

# **EFFECT OF PHOSPHATE SOLUBLIZING BACTERIA ON PLANT GROWTH**

A Dissertation

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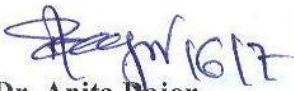
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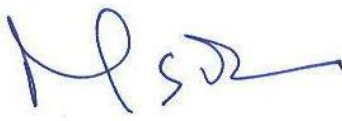
## CERTIFICATE


This is to certify that the dissertation titled: **“Effect of Phosphate Solublizing Bacteria on Plant Growth”** has been carried out by Monika Sharma, Roll No: 601001018, in the partial fulfillment of the requirements for the award of **Master of Technology** degree in **Environment Science and Technology** at **Thapar University, Patiala**, is an authentic piece of work carried out by her under our guidance and supervision.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/institute for award of any degree or any other qualification in India/abroad.

  
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## **ABSTRACT**

The phosphorous content in the soil can be increased by using phosphate solubilizing bacteria as inoculants, which further increase crop yield. The most powerful phosphate solubilizing bacteria genera are *Pseudomonas*, *Bacillus* and *Rhizobium*. The mechanism of phosphate solubilization includes production of organic acids in soil. Bacterial isolates number 3 and 6 were isolated from soil, which were able to increase the phosphatase activity. Acid phosphatase activity increased when rockphosphate and tricalcium phosphate were added in comparison to soil only. Phosphatase activity observed have maximum increase when isolate number 3 or 6 were added along with rockphosphate and tricalcium phosphate.

Acid phosphatase activity was 0.192 units/ml in wheat crop soil, and 0.218 units/ml in ladyfinger, whereas the acid phosphatase activity was observed to increase further when rockphosphate and isolate number 3 was added in soil. It is observed during the studies for acid phosphatase activity that isolate number 3 was more effective in comparison to isolate number 6. In case of alkaline soil activity it increases more in rockphosphate and bacterial isolate number 6 were added. Alkaline soil activity was 0.243 units/ml in wheat and 0.283 units/ml in lady finger in comparison to control and negative control.

Maximum efficiency for phosphate solubilization in tricalcium phosphahate was observed to be 98.7% in isolate no 3 and 6 both, in 72 hours. In case of rockphosphate maximum efficiency for phosphate solubilization was observed to be 98.5% which stabilized after 96 hours. Isolate number 3 showed maximum efficiency of 98.8% as compared to control which was 96.6% in 24 hours. Isolate number 6 shows maximum efficiency of 97.7% in 24 hours which increased to 98.5% in 72 hours. Chlorophyll content was also found to be more in soil in the presence of Isolate number 3 in comparison to Isolate number 6.

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## CHAPTER – 1

### INTRODUCTION

#### 1.1 INTRODUCTION

Industrialization and green revolution have brought about an increase in productivity but they have also resulted in massive abuse of environment. Extensive use of chemicals as fertilizers to improve plant health and productivity and for control of pathogens has disturbed the ecological balance of soil and has led to the depletion of nutrients. Hence there is a need to search for alternative strategies to improve soil health without causing damage to environment as well as soil. Biofertilizers are therefore gaining importance as they are eco-friendly, non hazardous and non toxic. Biofertilizer refers to products consisting of selected and beneficial living microbes, which are added to soil as microbial inoculants.

Several organisms such as *Cyanobacteria*, *Azolla*, phosphate solublizing microorganisms are presently being used as biofertilizers. These microbes are known to improve plant health by secretion of growth promoting substances and by increasing the availability of micronutrients. Phosphate solublizing bacteria especially are emerging as important organisms being used to improve soil health. *In-vitro* studies have demonstrated that phosphate solublizing bacteria bring about dissolution of rockphosphate, thereby reducing phosphate deficiency in soil.

Chemical fertilizer application is an effective method to increase yields, but is costly and may also lead to environmental problems. Phosphate exists in nature in variety of organic and inorganic forms. Phosphate availability is low in soils because of its fixation as insoluble phosphates of iron and aluminium. The bacteria used as phosphorus bio-fertilizers could contribute to increasing the availability of phosphates immobilization in soil and could enhance plant growth. Phosphate supply through biological means is a viable alternative, phosphate solublizing bacteria (PSB), phosphate solublizing fungi (PSF) and *Actinomycetes* have been reported to be active in conversion of insoluble phosphate to soluble phosphate.

Plants utilize fewer amounts of phosphatic fertilizers that are applied and the rest is rapidly converted into insoluble complexes in the soil. This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also

environmentally undesirable. Phosphorous is the second to nitrogen as an essential macronutrient for plant growth and development. Phosphorus is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available.

Phosphorus plays a significant role in several physiological and biochemical plant activities like photosynthesis, transformation of sugar to starch, and transporting of the genetic traits. The formation of strong bonds between phosphorous with calcium and magnesium in alkaline-pH and the same bonds with iron and aluminum in acidic soils. The mobility of phosphorus is very slow in the soil and cannot respond to rapid uptake by plants. This causes creation and development of phosphorus depleted zones near the contact area of roots and soil in rhizosphere. Therefore, the plants need an assisting system which could extend beyond the depletion zones and help to absorb the phosphorus from a wider area by developing an extended network around plant roots.

Now days, increase in the prices of phosphatic fertilizers coupled with low recovery (10 to 30%) of phosphorus from applied characteristics, the developing tropical countries are attempting to utilize their reactive ground rock phosphate as a cheap alternative source. Rock phosphate has been proposed for sustainability purposes. The problem with this less expensive but sparingly soluble form of phosphorus is its low effectiveness, particularly in non-acidic soils. Among microbial groups which can solublize mineral phosphates and improve plant phosphorus nutrition are *Arbuscular mycorrhizal* fungi. *Arbuscular mycorrhizal* fungal inoculations induce spectacular stimulations of the plant growth and phosphorus foliar content.

## **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 laying down the primordial for reproductive parts of plants. It is vital to seed formation and its content is higher in seeds than in any other part of the plant. It helps plants to survive winter rigors and also contributes to disease resistance in some plants. Phosphorus is also known for improving

the quality of many fruits, vegetables and grain crops. Biological Nitrogen Fixation depends appreciably on the available forms of phosphorus.

### **1.3 PHOSPHATE SOLUBLIZING BACTERIA (PSB)**

These are a group of beneficial bacteria capable of hydrolyzing organic and inorganic phosphorus from insoluble compound. Phosphate 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 the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids, through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms. In addition, some PSB produce phosphatase like phytase that hydrolyse organic forms of phosphate compounds efficiently. One or both types of PSB have been introduced to agricultural community as phosphate biofertilizer. Phosphorus is one of the major essential macronutrients for plants as discussed in the earlier section. Now a days, the main purpose in managing soil phosphorus is to optimize crop production and minimize phosphorus loss from soils. PSB have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield. When PSB is used with rock phosphate, it can save about 50% of the crop requirement of phosphatic fertilizer. The use of PSB as inoculants increases the phosphorus uptake by plants. Simple inoculation of seeds with PSB gives crop yield responses equivalent to 30 kg P<sub>2</sub>O<sub>5</sub> per hectare or 50 percent of the need for phosphatic fertilizers. Currently, different strains of these bacteria has been identified for use in biofertilizer, of all, three new strains, namely *Pantoea agglomerans* strain (P5), *Microbacterium laevaniformans* strain (P7) and *Pseudomonas putida* strain (P13) has been recently identified as the highly efficient insoluble phosphate Solublizer.

Microbial inoculants are in use for improving soil fertility for the last century; however, a meager work has been reported on phosphorus solubilization compared to nitrogen fixation. Soil phosphorus dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes. Large amount of phosphorus is applied as fertilizer. Efficiency of phosphorus fertilizer throughout the world is around 10 - 25 % and concentration of biologically available phosphorus in soil is very low reaching the level of 1.0 mg per kg of soil.

#### 1.4 PHOSPHORUS SOLUBLIZING MICROORGANISMS

Bacteria are more effective in phosphorus solubilization than fungi. Among the whole microbial population in soil, PSB constitute 1 to 50 %, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5 % in phosphorus solubilization potential (Chen et al., 2006). Number of PSB among total PSM in north Iranian soil was around 88 % (Fallah, 2006).

Microorganisms involved in phosphorus acquisition include mycorrhizal fungi and PSMs (Fankem et al., 2006). Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia have been described as effective phosphate solubilizers. Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus fungiare* the most powerful phosphorus solubilizers . *Bacillus megaterium*, *Bacillus circulans*, *Bacillus subtilis*, *Bacillus polymyxa*, *Bacillus sircalmous*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains . A *nematofungus Arthrobotrys oligospora* also has the ability to solubilize the phosphate rocks (Duponnois et al., 2006).

Dissolved inorganic phosphate exists in the sea mainly as ionic forms of orthophosphoric acid because of the negative charge of phosphate ions. The phosphorus cycle in soil is a system which involves soils, land and microorganisms. Major processes include the uptake of soil phosphorus by plants, recycling (the return of plant and animal residues), biological turnover (mineralization and immobilization), fixation to clay, solubilization. Phosphorus is 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 inorganic and organic already present in the soil.

Soil phosphatase plays a major role in the mineralization processes of organic phosphorus substrates. Enzymes in soils originate from animal, plant and microbial sources and the resulting soil biological activity includes the metabolic processes of all these organisms. The activity of soil phosphatase can be influenced by numerous factors and soil properties and farming systems play a key role among them.

## **1.5 PHOSPHATE AVAILABILITY IN SOIL**

Phosphorus is one of the major essential macronutrients for biological growth and development. It is present at levels of 400–1200 mg per kg of soil. Its cycle in the biosphere can be described as ‘open’ or ‘sedimentary’, because there is no interchange with the atmosphere. Microorganisms play a central role in the natural phosphorus cycle. This cycle occurs by means of the cyclic oxidation and reduction of phosphorus compounds, where electron transfer reactions between oxidation stages range from phosphine to phosphate.

