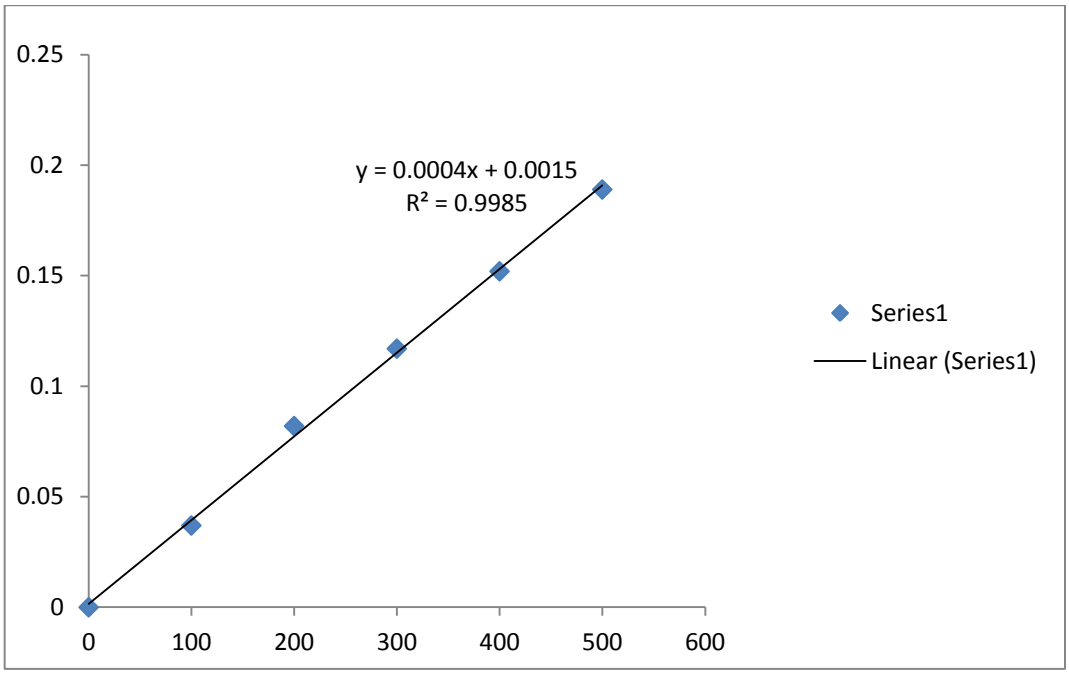

Figure 6.1 Standard graph of phytase activity

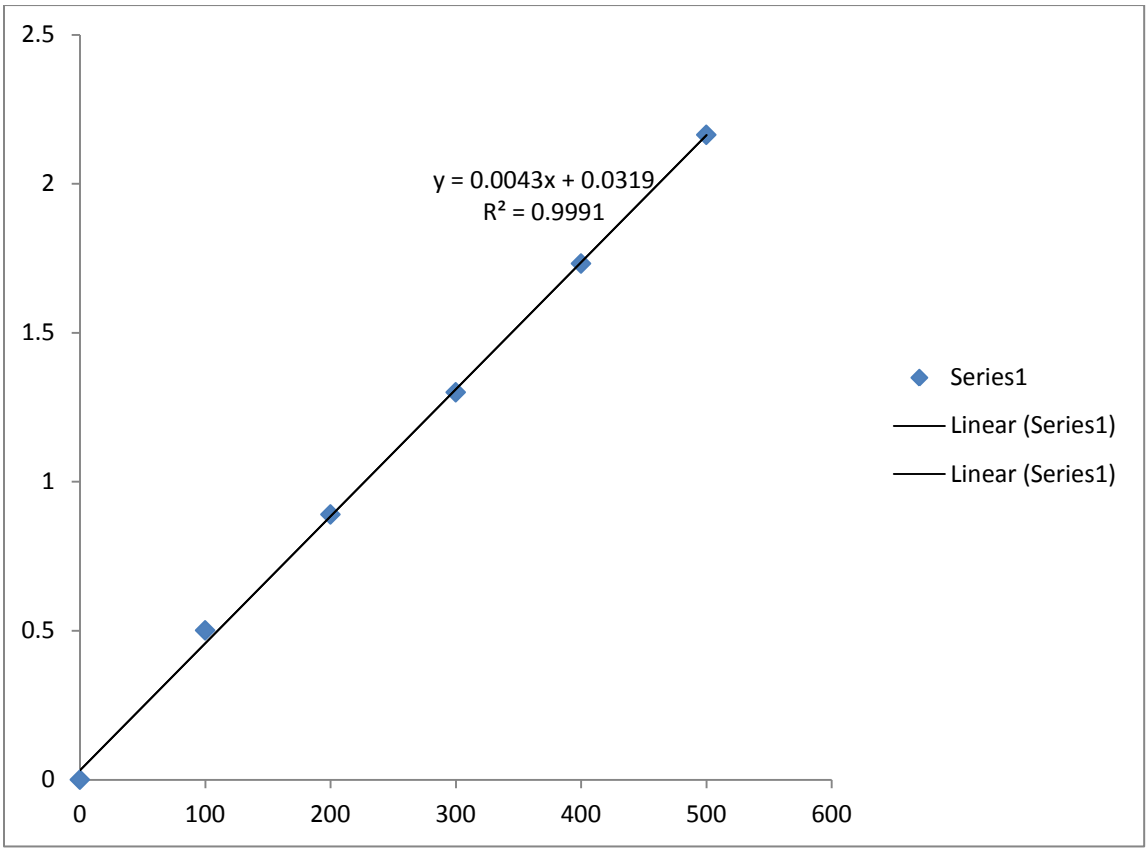

