2. REVIEW OF LITERATURE

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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 (Sharma *et. al.*, 2007).

Indiscriminate use of synthetic fertilizers has led to the pollution and contamination of the soil, has polluted water basins, destroyed micro-organisms and friendly insects, making the crop more prone to diseases and reduced soil fertility. Demand is much higher than the availability. It is estimated that by 2020, to achieve the targeted production of 321 million tonnes of food grain, the requirement of nutrient will be 28.8 million tonnes, while their availability will be only 21.6 million tonnes being a deficit of about 7.2 million tonnes. Search of microorganisms for their use as efficient biofertilisers is gaining importance nowadays due to a) Depleting feedstock/fossil fuels (energy crisis) and increasing cost of fertilizers which is becoming unaffordable by small and marginal farmers b) Depleting soil fertility due to widening gap between nutrient removal and supplies c) Growing concern about environmental hazards d) Increasing threat to sustainable agriculture. Besides above facts, the long term use of bio-fertilizers is economical, eco-friendly, more efficient, productive and accessible to marginal and small farmers over chemical fertilizers (Venkataraman and Shanmugasundaram, 1992).

Biofertilisers are gaining importance as they are ecofriendly, non-hazardous and non-toxic (Sharma *et. al.*, 2007). A substantial number of bacterial species, mostly those associated with the plant rhizosphere, may exert a beneficial effect upon plant growth (Valverde *et. al.*, 2006). Biofertilisers include mainly the nitrogen fixing, phosphate solubilising and plant growth promoting microorganisms (Shehata and El-Khawas, 2003; Geol *et. al.*, 1999). Biofertilisers are useful to reduce the pollution rate of soil and water (El-Assiouty and Abo Sedera, 2005).

2.1. Phosphorus in the Soil System and Its Availability to Plants

Only a small percentage of the total phosphorus in a soil is in a form available to plants, and an even smaller fraction in the soil solution. The remainder, excluding organically bound P, is in chemical forms that are, at best, only very slightly soluble (Stewart *et. al.*, 1980). Were it not for P-releasing mechanisms at work in the soil, biological immobilization and chemical precipitation would soon deplete the available-P supply, leaving very little available. While it is true that chemical equilibrium exist between P-containing minerals and P in solution, the levels involved cannot account for all the P used in conditions of high yield (Russell, 1973). Clearly, other mechanisms play a part.

Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa *et. al.*, 2002). Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N_2-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition. Although microbial inoculants are in use for improving soil fertility during the last century, however, a meager work has been reported on P solubilisation compared to nitrogen fixation. Soil P dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes. Large amount of P applied as fertilizer enters in to the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} in acidic, and Ca^{2+} in calcareous or normal soils (Gyaneshwar *et. al.*, 2002; Hao *et. al.*, 2002). Efficiency of P fertilizer throughout the world is around 10-25 % (Isherword, 1998), and concentration of bioavailable P in soil is very low reaching the level of 1.0 mg/ kg of soil (Goldstein, 1994). Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001).

However, many soils throughout the world are P deficient because the free phosphorus concentration (the form available to plants) even in fertile soil is generally not higher than 10 ppm even at pH 6.5 where it is most soluble (Gyaneshwar *et. al.*, 2002). Malakooti and Nafisi (1995) declared that the best pH for phosphorus uptake by plants is 6.5.

Phosphorus exists in soil in organic and inorganic forms. Each form is a continuum of many P compounds, existing in different phases and in equilibrium with each other. Availability of P ranges from soluble P (plant available) to very stable (plant unavailable) compounds (Fig. 2.1).