## **CHAPTER - 2**

### **LITERATURE REVIEW**

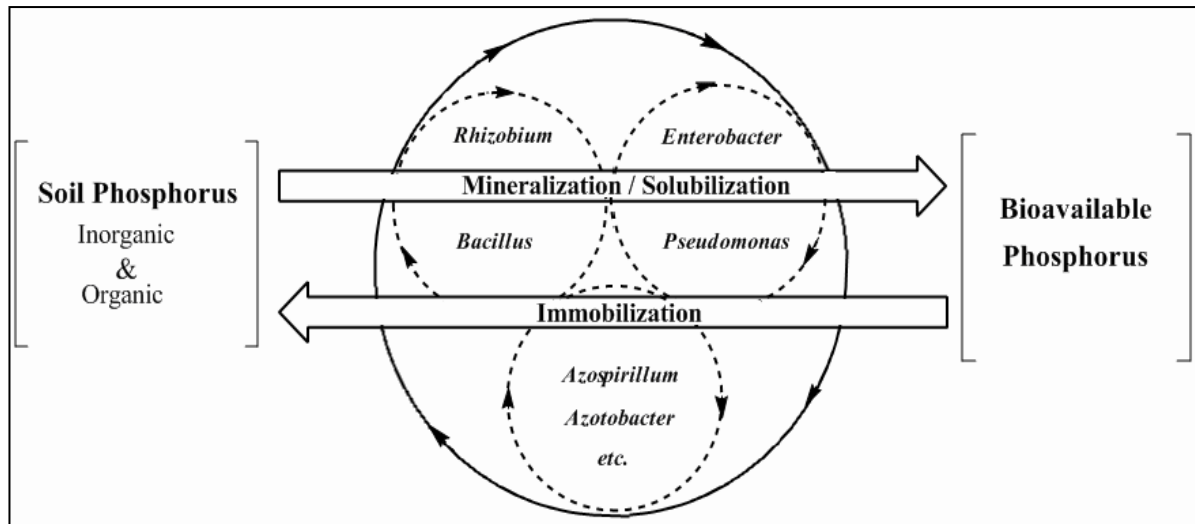
In the present chapter the detailed review of the literature relevant to the phosphate solubilizing bacteria has been presented. The literature studied has been subdivided in to various sections for the better presentation and understanding the various aspects of Phosphate Solubilizing Bacteria, as detailed below:

#### **2.1 PHOSPHATE SOLUBLIZERS**

Phosphate Solublizer are various bacteria and fungi which help in solubilization of phosphorus content in soil for better plant growth. The mechanism of phosphorus solubilization, phosphorus solublizing bacteria: occurrence, mechanisms and their role in crop production, and microbially mediated solubilization of insoluble phosphates have been discussed in this section as written below:

##### **2.1.1 Mechanism of Phosphorus Solubilization**

Some bacterial species have mineralization and solubilization potential for organic and inorganic phosphorus, respectively, Phosphorus solublizing 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). A wide range of microbial phosphate solubilization mechanisms exist in nature, and much of the global cycling of insoluble organic and inorganic soil phosphate is attributed to bacteria and fungi (Stevenson, 2005). The Phosphate Solublizing Bacteria dissolve the soil phosphate through production of low molecular weight organic acids mainly gluconic and keto gluconic acids (Goldstein, 1995; Deubel et al., 2000), in addition to lowering the pH of rhizosphere. The pH of rhizosphere is lowered through biotical production of proton bicarbonate release (anion cation balance) and gaseous (O) exchanges. Phosphorus solubilization ability of Phosphate solubilization bacteria has direct correlation with pH of the medium.



**Figure 2.1: Schematic diagram of soil phosphorus mobilization and immobilization by bacteria to CO<sub>2</sub>.** (Ahmad et al.,)

### 2.1.2 Phosphorus Solublizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production

Phosphorus can be released from organic compounds in soil by three groups of enzymes: (1) nonspecific phosphatases, which perform dephosphorylation of phosphor ester or phosphoanhydride bonds in organic matter, (2) Phytases, which specifically cause Phosphate release from phytic acid, and (3) Phosphonatas and Carbon–Phosphate Lyases, enzymes that perform Carbon–Phosphate cleavage in organo phosphonates. The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil. Availability of organic phosphate compounds for plant nutrition could be a limitation in some soils resulting from precipitation with soil particle ions. Therefore, the capability of enzymes to perform the desired function in the rhizosphere is a crucial aspect for their effectiveness in plant nutrition. Nevertheless, the efficiency of plant and microbial phosphatases on organic phosphate depletion in the rhizosphere and phosphate uptake by plants has been well documented (Tarafdar and Claassen, 1988).

The Organic acids produced by Phosphate solublizing bacteria solubilize insoluble phosphates by lowering the pH, chelation of cations and competing with phosphate for adsorption sites in the soil. Inorganic acids e.g. hydrochloric acid can also solubilize phosphate but they are less effective compared to organic acids at the same pH (Kim et al., 1997). In certain cases phosphate solublization is induced by phosphate starvation (Gyaneshwar et al., 1999). Gerretsen (1948) first showed that pure cultures of soil bacteria

could increase the Phosphate nutrition of plants through increased solubility of Ca-phosphates. Their solubility increases with a decrease of soil pH. Microorganisms through secretion of different types of organic acids e.g. carboxylic acid and rhizospheric pH lowering mechanisms (He and Zhu, 1988) dissociate the bound forms of phosphate like acidification of the microbial cell surroundings releases phosphate from apatite by proton substitution excretion of  $H^+$  (accompanying greater absorption of cations than anions) or release of  $Ca^{2+}$ . While, the reverse occurs when uptake of anions exceeds that of cations, with excretion of  $OH^-/HCO_3^-$  exceeding that of  $H^+$  carboxylic anions produced by phosphate solubilizing bacteria, have high affinity to calcium, solubilize more phosphorus than acidification alone. Complexion of cations is an important mechanism in phosphate solubilization if the organic acid structure favors complexion (Fox et al., 1990).

Organic anions associated protons are effective in solubilizing. Precipitated forms of soil iron and aluminium-Phosphate in acid soils Ca-P in alkaline soils, chelating. Metal ions that may be associated with complexed forms of Phosphate or may facilitate the release of adsorbed phosphate through ligand exchange reactions (Jones, 1998). Calcium phosphate (Ca-P) release results from the combined effects of pH decrease and carboxylic acids synthesis, but proton release cannot be the single mechanism (Deubel et al., 2000). Principal mechanism for mineralization of soil organic Phosphate is the production of acid phosphates (Hilda and Fraga, 2000). Release of organic anions, and production of siderophores and acid phosphates by plant roots microbes or alkaline phosphates enzymes hydrolyze the soil organic phosphate or split phosphate from organic residues. The largest portion of extracellular soil phosphates is derived from the microbial population (Dodor and Tabatabai, 2003).

### **2.1.3 Microbially mediated solubilization of insoluble Phosphates**

Although phosphate is quite abundant in many soils, frequent applications of phosphate fertilizers is needed to compensate. The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphates play a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings. The production of organic acids by phosphate solubilizing bacteria has been well documented. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Also, 2-ketogluconic acid is another organic acid identified in strains

with phosphate solubilizing ability. It is reported as the principal organic acid produced by phosphate solubilizing bacteria such as *Pseudomonas sp.*, *Erwinia herbicola*, *Pseudomonas cepacia* and *Burkholderia cepacia*. Another organic acid identified in strains with phosphate-solubilizing ability is 2-ketogluconic acid, which is present in *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Bacillus firmus* and other unidentified soil bacteria. *Bacillus amylolique faciens* were found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers.

Several reports have suggested the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate. In two thirds of all arable soils, the pH is above 7.0, so that most mineral phosphate is in the form of poorly soluble calcium phosphates (CaPs). Microorganisms must assimilate phosphate via membrane transport, so dissolution of CaPs to Pi ( $H_2PO_4$ ) is considered essential to the global phosphate cycle. Evaluation of samples from soils throughout the world has shown that, in general, the direct oxidation pathway provides the biochemical basis for highly efficacious phosphate solubilization in Gram-negative bacteria via diffusion of the strong organic acids produced in the periplasm into the adjacent environment. Therefore, the quinoprotein glucose dehydrogenase (PQQGDH) may play a key role in the nutritional eco physiology of soil bacteria. Mineral phosphate solubilizing bacteria may be used for industrial bio processing of rock phosphate ore (a substituted fluoroapatite) or even for direct inoculation of soils as a 'biofertilizer' analogous to nitrogen-fixing bacteria. Both the agronomic and ecological aspects of the direct oxidation mediated Mineral Phosphate Solubilizing trait. (Goldstein *et al.*, 2000).

In general, phosphate for agricultural applications is available in two forms: as conventional phosphate fertilizers and rock phosphate. Most phosphate fertilizers are produced by technologies based on the chemical processing of insoluble, inorganic, high-grade rock phosphate ore (RPO). This process is environmentally undesirable, not least because the release of contaminants into the main product, gas streams, and by-products. A biological approach for extracting phosphate from rock phosphate Ore was proposed as a less expensive and lower-energy technique compared with the conventional processes (Rogers and Wolfram 1993;). To improve the agricultural value of rock phosphate Ore, several methods were commonly proposed (Rajan and Watkinson 1993; including microbial based approaches

microorganisms (Vassileva et al 1998, 2000). The direct introduction of Rock phosphate ore solubilizing microorganisms normally confirms the microbial activity demonstrated in fermentation studies. Short-term reduction of the rhizosphere pH due to organic acid production and further complexation of cations resulting the release of soluble phosphate, which in turn increases its accessibility to the plant.

## 2.2 ROLE OF PHOSPHATE SOLUBLIZING BACTERIA AS BIOFERTILIZER

A considerable number of bacterial species are able to exert a beneficial effect upon plant growth. Mostly they are associated with the plant rhizosphere, so they are called as rhizobacteria. This group of bacteria has been termed plant growth promoting rhizobacteria, and among them are strains from genera such as *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, and *Serratia*. They are used as biofertilizers or control agents for agriculture improvement, and there are numerous researchers for the area with the agricultural environment conservation.

Detection and estimation of the phosphate solubilization ability of microorganisms have been possible using plate screening methods. Phosphate solubilizers produce clearing zones around microbial colonies in media as shown in figure 2.2. Insoluble mineral phosphates such as tri calcium phosphate or hydroxyl apatite are contained in the media. (Chen et al., 2006)

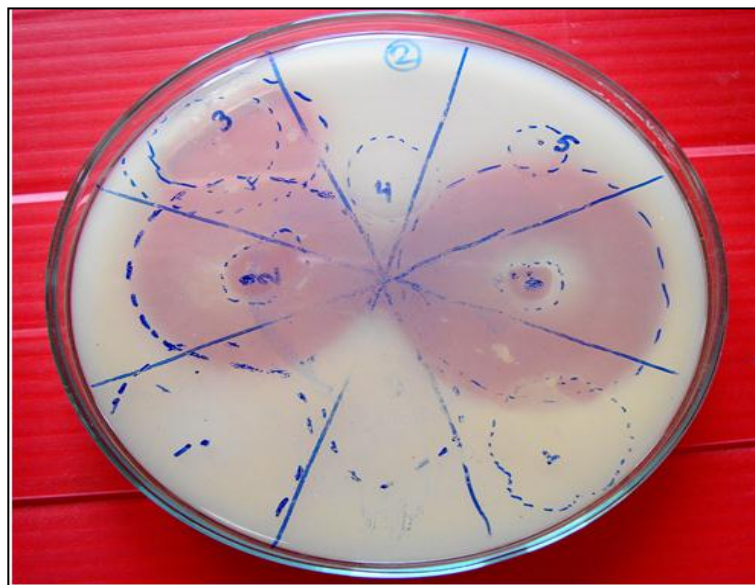


Figure 2.2: Clear zones on agar plate. (Chen et al., 2006)

Also the Bromo phenol blue method that produce yellow halos following pH drop through the release of organic acids is more reproducible and has greater correlation in comparison

with the simple halo method. However, clearing zones on agar plate method is generally used (figure 2.1). Pikovskays's medium (Table 2.1) is a general medium for selection of phosphate solubilizer.

Biofertilizers are organisms that enrich the nutrient quality of soil. The main sources of biofertilizers are bacteria, fungi, and cyanobacteria (blue-green algae). Plants have a number of relationships with fungi, bacteria, and algae. After the introduction of chemical fertilizers during the last century, farmers were happy of getting increased yield in agriculture. But slowly chemical fertilizers started displaying their ill-effects such as leaching out, polluting water basins, destroying flora and fauna including friendly organisms, making the crop more susceptible to the attack of diseases, reducing the soil fertility and thus causing irreparable damage to the eco system (Rodrigues and Fraga 1999). The principle behind this strategy is that microbes have various abilities which could be exploited for better farming practices. Some of them help in combat diseases while some have the ability to degrade soil complex compounds into simpler forms which are utilized by plants for their growth. They are extremely beneficial in enriching the soil by producing organic nutrients for the soil. To convert insoluble phosphates to a form accessible to the plants, like orthophosphate, is an important trait for a Plant Growth Promoting Bacteria for increasing plant yields. The use of microbial products has certain advantages over conventional chemicals: they are considered safer than many of the chemicals now in use; they do not accumulate in the food chain; the target organisms seldom develop resistance as is the case when chemical agents are used; and Biofertilizing agents are not considered harmful to ecological processes or the environment.