Figure 6.2 Standard Graph of Phosphatase activity

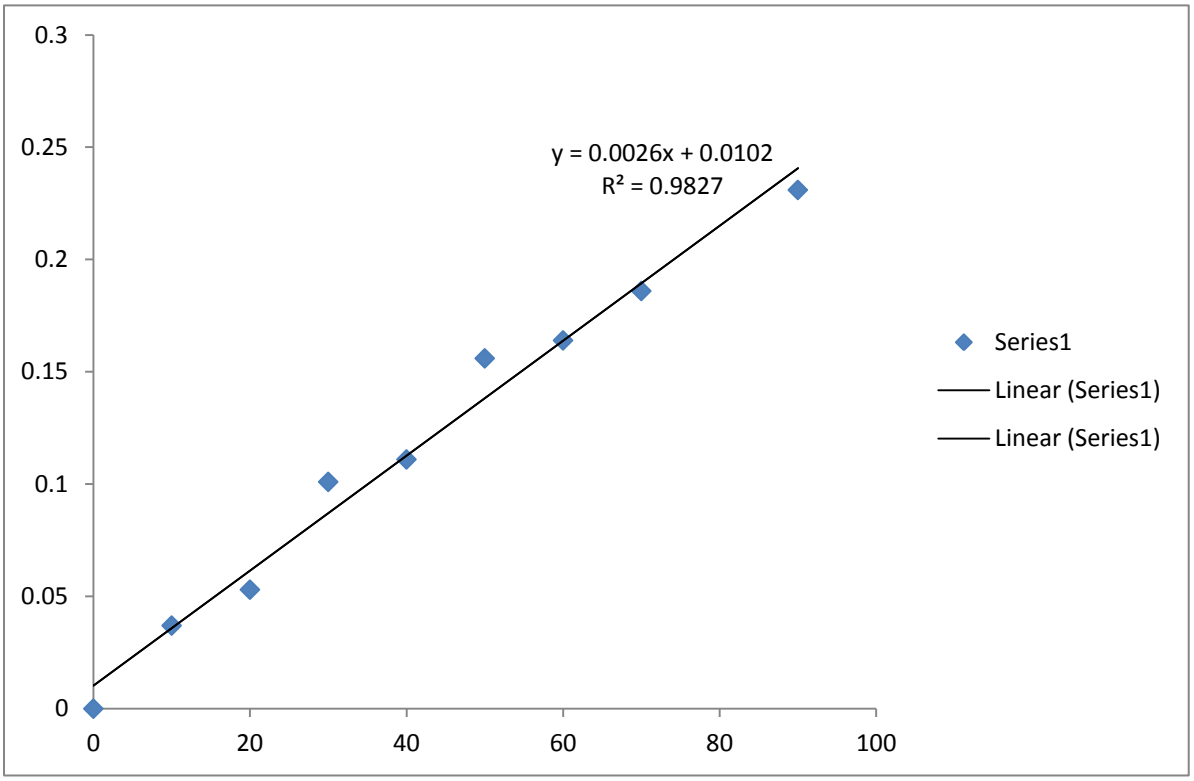

Figure 6.3 Standard for p solubilizing activity.