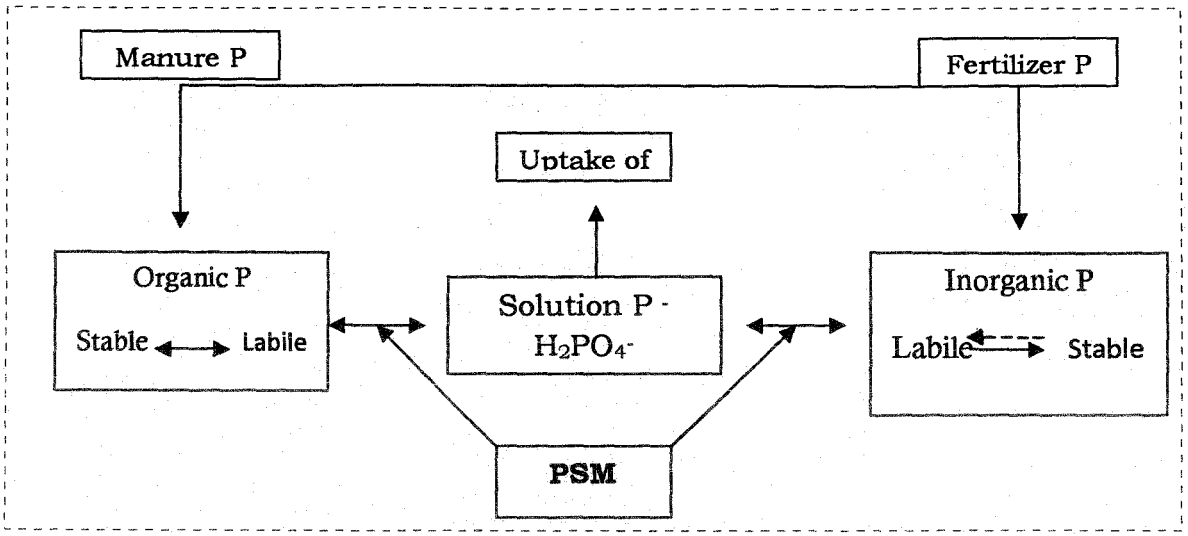

Figure 2.1 The soil phosphorus cycle (adapted from Sharpley, 2006), solid line indicates the conversion process. The dashed line means very slow conversion.

There is a dynamic and complex relationship among the different forms of P involving soil, plants and microorganisms. Organic P compounds are found in humus and other organic materials including decayed plant, animal and microbial tissues. Organic P is also the principal form of P in manure. Organic P is usually combined with oxygen to form ester compounds (Thompson and Troch, 1978). These esters make up about 50 to 70% of identified organic P (McGill and Cole, 1981).

Phosphorus in labile organic compounds can be slowly mineralized (broken down and released) as available inorganic phosphate or it can be immobilized (incorporated into more stable organic materials) as part of the soil organic matter (Tate, 1984; Mckenzie and Roberts, 1990). The process of mineralization or immobilization is carried out by microorganisms and is highly influenced by soil moisture and temperature. Mineralization and immobilization are most rapid in warm, well-drained soils (Busman *et. al.*, 2002).

Approximately 70 to 80% of P found in cultivated soils is inorganic (Foth, 1990). Phosphorus fertilizers are the main input of inorganic P in agriculture

soils. Despite its wide application, after N, P is the major nutrient limiting plant growth (Gyaneshwar *et. al.*, 2002; Fernandez, *et. al.*, 2007). Worldwide, 5.7 billion hectares contain too little available P for sustaining optimal crop production (Hinsinger, 2001). Phosphorus ion concentration in most soils ranges from 0.1 to 10 μM; P required for optimal growth ranges from 1 to 5 μM for grasses and 5 to 60 μM for high demanding crops such as tomato and pea (Raghothama, 1999; Hinsinger, 2001).

Phosphorus in fertilizers is converted to water-soluble Pi as orthophosphate ions $H_2PO_4^-$ and HPO_4^{2-} in soil within a few hours after application (Schulte and Kelling, 1996). As the fertilizer enters the soil, moisture from the soil begins to dissolve the fertilizer particles. The concentration of Pi in solution increases around the dissolved fertilizer particles and diffuses a short distance from the fertilizer particles (Busman *et. al.*, 2002). In most soils, orthophosphate ions $H_2PO_4^-$ and HPO_4^{2-} dominate at pH below 7 and above 7.2, respectively (Hinsinger, 2001). These negatively charged P ions attach strongly to the surfaces of minerals containing positively charged ions such as iron (Fe^{3+}) and aluminum (Al^{3+}) in acidic soils via sorption/desorption processes. Fe^{3+} and Al^{3+} act as the sorption sites for the negatively charged P (Sato and Comerford, 2005). These P anions also precipitate with the calcium (Ca^{2+}) in calcium carbonate minerals in calcareous soils forming relatively insoluble compounds. Both processes result in P being fixed or bound, thus removed from the soil solution and unavailable for plants (Banik and Dey, 1982; Foth, 1990; Schulte and Kelling, 1996).