### **2.3 PSBS AS PLANT GROWTH PROMOTERS**

The substantial number of bacterial species, mostly those associated with the plant rhizosphere, may exert a beneficial effect upon plant growth. This group of bacteria has been termed "plant growth promoting bacteria" or PGPB, and among them, some phosphate solublizing bacteria (PSB) are already used as commercial biofertilizers for agricultural improvements.

Among PGPR, phosphate solublizing bacteria (PSB) are considered as promising biofertilizers since they can supply plants with phosphorus (P) from sources otherwise poorly available. Beneficial effects of the inoculation with phosphate solublizing bacteria to many crop plants have been described by numerous authors (Peix *et al.*, 2001). Moreover,

synergistic interactions on plant growth have been observed by co-inoculation of phosphate solubilizing bacteria with other bacteria, such as *Azospirillum* and *Azotobacter* (Kundu and Gaur, 1984), or with vesicular arbuscular mycorrhizae (Toro *et al.*, 1997, 1998). In addition many *Pseudomonas* strains, promote plant growth by mechanisms such as the production of plant growth regulators and vitamins, enhancement of plant nutrient uptake and suppression of pathogenic or deleterious organisms (Davison, 1998; Glick, 1995; )

#### 2.4 INTERACTION OF PO<sub>4</sub> SOLUBLIZER WITH PLANTS

Phosphorus is one of the major plant nutrient limiting plant growth. It plays a key role in nutrition of plants as it promotes development of deeper roots. The average soil is rich in phosphorus as it contains about 0.05% (w/w) phosphorus (Barber, 1984) but only one tenth of this is available to plants approximately 95–99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants and due to its poor solubility and chemical fixation in the soil (Gaur and Gaiand, 1999) causing a low efficiency of soluble phosphate fertilizers. To increase the availability of phosphorus for plants, large amounts of fertilizer is used on a regular basis. But after application, a large proportion of fertilizer phosphorus is quickly transferred to the insoluble forms. Therefore, very little percentage of the applied phosphorus is used, making continuous application necessary. (Abd Alla, 1994).

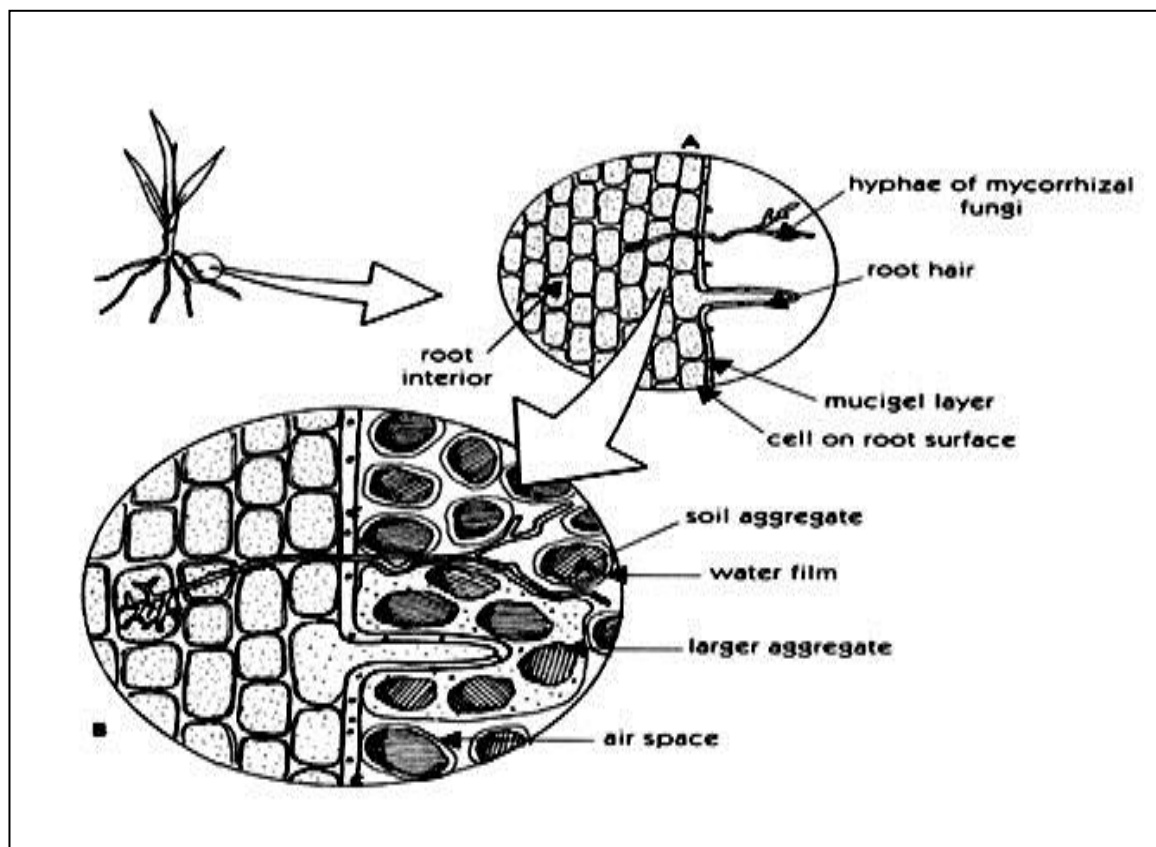


Figure 2.3: Microorganisms involved in Phosphate transformation

The microorganisms are involved in a range of processes that affect phosphate transformation and thus influence the subsequent availability of phosphate to plant roots. Free living phosphate solubilizing microorganisms (PSM) are always present in soils. The populations of inorganic phosphate solubilizing microorganisms are sometimes very low, less than  $10^2$  CFU per gram of soil as observed in a soil in Northern Spain.

The phosphate solubilization effect seems to be the most important mechanism of plant growth promotion in moderately to fertile soils. Strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizer. Rhizobia have well known beneficial symbiotic atmospheric nitrogen fixing symbiosis with legumes, have an excellent potential to be used as Phosphate growth promoters with non legumes. Plant Growth Promoters can also promote mycorrhizal functioning. Recently for example, (Villegas and Fortin (2001)) showed an interesting specific synergistic interaction between the Phosphate solubilizing bacterium *Pseudomonas aeruginosa* and the fungus *Glomus intraradices*. No synergistic effect was observed with the other two Phosphate solubilizing bacteria tested (*Pseudomonas putida* and *Serratia plymuthica*) showing the specificity of this interaction.

The amelioration of phosphate deficiency by the application of costly and environmentally hazardous phosphate fertilizers is not an ideal solution and has generated serious issues about the continued viability of current agriculture practice. This has led to a search for more environmentally friendly and economically feasible strategies to improve crop production in low phosphorus soils. In an ideal manner, such strategies should enable the efficient use of phosphate solubilizing microorganisms. Several scientists have reported the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tri calcium phosphate and di-calcium phosphate.

## **2.5 IMPROVEMENT OF AGGREGATE STABILITY**

Agricultural wastes, by-products, and other waste disposal amendments of high organic matter content are applied to soils to enhance their fertility and improve their biological and physicochemical properties. Numerous studies, both in the field and under controlled conditions, have demonstrated the important role of organic matter in the formation and stabilization of soil aggregates. Many authors suggest that it is the production of polysaccharides which is responsible for the soil structure improvement (Bearden and

Petersen, 2000). The microorganisms participate mechanically (union by hyphae) or by the excretion of polysaccharides into the medium. The symbiosis between fungi and plants has been shown to increase the stability of soil aggregate. Similar beneficial effect of our biotechnological product related to stabilization of soil aggregates was found in acidic semiarid mine tailings. Particularly after soil drying, aggregate stability was 66% higher than the control soil. The fermentation mixture of mineralized organic matter and solubilized rock phosphate raised the soil pH and increased soil water-soluble carbon under either watering or drying conditions.

## **2.6 EFFECT OF PHOSPHATE SOLUBLIZATION ON ORGANIC-AGRO WASTE ON PLANT GROWTH AND HEALTH**

A variety of beneficial microorganisms are applied to agricultural soils in order to control plant pathogens, increase symbiotic or associative nitrogen fixation, increase plant nutrient availability, and promote plant growth through the production of plant hormones. Solubilization of inorganic insoluble phosphates provides the plants with an organic amendment rich in polysaccharide compounds and available phosphate when Rock phosphate is applied in the fermentation process). In addition, this mineralized organic matter can be used as C and energy source for activities of soil microorganisms. This effect could be further extended to enhancement of the soil enzyme activities increase of soil and plant enzyme activities which are considered as major factors contributing to overall soil microbial activity and soil contributing to overall soil microbial activity and soil quality (Garcia et al. 1997).

## **2.7 ENZYMATIC ACTIVITIES OF PHOSPHATE-SOLUBLIZER**

In general, high enzymatic activities were related to the stimulating effect of root exudates. Root exudates have a variety of roles including that of metal chelators that may reduce the plant uptake of certain metals. The range of compounds exuded is wide and could play a role in plant metal tolerance (Hall 2002). These compounds are more abundant in rhizosphere of plants with a more developed root system as in our studies occurred with *A.niger*-treated Solubilizing bacteria /Rock Phosphate inoculated plants. Thus, the role of such treatments alleviating heavy metal toxicity in plant, possibly via exudates chelation, seems to be important. An indication supporting this speculation is the fact that the metabolic (enzymatic) activity of particular groups of rhizosphere microorganisms, involved in nutrient cycling, increased with the specific amendments applied to heavy metal contaminated soil. When assessing the overall effect of *A.niger*-treated organic matter/Rock Phosphate mixture, it should be bear in mind that in a recent study, we found high siderophores-like production

(with metal-chelating properties) by *A.niger* grown in conditions of solid state fermentation processes.

## **2.8 P-SOLUBLIZATION MICROBES ON IMPROVEMENT OF SOIL PROPERTIES**

Improvement of aggregate stability Agricultural wastes, by-products, and other waste disposal amendments of high organic matter content are applied to soils to enhance their fertility and improve their biological and physicochemical properties. Numerous studies, both in the field and under controlled conditions, have demonstrated the important role of organic matter in the formation and stabilization of soil aggregates. Many authors suggest that it is the production of polysaccharides which is responsible for the soil structure improvement . The microorganisms participate mechanically or by the excretion of polysaccharides into the medium. The symbiosis between fungi and plants has been shown to increase the stability of soil aggregates found in this degraded soil was also increased by *A. niger* treated Dry Olive Waste with Rock Phosphate.

The application of organic amendments to the soil increased enzyme ( $\beta$ -glucosidase and phosphatase) activities as well as the biodiversity of fungus populations. These results were further confirmed in other works which showed an increase in aggregate stability by the addition of the amendments (microbially treated solublizing bacteria- or SB+RP) to the soil, while no effect of Arbuscular Micorahizal fungus inoculation was observed in heavy metal (Zn)-contaminated soil (Medina et al. 2006). It was suggested that microbial population developed after the addition of the solid state fermentation processed wastes (that can be used as C and energy sources for activities of soil microorganisms) could be responsible for soil aggregate formation and stabilization. Similar beneficial effect of our biotechnological product related to stabilization of soil aggregates was found in acidic semiarid mine tailings. Particularly after soil drying, aggregate stability was 66% higher than the control soil. The fermentation mixture of mineralized organic matter and solubilized rock phosphate raised the soil pH and increased soil water-soluble carbon under either watering or drying conditions.