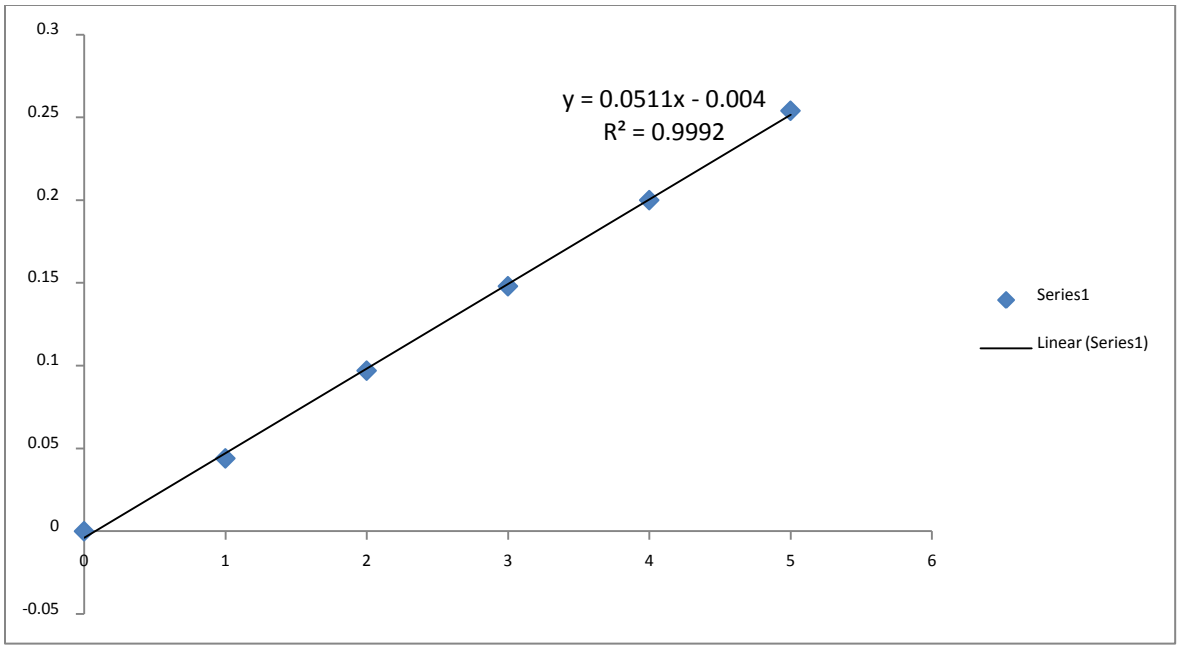

Figure 6.4 represents the standard for total 'p'