The conversion from stable P to labile P is a slow process and does not occur over the course of one growing season (Guo and Yost, 1998). However, the conversion from labile P to plant available P is a rapid process (Tate and Salcedo, 1988). Soil inorganic P exists as many compound species and the species distribution is controlled mainly by solution pH and the concentration of cations (Lindsay, 1979). In most soils, maximum P availability occurs between pH 5.5 to 7. Within this pH range, P is fixed by hydrous oxides of Fe, Al, and Mn. Between pH 6 to 8 and pH 6.5 to 8.5, P is fixed by silicate minerals and Ca, respectively. As a result, the most efficient use of P in neutral and calcareous soils occurs between pH 6 to 7 (Sharpley, 2006).

In neutral and calcareous soils, soil pH is between 7.3 and 8.5 depending on the amount of $CaCO_3$ presenting in the soil (Lindsay, 1979). With high levels of exchangeable Ca^{++}, available P ions react with solid phase $CaCO_3$ and precipitate on the surface of these particles to form Ca-P minerals: $Ca(H_2PO_4)_2$ (monocalcium P), $CaHPO_4.2H_2O$ (dicalcium phosphate dihydrate, DCPD, brushite), $CaHPO_4$ (dicalcium phosphate, DCP, monetite), $Ca_3(PO_4)_2$ (tricalcium phosphate, TCP), $Ca_4H(PO_4)_3.2.5H_2O$ (octacalcium P, OCP), $Ca_5(PO_4)_3.OH$ (hydroxyapatite) and least soluble apatites (Lindsay *et. al.*, 1989). The finer the size of solid phase $CaCO_3$ the higher the fixation of P. The solubility of Ca-P minerals is generally accepted as DCPD > DCP > TCP > hydroxyapatite. In alkaline soils, the initial products of reaction of fertilizer triple superphosphate are mainly DCPD and DCP (Russell, 1973; Whitelaw *et. al.*, 1999). Different phases of Ca-P compounds are transferable and, at a given pH, can be dissolved from unstable phases to become precipitated as stable phases. For example, a relatively soluble brushite when applied as fertilizer to calcareous soils can be transformed to monetite and slowly to octacalcium P. Octacalcium P can be stable for years if fertilizer is applied continually. The formation of hydroxyapatite is the ultimate result (Sposito, 1989).

Soil solution Pi concentration increases when water soluble P fertilizer applied to soil is readily dissolved. Over time, the soil fixes P by processes such as precipitation, thereby reducing its concentration in the soil solution. As a result, Pi in the soil solution is general low. In the United States, an average 29% of P added in fertilizer and manure is removed by harvesting crops (Sharpley, 2006). The Pi content is usually greater at surface horizons than in subsoils due to its immobility. The Pi accumulation in topsoil can be a problem especially in a reduced tillage system because of minimal or no mechanical incorporation when fertilizer is applied (Sharpley, 2006). Phosphate fertilizers can increase P availability initially, but will promote the formation of insoluble P minerals and consequently lead to P buildup. Therefore, P management is important both environmentally and economically. Phosphate solubilising microorganisms may be an answer for maintaining the supply of plant available P because PSM carry out the conversion from labile P to plant available P.

An adequate supply of P is essential for the earlier stages of plant growth. Early season deficiencies of P can lead to restriction on crop growth from which

the plant will not recover, even when the P supply is increased to an adequate level at a later stage (Grant *et. al.* 2005).

In the acid-weathered soils of the tropics, subtropics and temperate regions, P is fixed by free oxides and hydroxides of aluminium and iron, while in alkaline soils it is fixed by calcium, causing a low efficiency of soluble P fertilizers and limits crop production in those soils (Rodriguez and Fraga, 1999). Therefore phosphate solubilising bacteria have been used to enhance the solubilisation of fixed P for crop nutrition (Nautiyal *et. al.*, 2000).

Phosphate Solubilising Bacteria (PSB) solubilises insoluble phosphate and makes it available to the plants (Bhattacharya and Jain, 2000). Indian soils on an average contain 0.05% phosphorus that constitutes 0.2% of plant dry weight. Even applied phosphorus combines with metal ions, PSB are required for its release (Bagyaraj and Verma, 1995; Schachtman *et. al.*, 1998).

The organic P pool generally constitutes 30 to 80% of the total soil P (Oberson *et. al.*, 1996) and represents a labile P fraction that may supply P to plants through mineralization by the microbial biomass (Stewart and Tiessen, 1987). The microbial biomass is a small fraction of the total soil organic P, containing anywhere between 3 to 24% depending on cultivation (Brookes *et. al.*, 1984). However, it is significant in its role as recycler of P and as a relatively labile P source (Kwabiah *et. al.*, 2003).