Moreover, enhanced antioxidant enzyme activity was observed in amended soil-plant systems. Degenerative reactions related to heavy metal stress are mediated by reactive oxygen species such as free radicals (superoxide and hydroxyl radicals) but also  $H_2O$  and singlet oxygen. Hydroxyl radicals and singlet oxygen are so reactive that their production must be minimized. On the other hand, C and  $H_2O_2$  are synthesized at very high rates even

under optimal conditions and may trigger toxic reactions leading to lipid per oxidation, protein denaturation, and DNA mutations (Bowler et al. 1992). The ability of plants to increase antioxidative protection in order to combat the negative consequences of heavy metal stress appears to be limited as many studies have shown that exposure to elevated concentrations of redox reactive metals reduces rather than increases antioxidative enzyme activities (Schutzendubel and Polle 2002).

## **2.9 EFFECT OF P-SOLUBLIZING MICROBES ON SOIL REHABILITATION**

Experiments aimed at soil rehabilitation have been oriented to reclamation of soils contaminated with heavy metals and a forestation of decertified Mediterranean sites. Effect of P-solublizing microorganisms on heavy-metal-contaminates soils was studied. Elevated concentrations of heavy metals in soil from anthropogenic sources (fertilizers and amendments) or mining activities (Shetty et al. 1995) pose long-term risk to environmental and sustainable production. The deteriorated physical and biological characteristics of contaminated soils need to be improved to establish a vegetation cover.

Heavy metal-contaminated soil can be treated by operations based on chemical, physical, or biological techniques. It is generally accepted that these remediation techniques maybe grouped into two main categories: (1) ex situ techniques which are based on mechanical removal of the contaminated soil and subsequent disposal to a landfill site for further treatment and (2) in situ methods, which avoid excavation, transportation, and land-filling costs by treating the metal contaminated soil in place. In situ techniques are easy to perform, favored over the ex situ techniques, and preferred due to their lower cost and reduced impact on the ecosystem (Khan et al. 2004; Kumpiene et al. 2008).

An interesting approach applied in in-situ remediation of heavy metal-contaminated soil includes a variety of phosphate amendments such as soluble phosphate and Phosphate bearing insoluble sources, mainly rockphosphate. Showed that hydroxyapatite is a very efficient metal immobilizer in laboratory conditions. Most of the further studies on heavy metal stabilization using this approach were carried out with a wide range of natural and synthetic P-bearing amendments, such as hydroxyapatites (Arnich et al. 2003), synthetic and natural apatites , phosphate rock (Chen et al. 2007), as well as their combinations (including other P-containing compounds).

## CHAPTER - 3

### MATERIALS AND METHODS

This chapter discusses the materials used and methodology adopted during the study. All the chemicals used and reagents employed were of analytical grade with sufficient purity. The calibration curves were prepared prior to estimation of unknown concentration and used throughout the study.

#### 3.1 COLLECTION OF SOIL SAMPLES

Soil sample was collected at the depth of 2 cm from agriculture field of Thapar university campus soil was analyzed for major parameters before seedling and after harvesting of crop.

##### 3.1.1 Characterization of soil

The above sample was analyzed for alkalinity, chloride, hardness, sulphate, according to Trivedy et al.,(1967),

##### 3.1.2 Seedling and growth of crops

Plastic cup of size (9cm × 10cm) were purchased from market. 150 g of soil was weight and placed in side of cups. The seeds were washed with distilled water two to three times; dry them on the ordinary filter paper than places in the cup. Four seeds were placed in one cup in duplicate. The scheme adopted for seed germination has been presented in table 3.1 below:

**Table 3.1: Seed Germination Scheme**

S. No.	Treatment	Constituents
1	Control	soil alone
2	Positive control	soil + tricalcium phosphate
3	Positive control	soil + rock phosphate (10mg/kg)
4	Positive control	soil + rock phosphate(20mg/kg)
5	Treatment	soil + isolate no 3
6	Treatment	soil + isolate no 3 + tricalcium phosphate
7	Treatment	soil + isolate no 3 + rock phosphate 10 mg/kg
8	Treatment	soil + isolate no 3 + rock phosphate 20mg/kg
9	Treatment	soil + isolate no 6
10	Treatment	soil + isolate no 6 + tricalcium phosphate
11	Treatment	soil + isolate no 6 + rockphosphate 10mg/kg
12	Treatment	soil + isolate no + rock phosphate.(20mg/kg)

### 3.2 ISOLATION OF BACTERIA

1.0 gm of soil sample was added in 10.0 ml of distilled water and shaken at 150 rpm for 1 hour. Let the soil settle down for half an hour. The supernatant was added in the nutrient broth and incubated for 2 days at 37°C at 150 rpm. After that  $10^{-1}$  to  $10^{-6}$  dilutions were made and surface spreaded on nutrient agar plates ( $10^{-2}$  to  $10^{-6}$ ). Bacterial isolates from the soil samples were identified on nutrient agar plates. Repeat the process twice after that the isolated strains were inoculated in Pikovskaya's medium plates. Out of these the best grown isolates on Pikovskaya's media were selected on the basis of their growth. Finally the strains were preserved on Pikovskaya's media slant at 4°C.

**Table 3.2: Composition of medium for phosphate solubilizers (Pikovskay's medium)**

(Source: Nautiyal CSAgricultural Microbiology Division, National Botanical Research Institute, Lucknow, India)

S. No.	Components	Amounts (gm per liter)
1	Glucose	10
2	$\text{Ca}_3(\text{PO}_4)_2$	5
3	$(\text{NH}_4)_2\text{SO}_4$	0.5
4	NaCl	0.2
5	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1
6	KCl	0.2
7	Yeast Extract	0.5
8	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.002
9	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.002
10	pH	7.0

### 3.3 IDENTIFICATION OF SELECTED BACTERIAL ISOLATES

#### 3.3.1 Morphological and biochemical characterization

Selected isolates were characterized by colony morphology on nutrient agar, gram staining and morphological characteristics. Additional biochemical test were performed according to Bergey's Manual of Determinative Bacteriology for taxonomic characterization which

included gelatine liquefaction, lipase screening, casein hydrolysis, H<sub>2</sub>S production, catalase test, lipase test, xylanase test, pectinase screening, methyl red-voges proskauer (MR-VP), citrate utilization, carbohydrate utilization and amino acid utilization etc.

### 3.3.2 Biochemical Tests

#### 3.3.2.1 Starch Hydrolysis

**Table 3.3: Composition of starch hydrolysis test**

Composition	Concentration (gm per liter)
Starch	20
Peptone	5
Beef extract	3
Agar	15
Distilled Water	1000ml

**Procedure:** Plates of starch agar were inoculated with the bacteria's and were incubated at 37°C for 24-48 hours. The plates were flooded with iodine solution, a clear zone was observed around the colonies.

#### 3.3.2.2 Gelatin Agar Medium

**Table 3.4: Composition of gelatin agar medium**

Composition	Concentration (gm per liter)
Gelatin	40
Tryptic Soy broth	30
Distilled water	1000ml

**Procedure:** Stab small inoculums of bacterium about  $\frac{3}{4}$  of the way to the bottom of a tube of "deep" agar with the inoculating needle. Repeated with separate stab tubes for each of bacteria and incubated at 37°C for 24 to 48 hours. Place the incubated inoculated stab and uninoculated control into the refrigerator for approximately 30 minutes. The inoculated stabs were compared with the control by tapping the tubes gently.

### 3.3.2.3 H<sub>2</sub>S Production

**Table 3.5: Composition for the test of H<sub>2</sub>S Production**

Composition	Concentration (gm per liter)
Peptone	30
Beef extract	3
FAS	0.2
Sodium Thiosulphate	0.0210
Agar	15
Distilled Water	1000 ml

**Procedure:** An inoculated needle was used to stab small inoculums of each of bacteria approximately  $\frac{3}{4}$  of the way to the bottom into separate SIM agar tubes. The deep stabs were incubated at 37°C for 24 to 48 hours. The inoculated “deep” stab was compared with the control.

### 3.3.2.4 Lipase Screening

**Table 3.6: composition for lipase screening of test**

Composition:	Concentration (gm per liter)
Nutrient Broth	13
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.1
Tween 80	10 ml
Agar	20
Distilled water	1000 ml

**Procedure:** Medium was sterilized by autoclaving at 121°C for 15 minutes. Tween 80 was autoclaved separately and added to medium. Incubate inoculated plates at 37°C in an inverted position for 24 to 48 hours. The plates were observed till the formation of opaque zone around the growth.



### 3.3.2.5 Methyl Red-Voges Proskaver test (MR-VP broth)

**Table 3.7: Composition for MR-VP test**

Composition	Concentration (gm per liter)
Peptone	7
Glucose	5
Potassium Phosphate	5
Distilled Water	1000 ml

**Procedure:** separate tubes of MR-VP broth were inoculated with the bacteria and incubated the tubes at 37°C for 24 to 48 hours. After incubation, approximately 1/3rd of each culture was transferred into empty glass test tubes and was set aside for VP test. Five drops of the methyl red indicator was added to the remaining broths in the original tubes. Then 12 drops of VP reagent 1 (5% naphthol solution in absolute ethanol) was added to each of the broths that were set aside for the VP test. Each culture was shaken to mix the reagent with the rest of the broth. Then immediately 2-3 drops of VP reagent 2 (40% KOH in water) was added and the cultures were shaken again. The cultures were shaken again in every 3-4 minutes for approximately 15 minutes. The results were compared with the control.

### 3.3.2.6 Carbohydrate Utilization

**Table 3.8: Composition for carbohydrate utilization test**

Composition	Concentration (gm per liter)
Peptone	10
NaCl	15
Sugar (Sugar used are Glucose, Fructose, Sucrose, Mannitol, Maltose)	5
Distilled Water	1000 ml

**Procedure:** 0.1 ml of inoculum of each of the bacteria's were transferred into broths (glucose, sucrose, fructose, xylose, mannitol, matlose) and incubated the inoculated broths at 37°C for 24 to 48 hours in a rotator shaker at 150 rpm. Results were observed for each broth and compared to the uninoculated controls.

### 3.3.2.7 Xylanase screening

**Table 3.9: Composition for Xylanase screening test**

Composition	Concentration (gm per liter)
Yeast extract	1
Xylan powder	5
Agar	20
Distilled water	1000 ml

[1% Congo red, 100 ml and 1M NaCl, 250 ml]

**Procedure:** Inoculated the plates with the bacteria's and incubated at 37°C in an inverted position for 24 to 48 hours. After growth occur, flooded the plates with Congo red solution (1% solution) for 30 minutes, and then washed with NaCl. The plates were observed until formation of a zone around the growth.

### 3.3.2.8 Pectinase Screening

**Table 3.10: Composition for Pectinase screening test**

Composition	Concentration (gm per liter)
Ammonium Sulphate	1
K <sub>2</sub> HPO <sub>4</sub>	6
KH <sub>2</sub> PO <sub>4</sub>	3
Pectin	5
Yeast extract	1
Distilled Water	1000 MI

**Procedure:** The autoclaved medium, cooled to 45°C to 50°C was poured into sterile petri plates and allowed to solidify. Aseptically inoculated the plates and incubated at 37 °C for 24 to 48 hours. After growth flooded with CTAB buffer (1% solution) for 10 minutes and clear zone was observed around the growth on the media.