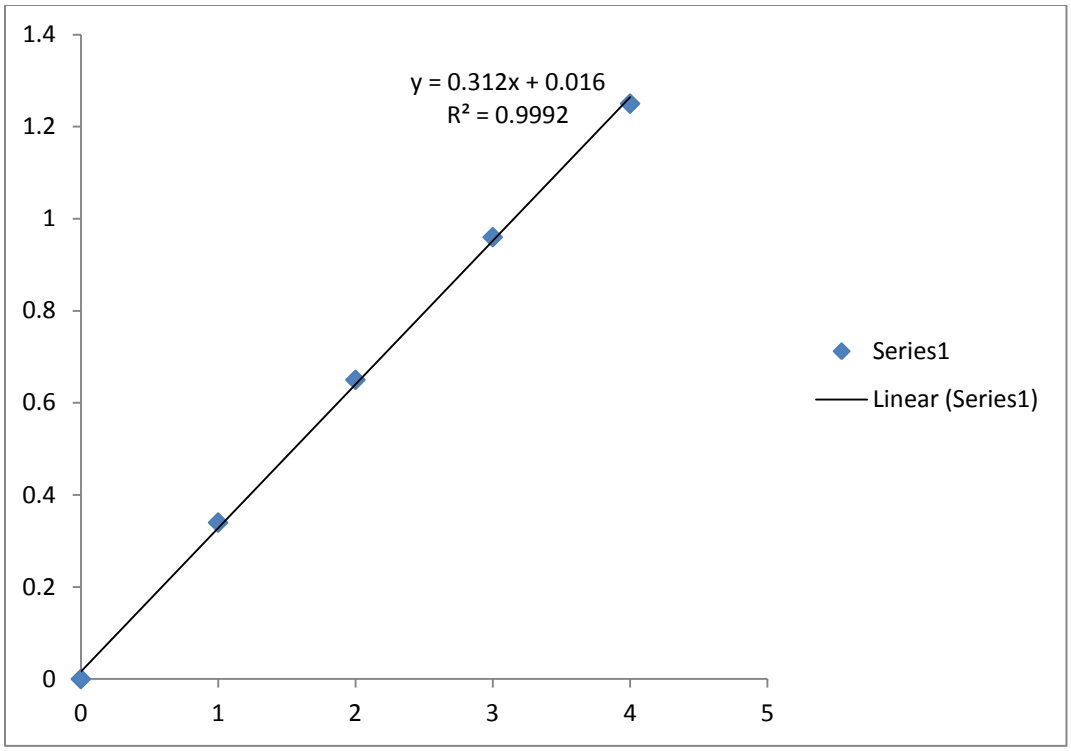

Figure 6.5 represents the standard of available 'p'

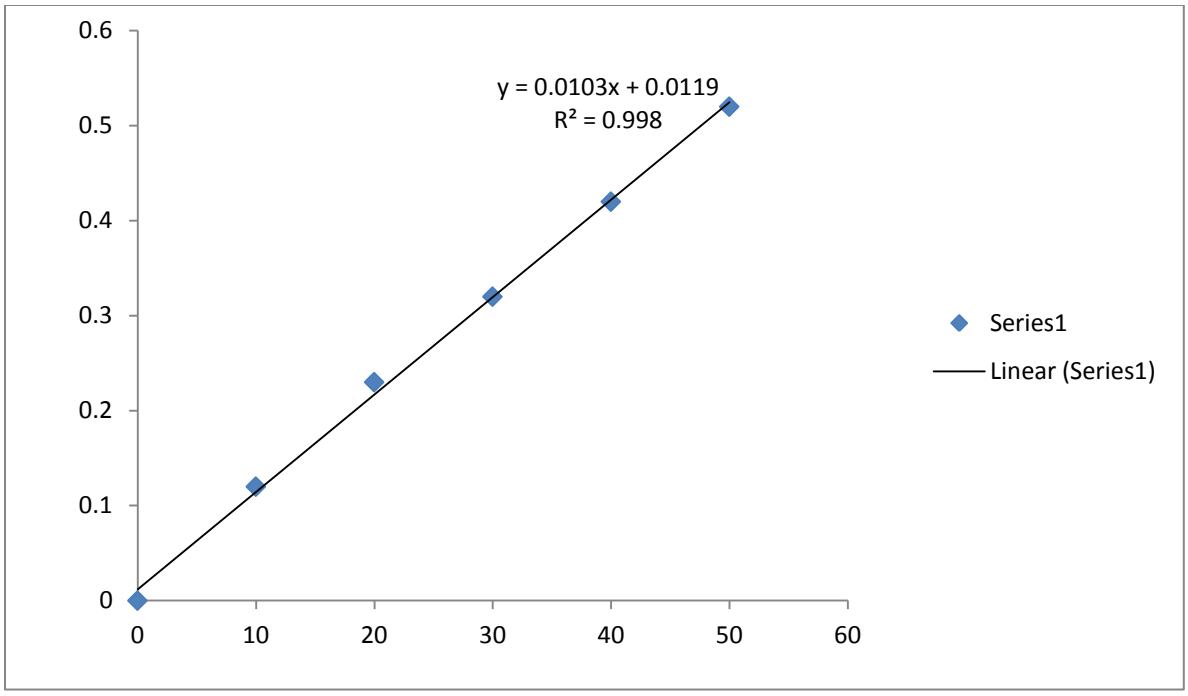

Figure 6.6 represent the standard graph of Indol Acetic Acid