Soils may contain a substantial quantity of organic P (Richardson 2001), and phosphatases from microorganisms may carry out mineralization of most organic phosphorus compounds. From 30 upto 63% of culturable soil bacteria can mineralize organic P in soils (Rodriguez and Fraga, 1999).

2.2. Plant Growth Promoting Rhizobacteria (PGPR) and its importance

Inappropriate application of mineral fertilizers in agriculture has resulted in pollution and salinisation of agricultural lands and water resources. The use of a nonhazardous biological method, like bacterial inoculants, in such regions to increase plant production is an important approach to help sustainable development. In particular, plant growth-promoting rhizobacteria (PGPR) have been reported to be key elements for plant establishment under nutrient-imbalance conditions. Their use in agriculture can favour a reduction in agro-

chemical use and support ecofriendly crop production (Herrera *et. al.*, 1993; Requena *et. al.*, 1997; Glick, 1995). PGPR can help the improvement of plant growth, plant nutrition, root growth pattern, plant competitiveness, and responses to external stress factors. They can also inhibit soil borne plant pathogens by producing growth-promoting chemical substances and inducing plant resistance (Lifshitz *et. al.*, 1987; Bothe *et. al.*, 1992; Hoflich *et. al.*, 1994). Different plant-growth promoting rhizosphere bacteria, including associative bacteria such as *Azospirillum, Bacillus, Pseudomonas, Enterobacter* group have been used for their beneficial effects on plant growth (Kloepper and Beauchamp, 1992). Several studies clearly showed the effect of plant growth-promoting bacteria on growth of different crops at different climates, soils and temperatures (Ruppel, 1987; Dobereiner, 1992; Boelens *et. al.*, 1993).

The use of biofertilisers or microbial inoculants for replacing the efficacy of chemical fertilizers has been found to be effective in reducing the cost of cultivation and maintaining the natural fertility of soil (Gothwal *et. al.*, 2006). In conventional agricultural practices, the increased use of agrochemicals including pesticides has however, led to the frequent and deliberate contamination of cultivated soils. These chemicals in turn may adversely affect the rhizospheric organisms including PGPR and associated biotic processes, which are governed by the rate of application, the activity spectrum of the pesticides and the persistence and availability of chemicals (Moorman, 1989; Srinivas *et. al.*, 2008).

The microbe-plant interaction in the rhizosphere can be beneficial, neutral, variable, or deleterious for plant growth. Rhizobacteria that exert beneficial effects on plant development are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). The term rhizobacteria is used for bacteria that aggressively colonize the rhizosphere (SubbaRao, 1999). Plant growth promoting rhizobacteria (PGPR) accounts for about 2-5% of total the rhizobacteria involved in plant growth promotion (Antoun and Kloepper, 2001).

In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have reported to enhance plant growth (Kloepper *et. al.*, 1989; Okon and Labandera-Gonzalez, 1994; Glick,

1995). The direct growth promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR prevent deleterious effects of one or more phytopathogenic microorganisms. The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene (Arshad and Frankenberger, 1991; Glick, 1995), (ii) asymbiotic N_2 fixation (Boddey and Dobereiner, 1995), (iii) antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982), antibiotics (Shanahan *et. al.*, 1992) and cyanide (Flaishman *et. al.*, 1996), (iv) solubilisation of mineral phosphates and other nutrients (DeFreitas *et. al.*, 1997; Gaur, 1990). Most popular bacteria studied and exploited as biocontrol agent includes the species of fluorescent *Pseudomonas* and *Bacillus*. Some PGPR may promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation or nodule occupancy (Fuhrmann and Wollum, 1989). However, role of cyanide production is contradictory as it may be associated with deleterious as well as beneficial rhizobacteria (Bakker and Schippers, 1987; Alstrom and Burns, 1989). In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil (Cattelan *et. al.*, 1999).

Unfortunately, the interaction between associative PGPR and plants can be unstable. The good results obtained *in vitro* cannot always be dependably reproduced under field conditions (Chanway and Holl, 1993; Zhender *et. al.*, 1999). The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effects on plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms (Bent *et. al.*, 2001). Therefore, it is necessary to develop efficient strains in field conditions.