### 3.3.2.9 Citrate Utilization

**Table 3.11: Composition for Citrate utilization test**

Composition	Concentration (gm per liter)
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.1
K <sub>2</sub> HPO <sub>4</sub>	1
NaCl	5
Sodium Citrate	2
MgSO <sub>4</sub>	0.2
Bromothymol blue	0.08
Agar	2
Distilled Water	1000 ml

**Procedure:** The surface of slants of Citrate agar was inoculated with the bacteria by using a small amount of inoculum and incubated at 37 °C for 48 hours. The inoculated slant was compared with the control.

### 3.3.2.10 Cellulase Screening

**Table 3.12: Composition for Cellulase screening**

Composition	Concentration (gm per liter)
Yeast extract	1
Carboxymethylcellulose sodium salt	5
Agar	20
Distilled water	1000 ml
Congo red solution	10

**Procedure:** Incubated the inoculated plates at 37°C in an inverted position for 24 to 48 hours and flooded the plates with Congo red solution (1% solution) after the growth occurred. The plates were observed till the formation of a zone around the growth.

### 3.3.2.11 Catalase Test

**Table 3.13: Composition for Catalase test**

Composition	Concentration( gm per liter)
Trypticase	15
Phytone	5
Sodium chloride	5
Agar	15

**Procedure:** Inoculated the trypticase soy agar slants and kept an uninoculated trypticase soy agar slant as control. The cultures were incubated at 37°C for 24 to 48 hours. Each culture was observed for the appearance or absence of gas bubbles.

## 3.4 PHYSIOLOGICAL TESTING

The optimal growth pH and salinity concentration for the two isolates were determined.

### 3.4.1 Effect of pH on growth.

Each species has ability to grow with in a specific pH range, that may be broad or limited, with the most rapid growth occurring within a narrow optimum range

**Procedure:** Inoculated each of a series of the tubes with bacterial cultures by adding 0.1 ml of the culture to each tube. Incubated the bacterial inoculated tubes for 24 to 48 hours at 37°C different pH (6-12). The growth was examined after 2 days by using spectrophotometer at 600 nm.

### 3.4.2 Effect of salt concentrations on growth.

**Procedure:**

Different salt concentrations (4%,8%, 12%,16%,20%of NaCl) were added to Nutrient broth tubes. Inoculated by each series of the tube and incubated the bacterial inoculated tubes for 24 to 48 hours at 37°C in a rotator shaker at 150 rpm. Growth was examined after two days with mass spectrophotometer at maximum wavelength of 600nm.

### 3.5 CHARACTERIZATION OF SOIL

Characterization of soil was done by using soil to water ratio 1:10. After shaking the soil for half an hour at 150 rpm flask was kept at stationary condition until the particle settle down. The supernatant was removed from the flask and then used for estimation of pH alkalinity chloride sulphate etc.

(i) **pH:** pH was determine by method given by (Razor ,2006)

(ii) **Hardness:** An excellent way to determine water hardness is to perform a complexometric titration using a ethylenediaminetetraacetic acid (EDTA) solution. Due to steric hindrance, EDTA will complex with calcium and magnesium in a one-to-one molar ratio. The end point in this experiment will be determined by using an EBT indicator. Hardness(Trivedy et al.,1967)

(iii) **Alkalinity:** Alkalinity of soil is its acid neutralizing capacity .It is the sum of all the titrable base. the measured value may vary with the end point pH used. (Trivedy et al.,1967)

(iv) **Chloride:** In a neutral or alkaline solution potassium chromate can indicate the end point if the silver nitrate titration of chloride. (Trivedy et al., 1967)

### 3.6 GRAVIMETRICAL DETERMINATION OF SULPHATE

2ml of HCL was added to 100ml of supernatant and was boiled. Then 20ml BaCL<sub>2</sub> was added, and the sample was cooled. Take initial weight of dry whatman filter paper no 42 and the sample was filtered. Precipitates were washed with warm water. After removing the filter paper from funnel, it was dried at 105°C for 15 minutes and then its final weight was measured.

### 3.7 ESTIMATION OF ORGANIC CARBON IN SOIL SAMPLE

The method developed by walkely and black (1939) was used for estimation of organic carbon in initial soil sample, as per the procedure mentioned below:

#### **Procedure:**

1. A sample of 1g of soil in 500ml of conical flask by addition of 10 ml of 1 NK<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>.
2. 30ml of H<sub>2</sub>SO<sub>4</sub> was added and allowed to stand for 30 mints and after this add 200 ml water.
3. Then 10ml Orthophosphoric acid, 0.5 g NaF and 1ml diphenalamine indicator was added
4. Content was titrated with freshly prepare 0.5N FAS until color changes from blue to green.

### **3.8 AVAILABLE PHOSPHOROUS IN ALKALINE SOIL (Olsen et.al., in 1982)**

#### **Procedure:**

1. 2.5 g of solution was put in a flask, followed by addition of 50 ml extracting solution.
2. Solution was kept on shaker for 30 minutes and the sample was filtered through whatman filter paper no 42.
3. 10 ml of aliquot of filtrate was taken in a conical flask and added 2.5 ml of H<sub>2</sub>SO<sub>4</sub>, 15.5 ml of distilled water, 8 ml of reagent B and added more 15.5 ml of distilled water.
4. For standard curve 0, 2, 5, 10, 15, 20ml of standard solution was placed in 50 ml of volumetric flask. Then 10 ml extracting solution, 1 ml of 2.5 concentrated H<sub>2</sub>SO<sub>4</sub> and 8 ml of reagent B volume make up to 50 ml. For blank add all reagents except PO<sub>4</sub>
5. Take absorbance at 882 nm.

### **3.9 BACTERIAL COUNTS**

Bacterial counts were carried out by standard plate count method using following composition of media:

Nutrient broth	1.3g
Agar	1g
Water	100ml

#### **Procedure:**

1. Soil sample serially diluted to 10<sup>-1</sup> to 10<sup>-6</sup> in sterile distilled water
2. 100 µl of sample from 10<sup>-5</sup> and 10<sup>-6</sup> was spread on agar plate.
3. Colony was counted with in 24hrs and 48 hrs

### **3.10 ESTIMATION OF PHOSPHATE SOLUBLIZATION ACTIVITY BY THE BACTERIAL ISOLATES**

The phosphate solublizing bacterial isolates were studied for the efficiency of percentage solublization of tri calcium phosphate (Gaur, 1990).

#### **Procedure:**

1. 200 µl of bacterial filtrate (obtained from each of the phosphate-solublizing Bacterial isolate) growing under shaker conditions at 28°C was transferred to 50 ml volumetric flask.

2. Added 10 ml of chloromolybdic acid along the sides of the flask and the contents were diluted to 40 ml.
3. Added 1 ml of Chlorostannous acid and the volume was immediately made up to 50 ml. The blue color intensity was measured at 690nm.
4. To prepare a standard curve, 0.0, 0.5, 2.5, 5.0, 7.5 and 10 ml was taken in 50 ml volumetric flasks and make total volume 50 ml take OD at 690 nm

### **3.11 ACID PHOSPHATASE ACTIVITY IN SOIL**

As reported by Bremner(1969), 1 g of fresh soil (<2 mm) was put in a 100 ml Erlenmeyer flask, added 4 ml of modified universal buffer (pH 6.5) and 1 ml of 100 mM p-NPP. Substrate was dissolved in the buffer, and swirled the flask slightly for a few seconds to mix the contents. The flask was then sealed and incubated at 30 ° C for 30 min. After incubation, the flask was immediately placed on ice and followed by addition of 1 ml of 2 M CaCl<sub>2</sub> and 4 ml of 0.2 M NaOH to terminate the reaction and they extract the para-nitrophenol (p-NP) formed. The sample was diluted with 90 ml of deionized water and then filtered through Whatman-42 filter paper. Absorbance of released pNP was determined Spectrophotometrically at 400 nm. Four replicates including one blank were used for each soil sample. The acid phosphatase activity was expressed as μmol p-NP per gram dry soil and incubation time (unit/mol).

### **3.12 ALKALINE PHOSPHATASE ACTIVITY IN SOIL**

**Procedure:** Further in his work Bremner (1969), took 1 g of fresh soil (<2 mm) in a 100 ml Erlenmeyer flask, added 4 ml of modified universal buffer (pH 10) and 1 ml of 100 mM p-NPP substrate dissolved in the buffer, and swirled the flask slightly for a few seconds to mix the contents. The flask was then sealed and incubated at 30 ° C for 30 min. After incubation the flask was immediately placed on ice and then added 1 ml of 2 M CaCl and 4 ml of 0.2 M NaOH to terminate the reaction and extract the para-nitrophenol (p-NP) formed. The sample was diluted with 90 ml of deionized water and then filtered through Whatman-42 filter paper. Absorbance of released pNP was determined spectrophotometrically at 400 nm. Four replicates including one blank were used for each soil sample. For the blank, p-NPP was added after (instead of before) the incubation.

### **3.13 CHLOROPHYLL ESTIMATION**

#### **Procedure:**

1. 0.05gm of leaves are taken in screw cap tube
2. Added 20ml of 80% of acetone and placed the test tube on refrigerator for 24 hours.
3. The leaves were removed from acetone and optical density (OD) were measured on 663nm and 645nm on spectrometer (Sadavisiom & Manickam 1996)).

## CHAPTER - 4

### RESULTS AND DISCUSSION

The result of study performed and discussion on results has been presented in this chapter. The composition of soil was done before and after conducting the studies on plant growth. The soil used for plant growth shows pH 7.2, NPK level 1.96%, total phosphorous 0.102 and available potassium 0.080% respectably. This indicated that the nutritional level of soil is appropriate for this study. Also soil is not showing any metals like Fe, Cr, Ni and thus contamination due to metal is not possible. Soil selected has a high water holding capacity of 9.65% and organic carbon of 0.7% which makes soil suitable for growing crops. The table 4.1 below shows the result of soil analysis for physicochemical characteristics.

**Table 4 .1: Physicochemical characteristics of soil**

S. No.	Physicochemical characteristics	Value
1	pH	7.2
2	Soil conductivity	0.025mho/cm
3	Water holding capacity	9.65% dry weight
4	Organic carbon	0.7
5	Chromium	Nil
6	Iron	Nil
7	Total nitrogen	1.96%
8	Total phosphorous	0.102%
9	Available potassium	0.080%
10	Alkinity	2000mg/l
11	Chloride	37mg/l
12	Sulphate	605.05mg/l

#### 4.1 PLANT GROWTH

Germination of seeds observed 100% within 2 days in all replicates mention in case of wheat except control soil only where it took 6 days, in case of positive control where soil +rock phosphate 20mg /kg were added also took six days these data showed that rock phosphate took some time for solublization .in case of lady finger maximum time period taken for 100% growth was observed in 10days.

The germination period seeds and harvesting period of crops depend upon atmospheric temperature moisture availability soil characteristic and nature of crop and type of seeds some crop like paddy sugarcane take 1year and bamboo takes several years for yield the harvest.

**Table 4.2: Total weight of shoot and root of plant**

Treatment	Wheat			Ladyfinger		
	plant total weight	height of shoot	Height of root	plant total weight	Height of shoot	Height of root
Soil +crop	0.8	7	6	0.7	2.8	1.6
Soil + tricalcium phosphate	0.15	8	5	.23	8	3
Soil + rockphosphate(10mg/kg)	1.09	6	5	0.42	8	5
Soil + rock phosphate(20mg/kg)	0.85	18	10	0.21	6	2
Soil + isolate no 3	2.24	20	15	1.02	7.82	7
Soil + tricalcium phosphate + isolate no 3	0.736	20	21	1.46	7.5	2.3
Soil + rockphosphate (10mg/kg) + isolate no 3	2.24	15	12	2	9	5.4
Soil + rock phosphate(20mg/kg) + isolate no 3	1.74	21	18	0.81	7.5	5
Soil + isolate no 6	3.29	23.37	17	1.85	8.5	5
Soil + tricalcium phosphate +isolate no 6	3.84	13.25	27	.53	8.12	2.75
Soil + rock phosphate(10mg/kg) + isolate no 6	3.90	34.37	25	2.16	8	4.25
Soil + rock phosphate(20mg/kg) + isolate no 6	1.1	14	19	0.88	6.37	3

Total plant weight, their height, shoot weight are the average of 4 plants

Growth of seedling and crop growth, the observation from 4 day to 12 days majority include length measurement of plumule and radicals and number of leaves but in our case ladyfinger took 10 days for germination so all the observation taken after 30 days of growth.



**Figure 4.1: All Samples of plants of wheat and ladyfinger after 30 days**

The crop were irrigated with 15ml of water every after alternate days if required different treatment was given to the soil as discuss in (table 4.2) there are positive and negative control replicates with different combination of rock phosphate and calcium triphosphate (Tomar et al.,2002) discuss that high temp and pH huge concentration of in Inorganic salts organic matter caused high salinity and high osmotic pressure in soil which decreased seed germination drastically.



**Figure 4.2: Growth of Wheat plant after 30 days**

The crop wheat showed better in term of total height of plant as it showed 34.37cm when isolate no 6 was added with 10mg /kg of rockphosphate in treatment followed by soil + isolate no 6 23.27cm, and soil isolate no 3, 19.25cm, soil + tricalcium phosphahate with isolate no 6, 13.25cm. The maximum weight 3.90g observed in soil + RP10mg/kg + isolates no 6

In case of ladyfinger maximum plant weight was observed in 10mg/kg of rockphosphate with isolate no 6,2.16cm. The height of shoot was ranges in between 6 to 9 cm in ladyfinger and root ranges from 1.6 to 7 cm.



**Figure 4.3: Growth of ladyfinger plant after 30 days**

In India 260 million tons of rock phosphate deposit and utilized in agriculture in better ways for sustainable agriculture system, Microbial solublization of rock phosphahate, Its use in agriculture receive greater attention because many fungi and bacteria can solublize inorganic phosphate in to soluble form through the process of acidification. (Steven et al., 2004).

Our results showed that rockphosphate when solublize with bacteria permote the growth of plant. (Saber et al., 2009 Omar 1998) also indicating their results that utilization of rockphosphate as fertilizer along with phosphate solublizing bacteria are scare especially in field conditions Sing and Reddy 2011 used phosphate solublizing fungus on growth of maize and wheat plants with rockphosphate. The growth parameters were higher in rockphosphate fertilize by bacteria or fungus as phosphorous is known to initiate cell division and enlargement process the increase in shoot height could be probable due to increase

mobilization of phosphorous made soluble by phosphorous solubilizing microbes. (Reyes et al., 2002)

#### **4.2 Isolation of bacteria:**

Bacteria were isolated on nutrient broth and finally checked on Pikosky medium

##### **4.2.1 Screening of bacteria**

Screening of bacteria was done by using nutrient broth and incubate for 24 to 48 hrs at 37°C on rotator shaker at 150 rpm. After 48 hrs culture serially diluted and spread on nutrient agar plate and incubate at 37°C. Total 8 colonies were selected from nutrient agar plates and isolated colonies were streaked on Pikovskaya's medium and out of 8 colonies, 5 colonies were grown on Pikovskaya media, All the five colonies were tested for phosphate solubilization activity out of them, two colonies were selected for further experimentation.

##### **4.2.2 Identification of selected isolates**

The selected two isolates no 3 and no 6 were characterized by colony morphology on nutrient agar. Gram staining and additional biochemical tests were performed according to (Aneja 2008) which includes gelatin liquefaction, lipase screening, H<sub>2</sub>S production, casein hydrolysis, MR-VP test, citrate utilization, carbohydrate utilization and release of enzyme etc.

##### **4.2.3 Biochemical activity of microorganism**

Microorganism must be selected, separated and identified for wide variety of reasons such as

1. Determination of pathogens.

2. Selection and isolation of strain of fermentative microorganism necessary for industry vitamins, organic acid, antibiotics and industrial enzyme.

3. Comparison of biochemical activities for taxonomic purpose

The chemical reaction is defined as cellular metabolism and biochemical transformation that occur both inside and outside the cell. Most high molecular weight substances are not able to pass through cell membrane therefore material food stuff such as polysaccharide and lipid and protein must be degraded to low molecular weight material. Nutrient before they can be transported into cell these tests are starch, casein, lipid, gelatin hydrolysis.

##### **4.2.4 Intracellular enzyme**

These enzymes function inside the cell and are mainly responsible for synthesis of new protoplasmic requirement and production of cellular energy from assimilated material tests are

carbohydrates fermentation, H<sub>2</sub>S production, Nitrate and catalase reaction, Urease and Oxidase test, special test, Indole, Methylred, Vogesproskauer and Citrate utilization.

### **4.3 Biochemical Tests:**

#### **(a) Starch Hydrolysis**

The starch hydrolytic activities include degradation of macro molecules which require co-enzyme. The starch which serves as the polysaccharides substrate. The detection of hydrolytic activity of starch, the starch test to determine the presence and absence of starch medium. Starch in the presence of iodine will impart blue color to medium and clear zone around the colony indicating presence of starch hydrolyzing bacteria. The isolate number 3 showed positive results.

#### **(b) Gelatin hydrolysis test**

Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue. Below temperature 25<sup>0</sup>C gelatin will maintain its gel properties, and exist as solid, temperature above 25<sup>0</sup>C gelatin is liquid. Liquefaction is accomplished by some micro organisms capable of producing a photolytic extra cellular enzyme called gelatinase which act to hydrolyze this protein to amino acid. Once degradation occur even very low temperature of 4<sup>0</sup>C will not restore the gel characteristics. The isolate number 3 shows the positive results when the culture as placed in a refrigerator at 4<sup>0</sup>C for 30 minutes. Culture remain liquefied produce gelatenase and called as rapid hydrolyse. Culture remain liquefied produce gelatenase and called as rapid hydrolyser, whereas isolate number 6 showed gelatenase negative results.

#### **(c) Pectinase Screening**

Collective term for raw enzymes that is able to transform pectins. some fruits form pectinase during natural gestation. A commercial pectinase might typically be activated at 45 to 55<sup>0</sup>C and work well at pH of 4.5 to 5.5. CTAB buffer(1% solution) was flooded on petriplates for 10 minutes and clear zone was observed around the growth for positive results on the media. In our case both isolates shows negative results

#### **(d) Lipase Activity**

Lipase and high molecular weight compounds processing large amount of energy. The degradation of lipids such as triglycerides is accomplish by hydrolyzing enzymes called lipases that cleave the ester bond in the molecule by the adding of water to form a building blocks glycerol and fatty acid Lipase showed positive result in isolate no 3, where as isolate no 6 showed negative results

**Table 4.3: Results of biochemical tests**

<b>Isolates</b>	<b>Starch agar media</b>	<b>Gelatin agar media</b>	<b>H<sub>2</sub>S production</b>	<b>Citrate utilization</b>	<b>MR-VP</b>
3	-	+	-	-	+
6	-	-	-	-	-

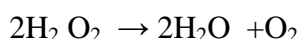
**(e) Carbohydrate utilization**

Most organism obtained energy through a series of orderly and integrated enzymatic reaction leading to biooxidation of a substrate. Some organisms are capable of fermenting of sugar such as glucose aerobically and anerobically. Aerobic biooxidation in which molecular oxygen can serve as final electron acceptor. Whereas anaerobic biooxidation in which inorganic ions other than oxygen serve as final electron acceptor.

In carbohydrate utilization xylose, mannitol, maltose, fructose and glucose show positive results. Means they permote the growth of isolate no 3 or isolate no 6 where as sucrose show negative results which indicate that sugar sucrose is not suitable for growth of these isolates when it mixed with nutrient broth(4.4).

**(f) Catalase production**

During aerobic respiration microorganism produce hydrogen peroxide and in some case an extremely toxic super oxide was produced. Accumulation of these substances will results in death of organism unless they can be enzymatically degraded. Under anaerobic condition carbohydrate degraded for energy production organisms capable of producing catalase and rapidly degraded hydrogen peroxide as written below:



Catalase production can be determined adding the substrate H<sub>2</sub>O<sub>2</sub> on agar plate (table .4.4).If catalase is present the chemical reaction mention is indicated by bubbles of free oxygen gas this show a positive catalase test and the absence of bubble formation show negative catalase test. In our isolate no 3 and isolate no 6 catalase show negative results which show non toxicity in the process.

**Table 4.4: Results of biochemical tests**

Isolates	Xylose	mannitol	Maltose	Sucrose	Fructose	glucose	Catalase
3	+	+	+	-	+	+	-
6	+	+	+	-	+	+	-

**Table 4.5: Results of biochemical tests**

Isolates	Xylanase	Cellulase	Lipase	Pectinase
3	-	-	+	-
6	-	-	-	-

**(g) Citrate utilization process**

In absence of fermentable glucose or lactose some microorganism are capable of using citrate as a carbon source for their energy. This ability depends on the presence of citrate. During the growth of bacteria, reaction take place and medium become alkaline. Oxygen that is generated combines with sodium and water and form sodium carbonate, an alkaline product. The presence of sodium carbonate change the bromo-thymol blue indicator incorporated into the medium from green to deep Prussian blue. In this experiment as already shown (table 4.4) that almost all the carbohydrates were utilize by isolate no 3 and isolate no 6, So the citrate utilization result found negative .

**(h) Hydrogen sulphide production**

There are two fermentative path ways by which some microbes are able to produce H<sub>2</sub>S.

1. H<sub>2</sub>S may be produced by the reduction of organic sulpher present in the amino acid which is a component of peptones contained in the medium which degraded enzymatically.
2. By the reduction of inorganic sulpher compound such as thiosulphate, sulfate and sulphides The SIM medium contain peptone and sodium thiosulphate as the sulpher substrate. The ferrous ammonium sulfate in the medium serve as indicator by combining with the gas H<sub>2</sub>S forming black ferrous sulfide precipitate that was seen along the line of stab inoculation the isolate no 3 and isolate no 6 are not producing any gas so both the culture showed negative results for H<sub>2</sub>S production.

**(i)Cellulose activity**

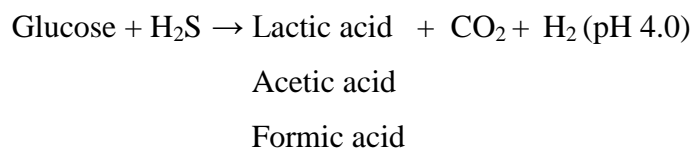
In cellulase activity, the enzyme complex break down cellulose to beta-glucose. cellulase action is considered to be synergistic. Cellulase activity can be determine by flooded the plates with congo red solution (1% solution) for 30 minutes, than washed with NaCl, , if zone around the growth was observed. Showed positive results. In our case the isolate no 3 or 6 shows negative results

#### **(j) Xylanase screening**

Xylanase are genetically single chain glycoprotein, ranging from 6-80kDa, active between pH 4.5-6.5 and temperature between 40-60<sup>0</sup> C. xylanase from different sources differ in their requirement for temperature and pH. xylanase production was determine by flooded the plate with Congo Red solution (1% solution for 30 minutes, washed with NaCl , a zone around the growth was observed. The isolates no 3 or 6 shows negative results

#### **(k) Methyl red**

The hexose monosacride glucose is the major substrate oxidized by bacteria for energy production.. In this test methyl red indicator was used which give red colour .Isolate 3 show positive results in methyl red.