Although the mechanisms by which PGPR promote plant growth are not yet fully understood, many different traits of these bacteria are responsible for growth promotion activities (Cattelan *et. al.,* 1999). It includes the ability to produce or change the concentration of the plant hormones indole acetic acid (IAA), gibberellic acid, cytokinins, and ethylene; fix dinitrogen; suppress the growth of deleterious microorganisms by production of siderophore, β-1,3-glucanase, chitinases, antibiotics, and cyanide; and dissolve phosphates and other nutrients. Initially, *Azotobacter* and *Azospirillum* were believed to promote plant growth due to their ability to fix dinitrogen. Later, it was known that other plant growth stimulating hormones such as IAA was also involved (Kennedy, 1998). The use of P-solubilising bacteria was reported to increase plant growth in some cases, but in other cases it was not. It indicated that other mechanisms may involve in growth response (DeFreitas *et. al.,* 1997). Ample studies on the biological dinitrogen fixation have been documented. A number of free-living bacteria have the ability to fix dinitrogen and increase nitrogen availability for plant. IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots (Okon and Kapulnik, 1986). Microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells (Frankenberger and Arshad 1991; Benizri *et. al.,* 1998). P-solubilising bacteria are potential to increase available P for plant, especially in soils with large amounts of precipitated phosphate (Goldstein, 1986). These bacteria release bound phosphate by secreting a number of organic acids although it is not the only way by which Phosphate is solubilised (DeFreitas *et. al.,* 1997; Kim *et. al.,* 1997a). Siderophore-producing bacteria promote plant growth indirectly by sequestering the limited iron in the rhizosphere, especially in neutral and alkaline soils, and thereby reduce its availability for the growth of pathogen (Alexander and Zuberer, 1991; SubbaRao, 1999).

Azospirillum and phosphorus solubilising bacteria (PSB) are two groups of PGPR, which enhance plant growth by several mechanisms such as nitrogen fixation, Phosphate solubilisation and hormone production in crop rhizosphere (Okon, 1985; Asea *et. al.,* 1988). Due to endemicity of beneficial bacterial groups, the isolation and characterization of beneficial PGPR from dominant crops of a region may be necessary in development of bioinoculants for local crop production system (Kole & Hazra, 1998, Talukdar *et. al.*, 2001 and Cho & Tiedje, 2000).

2.3. Diversity of Phosphate solubilising microorganisms

The immediate vicinity of root surface which constitute the rhizosphere is an extremely important habitat for microbes. Roots secrete a number of compounds into the soil which may either enhance or inhibit the growth of microorganisms (Chakraborty and Chakraborty, 1997). Many microorganisms in the soil are able to solubilise "unavailable" forms of calcium-bound P by excreting organic acids which either directly dissolve rock phosphate or chelate calcium ions to bring the P into solution (Katznelson and Bose, 1959; Sperber, 1958b). Studies have shown that these microorganisms are present in the soil in different numbers (Katznelson *et. al.,* 1962; Khan and Bhatnagar, 1977; Louw and Webley, 1959), and that a large proportion of the bacterial phosphate-solubilising (PS) population is found in the rhizosphere of plants (Sperber, 1958a). However, the PS bacteria, when viewed as a percentage of the total soil microbial population, were not found to constitute a significantly larger proportion of the rhizosphere microbial population (Sperber 1958a; Katznelson and Bose 1959). Kobus (1962) reported that the numbers of PS bacteria in a soil were influenced more by soil type and the manner of its cultivation than by the physical composition or content of humus, N or P in the soil.

In the frame of agriculture, the microflora is of great significance because it has both beneficial and detrimental influence upon man's ability to feed himself (Gaur, 1990; Motsara *et. al.,* 1995; White law, 2000).

Microorganisms having PS ability include bacteria, fungi, and actinomycetes (Sperber, 1958b) and, naturally, the range of PS ability within such a heterogeneous group is very wide. Fungi isolated from southern Alberta soils were found to be more active phosphate-solubilisers than bacterial isolates (Kucey, 1983).

Evidence of naturally occurring rhizospheric phosphorus solubilising microorganism (PSM) dates back to 1903 (Khan *et. al.,* 2007). Bacteria are more effective in phosphorus solubilisation than fungi (Alam *et. al.,* 2002). Among the whole microbial population in soil, PSB constitute 1 to 50 %, while phosphorus solubilising fungi (PSF) are only 0.1 to 0.5 % in P solubilisation potential (Chen *et. al.,* 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 solubilisers (Igual *et. al.*, 2001). Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilisers (Whitelaw, 2000). *Bacillus megaterium*, *B. circulans*, *B.subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains (SubbaRao, 1988; Kucey *et. al.*, 1989). A nematofungus *Arthrobotrys oligospora* also has the ability to solubilise the phosphate rocks (Duponnois *et. al.*, 2006). Many soil bacteria and fungi have the ability to solubilise P and make it available to growing plants (Antoun *et. al.*, 1998). Microorganisms are central to the soil P cycle and play a significant role in mediating the transfer of P between different inorganic and organic soil P fractions, subsequently releasing available P for plant acquisition (McLaughlin *et. al.*, 1988; Oberson *et. al.*, 2001). There are two aspects in microbial P solubilisation: 1) P released by solubilisation processes (Rodriguez and Fraga, 1999), and 2) P released from accumulated P in biomass of microorganisms (Oehl *et. al.*, 2001). Inorganic phosphate solubilising microorganisms (PSM) constitute various portions of the soil microbial population and vary from soil to soil (Banik and Dey, 1982; Kucey *et. al.*, 1989). The populations of PSM are reportedly varied and ranged from very low (less than 10^2 cfu /g of soil) in a soil in Northern Spain to very high (3×10^6 cfu g^{-1} of soil) in Quebec, Canada (Chabot *et. al.*, 1993; Peix *et. al.*, 2001). Phosphate solubilising microorganisms were isolated from rhizosphere soils of different crops of India (Ponmurugan and Gopi, 2006). The numbers of PSM are more important in rhizosphere than non-rhizosphere soil (Kucey *et. al.*, 1989). The PSM represented 0.1 to 0.5% of total bacterial and fungal populations in 29 Alberta soils (Kucey, 1983). PSM occur in both fertile and P-deficient soils and the fastest initial rates of P incorporation were observed in P-deficient soils (Oehl *et. al.*, 2001).

Phosphate solubilising fungi are superior to their bacterial counterpart for P solubilisation both on precipitated agar and in liquid (Kucey, 1983). Fungal hyphae in liquid culture were attached to P mineral particles shown by scanning electron microscopy, whereas bacteria were not (Chabot *et. al.*, 1993). Furthermore, because of their hyphae, fungi are able to reach greater distances

more easily in soil than bacteria. JumpStart ®, a product of Canada was the first P-solubilising seed inoculant in the market and the active ingredient was the fungus *Penicillium bilaiae* formerly known as *Penicillium bilaji* and *Penicillium bilaii* which is said to increase the availability of fertilizer and native soil P to plant roots. P. *bilaiae* is known for its superior ability in Ca-P solubilisation (Kucey, 1988; Sanders, 2003). P. *bilaiae* had a high solubilisation for Idaho rock phosphate in solution culture (Kucey, 1983; Asea *et. al.*, 1988). In addition to *P. bilaiae, P. aurantiogriseum* and *Pseudomonas* species solubilised Ca-P (Illmer and Schinner, 1995), and *Pseudomonas striata* and *Penicillium oxalium* solubilised Al-P and Fe-P (Gadagi and Sa, 2002). *Penicillium regulosum* strains utilised rock phosphate and stimulated the growth of maize plants with 3.6 to 28.6% increase in dry matter yields in a low fertility soil at pH 6.25 (Reyes *et. al.*, 2002). *Penicillium* and *Aspergillus* sp. are the dominant P solubilising fungi found in rhizosphere (Kucey, 1983).

In addition to Phosphate solubilising fungi, Phosphate solubilising bacteria are present in soil and plant rhizospheres. The populations of these bacteria are higher in rhizosphere than non-rhizosphere soils (Katznelson *et. al.*, 1962). The most important P solubilising bacterial genera are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium and Erwinia* (Rodriguez and Fraga, 1999). According to Babenko *et. al.*, (1984), the phosphate solubilising patterns of bacteria were grouped into two categories: 1) soluble P increased linearly along with the growth of the bacterial culture; 2) soluble P increased at different points of the growth stage but not throughout the whole incubation period, which the authors attributed to induction and repression of the enzyme systems responsible for solubilisation. Rodriguez and Fraga (1999) also compared 13 bacterial strains of different genera for their solubilising abilities on different insoluble mineral phosphate substrates and indicated that *Rhizobium, Pseudomonas* and *Bacillus* species were among the most powerful P solubilisers.