End point: Methyl red indicator gives red colour.

#### **(l) Voges-proskour test**

This test determine the capability of some organism to produce non acidic or neutral end product such as acetyl, methyl,carbonyl from the organic acid. That result from glucose metabolism this reaction occur in the presence of the alphanepthol of catalyst and a guanidine group that is present in peptones of the MR-VP medium as a pink complex is formed.In isolate no 3 MR-VP test are positive where as isolate no 6 showed negative result .

### **4.3 PHYSIOLOGICAL TEST**

#### **4.3.1 Effect of pH on bacterial growth**

From the table (4.6) it was observed that isolate no 3 showed maximum growth 1.251 at pH 7. Whereas isolate no 6 showed maximum growth at pH 8. Same minimum growth at 11 pH is 0.052 in isolate no 3 and 0.073 in isolate no 6. These results indicate that up to pH 9 the promotion of growth is found which started decreasing after pH 9. This result shows that both of the isolates were pH sensitive. The results have been observed taking optical density (OD) at 600nm.

**Table 4.6: Effect of pH on bacterial growth**

<b>pH</b>	<b>Isolate no 3</b>	<b>Isolate no 6</b>
7	1.251	0.562
8	0.573	1.054
9	0.921	1.025
10	0.627	0.098
11	0.052	0.075
12	0.072	0.073

OD has been measured at 600nm

#### **4.3.2 Effect of salt concentration on Growth.**

Bacteria can divide into four groups depending on their ability to at various sodium chloride concentrations; Non halophilic (grow at less than 2% sodium chloride concentration). Halophilic may further be of three type: slightly halophilic (grow between 2 to 5%NaCl) moderately halophilic (grow up to 10%) and extremely halophilic (grow at more than 20% NaCl)

Isolate no 3 shows maximum growth at 12% salinity and then start decreasing with increase in salt concentration. It indicated that isolate 3 can tolerate upto 12% salinity, whereas isolate 6 show maximum growth at 8% salinity. Tomer et al., (2002) also reported buildup of soil salinity and high osmotic pressure in soils treated with distillery effluents which also effect on germination of growth from the (table 4..7) it was concluded that salinity not required for growth of these two isolates no 3 and isolate no 6.

But if they grow in high salinity, they can survive easily.

**Table 4.7: Effect of salinity on bacterial growth**

<b>Salt%</b>	<b>Control</b>	<b>Isolate no3</b>	<b>Isolate no 6</b>
0	0.002	0.367	0.463
4	0.001	0.458	0.601
8	0.004	0.538	0.672
12	0.003	0.555	0.534
16	0.002	0.401	0.410
20	0.003	0.226	0.405

#### 4.5 PHOSPHATE SOLUBLIZATION ACTIVITY IN BACTERIAL ISOLATES

The phosphate solubilization activities in both isolates were studied up till 5 days. Maximum efficiency was observed in 98.7% in isolate no 3 in 72 hrs. There are marginal difference between 48 & 72 hour values. There are 96.5 % increases in 24 hrs as compared to zero hour. Whereas in 48 to 72 hour marginal difference was observed. There is almost same efficiency was observed in 96 hours in case of isolate no 6. The maximum efficiency was 98.7% in 72 hr and removal also maximum with in 24 hr 96.5% (Table 4.8.) In 96 hrs in both isolates decreased to 97.4% which indicates the study can be terminated within 48 to 72 hour.

**Table 4.8 Percent solubilization of bacterial isolates in tricalcium phosphate.**

<b>Tricalcium Phosphate</b>				
<b>Days</b>	Isolates 6OD	Isolate no 6 at % solubilization	Isolates no 3 OD	Isolate no 3 at % solubilization
0 hour	0.04	0.04	0.06	0.06
24 hour	1.16	96.5	1.34	96.5
48 hour	2.12	98.6	2.9	98.6
72 hour	3.24	98.7	3.9	98.7
96 hour	1.58	97.4	2.4	97.4

**Table 4.9 Percent solubilization of bacterial isolates in rockphosphate**

<b>Rock phosphate</b>				
<b>Days</b>	Isolates no 6 OD	Isolate no 6 at % solubilization	Isolates no 3 OD	Isolate no 3 at % solubilization
<b>0 hour</b>	0.04	0.04	0.04	0.04
<b>24 hour</b>	1.18	96.6	1.78	97.7
<b>48 hour</b>	1.92	97.9	2.92	98.6
<b>72 hour</b>	2.68	98.5	3.58	98.8
<b>96 hour</b>	2.02	98.0	2.59	98.4

In case of rock phosphate maximum efficiency was observed 99.4 which is almost stable for 96 hrs. These result showed that maximum efficiency as compared to control 96.6% was in 24 hr which decreased linearly, same observation was found in isolate no 6 maximum

difference was observed compared to control in 24 hrs 97.7% after that there is marginal difference found in 48 hours to 96 hours.

The means of solubilization by which insoluble phosphorous compounds are mobilized mineralize by the production of organic acid .These organic acids are converted in to tricalcium phosphate to monobasic phosphate .There by enhancing the availability of the element to plants all the carbonaceous substrate available to microbes converted to organic acid like formic, acetic, succinic and lactic acid.

#### **4.6 ACIDIC AND ALKALINE PHOSPHATASE ACTIVITY**

Soil enzyme activity serve several important functions .It reflect the microbiological activity in soil and act as indicator of soil change. One of the most important soil property is pH. It effects availability of nutrient, control the composition, and diversity of microbial community, alter the equilibrium solid phase and impact plant response .Soil pH also effect the activity of enzyme due to pH sensitivity of amino acid functional group.

##### **4.6.1 Acid phosphatase activity**

Acid phosphatase activity 0 .154 unit /ml was found maximum before growth of crop in soil + rockphosphatase (20mg/kg )+isolate no 3 and 0.112 in 20mg/kg rock phosphate +isolate no 6 minimum value found in control was 0.054.

The bacterial isolate no 3 showed increase in activity (100%) in wheat where as 61.7% in ladyfinger isolate no 6 showed 46% in wheat and 21.35% in ladyfinger. The acid phosphatase activity maximum increased 0.192 unit/ml in wheat crop soil and 0.218 units /ml in lady finger crop in where 20 mg/kg rockphosphate and isolate no 3 was added in soil, which was higher than soil before plant growth. The increased value indicated that microbes solublize phosphorous and activity was increased after 30days growth of plant in their soil. (Richardson et al.,2005) also found similar results . He indicated that increase activity due to reduction of pH also.

Burns (1983) studied the activity of various phosphatase in rhizosphere maize, barley and wheat which showed that phosphatase activity found good at acidic soil and neutral soil pH. The soil bacteria expressing a significant level of acid phosphatase .the increasing enzyme activity would respond to meet the increasing phosphate demand by plant and microbe growth. The better is soil nutrient condition, higher is soil phosphatsae activity (Chen et al.,

2008). As soil phosphatase activity is a phosphatase prevailing indicator of degree in phosphate limitation (Turner et al., 2005)

**Table 4.10: Acid phosphatase activity**

S. No.	Treatment	Before treatment	Acid Phosphatase Activity after 30 days of plant growth	
			After treatment in wheat	After treatment in ladyfinger
1	Soil + crop	0.054	0.077	0.089
2	Soil + tricalcium phosphate	0.054	0.097	0.102
3	Soil + rockphosphate(10mg/kg)	0.063	0.078	0.090
4	Soil + rock phosphate(20mg/kg)	0.073	0.097	0.089
5	Soil + isolate no 3	0.069	0.154	0.128
6	Soil + tricalcium phosphate + isolate no 3	0.079	0.165	0.154
7	Soil + rockphosphate (10mg/kg) + isolate no 3	0.081	0.170	0.207
8	Soil + rock phosphate(20mg/kg) + isolate no 3	0.089	0.192	0.218
9	Soil + isolate no 6	0.075	0.113	0.108
10	Soil + tricalcium phosphate +isolate no 6	0.065	0.108	0.144
11	Soil + rock phosphate(10mg/kg) + isolate no 6	0.059	0.149	0.136
12	Soil + rock phosphate(20mg/kg) + isolate no 6	0.079	0.135	0.174

Our result show good activity where bacterial isolate were added as compared to soil where no bacteria was added. (Huang et al., 2011) showed effect of precipitation on soil acid phosphatase activity in three successional forest in the china, he stated that soil acid phosphatase plays a vital role in controlling phosphatase mineralization and its activity reflect the capacity of organic phosphorous mineralization potential in soil.

#### **4.5.2 Alkaline phosphatase activity**

The sensitivity of soil enzyme at pH should make it possible to evaluate the effective pH status of soil by presence of relative activity of certain enzymes. As acid phosphatase activity

was predominantly in acidic soil and alkaline phosphatase in alkaline or neutral soil. (Dick and Tabatabai1992).

**Table 4.11: Alkaline phosphatase activity**

S. No.	Treatment	Before treatment	Alkaline phosphatase activity after 30 days of plant growth	
			After treatment in wheat	After treatment in ladyfinger
1	Soil only	0.059	0.067	0.081
2	Soil + tricalcium phosphate	0.063	0.081	0.099
3	Soil + rockphosphate(10mg/kg)	0.061	0.102	0.116
4	Soil + rock phosphate(20mg/kg)	0.063	0.108	0.116
5	Soil + isolate no 3	0.072	0.114	0.163
6	Soil + tricalcium phosphate + isolate no 3	0.071	0.136	0.199
7	Soil + rockphosphate (10mg/kg) + isolate no 3	0.075	0.155	0.178
8	Soil + rock phosphate(20mg/kg) + isolate no 3	0.095	0.171	0.225
9	Soil + isolate no 6	0.075	0.106	0.122
10	Soil + tricalcium phosphate +isolate no 6	0.063	0.108	0.171
11	Soil + rock phosphate(10mg/kg) + isolate no 6	0.071	0.138	0.159
12	Soil + rock phosphate(20mg/kg) + isolate no 6	0.072	0.243	0.280

Some attempt has made to see both activity (acid/alkaline) in this work given in table (4.11). It was found that maximum alkaline phosphatase activity was found in soil + isolate no 6, before plant growth 0.079 unit/g where as minimum in control (only soil) was 0.059. This

value showed that activity increase due to the addition of tricalcium phosphate as compared to control. Dick et. Al., (2000) worked on soil acid or alkaline phosphates activity and stated that in general pH of approximately 6.0 was required to achieve alkaline or acidic phosphate ratio, with sufficient application of tricalcium phosphate and adjustment compared to those soil which required additional pH adjustment.

Alkaline and acidic phosphate activity primarily derived from the microbial sources and not from higher plant material (Tabatabai 1994) so to stimulate microbial activity we added two bacterial strains isolate no 3 and isolate no 6 other than tricalcium phosphate and rock phosphate (table 4.10 &4.11).

#### **4.7 AVAILABLE PHOSPHOROUS**

Most of the agricultural soil contains large reserves of phosphorous some part of which are accumulated as a consequences of regular application of phosphorous fertilizer. This excess phosphorus immobilized soon after application and unavailable to plants. The fixation and precipitation of phosphorus in soil is highly dependent upon pH and soil type. Thus in acid soil phosphorous is fixed by free oxides and hydroxides of aluminium and iron. While in alkaline soil it is fixed by calcium causing a low efficiency of phosphorous fertilizer such as super calcium (Goldstein 1986 and Jones et al.,1991).