Rhizobium leguminosarum is of particular interest because of its dual function: its ability to fix N and to solubilise P (Wood and Cooper, 1984; Chabot *et. al.*, 1996a; Hara and de Oliveira, 2004). Lettuce and maize inoculated with two strains of P solubilising *R. leguminosarum* are better in root colonization and growth. Additionally, rhizobia exhibited an ability to promote plant growth

for non-legumes (Chabot *et. al.*, 1996b, 1998). The multi-functionality exhibited by *R. leguminosarum* makes it important in food production in terms of reducing cost and improving efficiency of P fertilization, especially in P-limited soils, particularly in countries such as Australia, Brazil and India where soil available P is generally low. Roychoudhury and Kaushik (1989) reported that phosphate rock deposits are estimated at approximately 40 million tons in India. The phosphate rock deposits could be an inexpensive source of phosphate fertilizer for crop production if these deposits became available for plant growth (Halder *et. al.*, 1990).

Despite the beneficial influences by the PSM, some cases of inconsistent results have been reported. *Bacillus megaterium* var. *phosphoricum* performed inconsistently in soils as inoculants in India, former Soviet Union and the United States (Rodriguez and Fraga, 1999). Furthermore, instability of P solubilising character was reported for some organisms (Halder *et. al.*, 1990; Illmer and Schinner, 1992).

It has been observed by many investigations that a high proportion of P solubilising microorganisms are concentrated in the rhizosphere of plants (Gaur, 1990). Since phosphate activities are found to be in much higher in rhizosphere soil than in bulk soil (Seeling and Jungk, 1992), inorganic phosphorus solubilising microorganisms are more concentrated in rhizosphere plants than in bulk soil (Vazquez *et. al.*, 2000).

Phosphate solubilising microorganisms (PSM) like bacteria and fungi, can grow in media containing tricalcium, iron and aluminium phosphate, hydroxyapatite, bonemeal, rock phosphate and similar insoluble phosphate compounds as the sole phosphate source. Such microbes not only assimilate P but a large portion of soluble phosphate is released in quantities in excess of their own requirement (Gaur, 1990). The most efficient PSM belong to genera *Bacillus* and *Pseudomonas* amongst bacteria and *Aspergillus* and *Penicillium* amongst fungi. The reported bacilli include, *B. brevis*, *B. cereus*, *B. circulans*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. mesentricus*, *B. mycoides*, *B. polymyxa*, *B. pumilis*, *B. pulvifaciens* and *B. subtilis* from the rhizosphere of legumes, cereals (rice and maize), arecanut palm, oat, jute and chilli (Sundara Rao and Sinha, 1963; Taha *et. al.*, 1969; Barea *et. al.*, 1976; Banik and Dey, 1981; Venkateswarlu *et. al.*, 1984; Sattar and Gaur, 1985; Ali *et. al.*, 1989;

Gaind and Gaur, 1999; Rajarathinam *et. al.*, 1995; Bhattacharya *et. al.*, 1998; Kole and Hazra, 1997, 1998).

Pseudomonas striata, P. cissicola, P. fluorescens, P. pinophillum, P. putida, P. syringae, P. aeruginosa, P. putrefaciens and *P. stutzeri* have been isolated from rhizosphere of *Brassica*, chickpea, maize, soybean and other crops, desert soils and Antarctica lake (Kole and Hajra, 1997; Bardiya and Gaur, 1974; Nair and Rao, 1977; Jisha, 1997; Pal *et. al.*, 2000; Gupta *et. al.*, 1998). In addition, *Escherichia freundii, E. intermedia, Serratia phosphaticum* and species of *Achromobacter, Brevibacterium, Corynebacterium, Erwinia, Micrococus, Sarcina* and *Xanthomonas* are active in solubilising insoluble phosphates.

Cyanobacteria, viz. *Anabaena* sp., *Calothrix brauni, Nostoc* sp., *Scytonema* sp. and *Tolypothrix ceylonica* can also solubilise phosphate (Gupta *et. al.*, 1998).