The available phosphorous before treatment (no crop was grown) was showed in( table 4.12). It was ranged from 141ppm to 235.9 ppm in soil before growth of plants. The available phosphorous increased relatively when rock phosphate tricalcium phosphate and bacterial isolate were added.

In case of wheat crop the sample were having maximum available phosphorous , 266.30ppm where tri calcium was used with isolate no 6 and minimum 154.34 was 20mg/kg of rock phosphate alone were used. Again available phosphorous increased when isolate no 3and isolate no 6 were added alone with tricalcium phosphate and rock phosphate the range was in between 194.5 to 266.3 ppm. In case of lady finger similar trend were observed where as in case of control where only soil was added the value were smaller than treatment.

**Table 4.12: Available phosphorous before and after 30 days of plant growth**

S. No.	Treatment	Available phosphorous in soil before plant growth	Available PO <sub>4</sub> after 30 days of plant growth	
			Wheat	Ladyfinger
1	Soil+crop	157.6	154.3	152.26
2	Soil + tricalcium phosphate	167.4	191.30	176.00
3	Soil + rock phosphate(10mg/kg)	176.1	186.9	177.17
4	Soil +rock phosphate(20mg/kg)	141.3	154.34	152.17
5	Soil+ isolate no 3			
6	Soil+ tricalcium phosphate + isolate no3	213.04	244.5	220.6
7	Soil + rock phosphate(10mg/kg) + isolate no 3	210.8	225	215.2
8	Soil + rock phosphate(20mg/kg) + isolate no 3	220.65	248.9	228.26
9	Soil + isolate no 6	172.9	194.5	177.17
10	Soil + tricalcium phosphate +isolate no 6	235.2	266.30	257.6
11	Soil + rock phosphate(10mg/kg) + isolate no 6	200	213.04	208.7
12	Soil + rock phosphate(20mg/kg) + isolate no 6	215.2	233.36	222.82

In case of isolates when tricalcium phosphate is mixed with bacterial isolate no 6. Then available phosphorous is increased more 266.3ppm as compared to isolate no 3 which was 244.5ppm. The reverse result were obtained when 10mg /kg rockphosphate were added with bacterial isolate no 3. The value increases more as compared to isolate no 6 in both the crop soil. When rockphosphate 10mg/kg added in isolate no 3 the phosphorous increase to 225ppm 215.2ppm which further increase to 248.9ppm and 228.3ppm. When rock phosphate 20mg/kg added with isolate no 3. But compared to isolate no 3 isolate no 6 show less

available phosphorous which is 213.04 and 208.7ppm respectively with rockphosphate 10mg/kg while with 20mg/kg it is 233.4 and 222.8ppm respectively with wheat and ladyfinger but all these values are lesser than control (only soil) .These result indicate that the variability of available phosphorus become increased after addition of bacterial isolates which solublize the phosphorous, and isolate no 3 was found more active in solublization of phosphorous as compared to isolate no

Table 4.12 showed that increase in available phosphorous and organic carbon was greater in inoculated treatment as compared to non inoculated bacteria replicates. This may be attributed to ability of such microorganism to excrete organic acid. There by decrease the pH and increase the concentration of phosphorous in soil. (Vassilve et al., 1996). It was assumed by the authors that these organic acid Solublize insoluble form of phosphate to useable form this increase the potential availability of phosphate for plant observed by (Kucey et al., 1989)

#### **4.8 TOTAL ORGANIC CARBON**

The organic carbon present before treatment in the range of 0 .04 % to0 .68% which indicate soil is rich in carbon. The presence of organic carbon support the water holding capacity of the sample, Initially maximum organic carbon 0.68%found in soil + rockphosphate10mg/kg + isolate no 3 whereas after treatment it increases to 0 .81% in wheat crop and 0 .75% in ladyfinger(table 4.13). When isolate no 3 and isolate no 6 were added to soil than value increase to 0.68%and0 .57% in wheat and ladyfinger, which will be more or less same when isolate no 6 added 0.75% and 0.58% respectively. The organic carbon was increased initially when tri calcium phosphate and rock phosphate mixed with both the strains isolate no 3 and isolate no 6 .These results indicate that organic carbon had close relationship with soil acid phosphatase activity(Turner and Haygarth 2005).

**Table 4.13: Total organic carbon in soil before and after 30 days of plant growth**

S. No.	Treatment	Before plant growth	% organic carbon after 30 days of plant growth	
			wheat	Ladyfinger
1	Soil+crop	0.40%	0.27%	0.19
2	Soil + tricalcium phosphate	0.40	0.60	0.68
3	Soil + rock phosphate(10mg/kg)	0.40	0.34	0.13
4	Soil +rock phosphate(20mg/kg)	0.27	0.17	0.20
5	Soil+ isolate no 3	0.47	0.68	0.57
7	Soil+ tricalcium phosphate + isolate no3	0.40	0.60	0.68
8	Soil + rock phosphate(10mg/kg) + isolate no 3	0.68	0.81	0.75
9	Soil + rock phosphate(20mg/kg) + isolate no 3	0.51	0.64	0.55
10	Soil + isolate no 6	0.57	0.75	0.58
11	Soil + tricalcium phosphate +isolate no 6	0.51	0.68	0.54
12	Soil + rock phosphate(10mg/kg) + isolate no 6	0.57	0.68	0.77
13	Soil + rock phosphate(20mg/kg) + isolate no 6	0.40	0.43	0.68

#### 4.9 CHLOROPHYLL

Total chlorophyll was estimated after 30 days of the growth after termination of experiment. The maximum growth was observed 35.103 in crop of wheat with isolate no 3 where as minimum 11.154 in soil only. Whereas 46.356 in ladyfinger with rock phosphate(20mg/kg) with isolate no 6 and minimum 23.018 mg/g with wheat crop.(table 4.14) so chlorophyll also get affected. The chlorophyll content increase in treatment where bacterial isolates were added compared to positive control soil with combination of tricalcium phosphate and rockphosphahate (10mg/kg and 20mg/kg) 12.955, 13.497,12.388 mg/g respectively. Both isolates no 3 and isolate no 6 with treatment give equally good chlorophyll which ranges from 23.018 to 35.103 respectively. The total chlorophyll observed lesser in ladyfinger than

wheat crop. In ladyfinger and even control only. Soil which indicated that may be solublization of phosphate was less, in ladyfinger and even total plant growth also affected (table 4.2).

In ladyfinger the minimum chlorophyll observed 4.3068 mg/g in soil alone. and maximum 46.3561 mg/g in combination where rock phosphate added with isolate no 6 which Is exceptionally higher (reason was not known).other trends are same in lady finger observed in crop of wheat (table4.14) only the values are less compared to wheat crop .the overall result indicated from table 4.14 that when bacterial isolate were added the growth of plant were good because of the availability of  $PO_4$ , so the chlorophyll was also higher compared to negative and positive control.

**Table 4.14: Total chlorophyll in wheat and lady finger after 30 days of plant growth**

S. No.	Treatment	Chlorophyll after 30 days	
		Wheat	Ladyfinger
1	Soil+crop	11.1547	4.3068
2	Soil + tricalcium phosphate	12.955	5.8415
3	Soil + rock phosphate(10mg/kg)	13.497	8.896
4	Soil +rock phosphate(20mg/kg)	12.388	7.137
5	Soil+ isolate no 3	35.103	20.393
6	Soil+ tricalcium phosphate + isolate no3	27.095	17.318
7	Soil + rock phosphate(10mg/kg) + isolate no 3	19.657	18.502
8	Soil + rock phosphate(20mg/kg) + isolate no 3	26.026	19.627
9	Soil + isolate no 6	27.836	10.064
10	Soil + tricalcium phosphate +isolate no 6	20.579	20.221
11	Soil + rock phosphate(10mg/kg) + isolate no 6	18.415	18.716
12	Soil + rock phosphate(20mg/kg) + isolate no 6	23.018	46.35606

#### 4.10 TOTAL CFU COUNT IN SOIL

Total CFU count was done by the spread plate count method. Initially ranges from  $1.1 \times 10^5$  to  $10 \times 10^5$  variations in no may be due to experimental error. But overall no of microbes are very high with all treatment replicates. After 30 days no was not decreased it ranges from  $2.3 \times 10^5$  to  $10 \times 10^5$  with wheat crop soil  $1.2 \times 10^5$  to  $9 \times 10^5$ . In ladyfinger these high number of cells indicted that soil after 30 days of enzyme activity give good no of cells which can performed solublization activity and enzyme activity very well if they would grown after 30 days also.

The control soil is having total number  $1 \times 10^6$  -  $2 \times 10^6$

**Table 4.15: Total bacterial count**

S. No.	Treatment	Before plant growth CFU	CFU after 30 days of plant growth	
			Wheat	Ladyfinger
1	Soil+crop	$5 \times 10^6$	$10 \times 10^6$	$9 \times 10^6$
2	Soil + tricalcium phosphate	$2 \times 10^6$	$5 \times 10^6$	$1.2 \times 10^5$
3	Soil + rock phosphate(10mg/kg)	$4 \times 10^5$	$5 \times 10^5$	$7 \times 10^5$
4	Soil +rock phosphate(20mg/kg)	$5 \times 10^6$	$10 \times 10^5$	$6 \times 10^5$
5	Soil+ isolate no 3	$10 \times 10^5$	$2.9 \times 10^5$	$2 \times 10^5$
6	Soil+ tricalcium phosphate + isolate no3	$2 \times 10^5$	$3.2 \times 10^5$	$2.5 \times 10^5$
7	Soil + rock phosphate(10mg/kg) + isolate no 3	$3 \times 10^5$	$6 \times 10^5$	$5 \times 10^5$
8	Soil + rock phosphate(20mg/kg) + isolate no 3	$1.1 \times 10^5$	$3.2 \times 10^5$	$4 \times 10^5$
9	Soil + isolate no 6	$2.2 \times 10^5$	$4 \times 10^5$	$5 \times 10^5$
10	Soil + tricalcium phosphate +isolate no 6	$1.9 \times 10^5$	$2.3 \times 10^5$	$3 \times 10^5$
11	Soil + rock phosphate(10mg/kg) + isolate no 6	$1.8 \times 10^5$	$3 \times 10^5$	$2.7 \times 10^5$
12	Soil + rock phosphate(20mg/kg) + isolate no 6	$2 \times 10^5$	$2.9 \times 10^5$	$2.7 \times 10^5$

## **CHAPTER - 5**

### **CONCLUSION**

Phosphate-solubilizing microorganisms pose wide variety of functions which facilitate their implications in large number of agro-, bio-, and environmental technological strategies. These microbial features can be evidenced and studied in fermentation conditions and soil-plant system as well.

The results presented here emphasize the important combination of soil bacteria with well-defined Phosphate-solubilizing activity. But which use different nutrient sources and/or demonstrate optimum metabolic capacity at different environmental parameters (temperature, pH, humidity, etc.) would probably enhance the multifaceted characteristics of all microbial mixture participants applied in different soil-plant.

1. Present study concluded that bacterial isolate no 3 and 6 are able to solublize phosphate in soil.
2. Both isolates are able to decrease the pH and increase the phosphatase activity in soil as compared to soil which is without bacterial isolates.
3. Phosphate solublizing bacteria Secrete organic acids and lower the pH in their vicinity to bring about dissolution of bound phosphates in soil. Increased growth of plant.
4. These results have an agronomic importance for crop cultivation and yield which may assist in solving problems encountered with food shortage and crop production economy. Thus, the application of Phosphate solubilizing bacteria is recommended as a sustainable way for increasing crop yield and also improving the physio-chemical properties of the soil.

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