Among phosphate solubilising fungi, *Aspergillus niger, A. flavus, A. nidulans, A. awamori, A. carbonum, A. fumigatus, A. terreus* and *A. wentii* have been reported from the rhizosphere of maize, soybean, chilli, tista soils, acidic lateritic soils and compost (Subba Rao and Bajpai, 1965; Chhonkar and Subba Rao, 1967; Prerna *et. al.*, 1997). *Paeciliomyces fusisporus, Penicillium digitatum, P. simplicissimum, P. aurantiogriseum, Sclerotium rolfisii* and species of *Cephalosporium, Alternaria, Cylindrocladium, Fusarium* and *Rhizoctonia* are other solubilisers of insoluble phosphate. Amongst yeasts, *Torula thermophila, Saccharomyces cerevisiae* and *Rhodotorula minuta* can solubilise inorganic phosphate (Varsha-Narsian *et. al.*, 1994). PSM inoculants include species of *Aspergillus, Bacillus, Escherichia, Arthrobacter* and *Pseudomonas* (Mishra, 1985; Datta *et. al.*, 1982) which can add 30–35 kg P_2O_5 /ha (Gaur *et. al.*, 2004). Due to the ecotype diversity of PSMs and its tolerance in some environmental stresses, these bacteria are of special importance as a biological fertilizer (Sharma, 2002).

2.4. Occurrence of Phosphate Solubilising Bacteria

High proportion of PSM is concentrated in the rhizosphere, and they are metabolically more active than from other sources (Vazquez *et. al.*, 2000). Usually, one gram of fertile soil contains 10^1 to 10^{10} bacteria, and their live

weight may exceed 2,000 kg ha^{-1}. Soil bacteria are in cocci (sphere, 0.5 μm), bacilli (rod, 0.5–0.3 μm) or spiral (1-100 μm) shapes. Bacilli are common in soil, whereas spirilli are very rare in natural environments (Baudoin *et. al.* 2002). The PSB are ubiquitous with variation in forms and population in different soils. Population of PSB depends on different soil properties (physical and chemical properties, organic matter, and P content) and cultural activities (Kim *et. al.*, 1998). Larger populations of PSB are found in agricultural and rangeland soils (Yahya and Azawi, 1998).

2.5. Mechanisms of Phosphate Solubilisation

Apart from fertilization, mineralization and enzymatic decomposition of organic compounds, microbial P solubilisation is the main contributor increasing plant available P (Illmer and Schinner, 1992). Several theories exist explaining the mechanisms of microbial P solubilisation (Kucey, 1983, Asea *et. al.*, 1988; Cunningham and Kuiack, 1992; Illmer and Schinner, 1995): the sink theory (Halvorson *et. al.*, 1990), the organic acid theory (Cunningham and Kuiack, 1992), and the acidification by H^+ excretion theory (Illmer and Schinner, 1995).

In the sink theory, P solubilising organisms are able to remove and assimilate P from the liquid and therefore stimulate the indirect dissolution of Ca-P compounds by continuous removal of P from broth (Halvorson *et. al.*, 1990). Illmer and Schinner (1995) demonstrated P content in the biomass of two P solubilising organisms (*Pseudomonas* sp. and *P. aurantiogriseum*) were the same as that in non-P solubilising organisms. They further argued that only about 1% of total P was absorbed by organisms despite all of the P solubilised in broth. The sink theory, however, can be used to explain mineralization of organic P compounds in which the P content in biomass of organisms is consistently correlated with the decomposition of P-containing organic substrates (Dighton and Boddy, 1989).

Microbial metabolic product i. e., organic acid theory is recognized and accepted by many researchers. In this theory, insoluble sources of inorganic P in liquid broth are solubilised by PSM either by lowering the pH or by enhancing chelation of the cations bound to P. Chelation involves the formation of two or more coordinated bonds between a molecule (the "ligand") and a metal

ion resulting in a ring structure complex. Chelation by an organic acid ligand occurs via oxygen contained in hydroxyl and carboxyl groups (Whitelaw, 2000). The solubilisation of 837 mg /L $CaHPO_4$ by *P. bilaiae* was achieved at pH 4.5 in the presence of citrate, but no $CaHPO_4$ solubilisation occurred at the same pH in the presence of the inorganic acid alone indicating that chelation involved citric acid (Cunningham and Kuiack, 1992). Gluconic acid or *P. radicum* inoculation alone solubilised more amorphous Al-P than HCl at the same pH (Whitelaw *et. al.*, 1999). The insoluble sources of inorganic P in liquid broth are solubilised by PSM accompanied by the production of organic acids: the action of organic acids synthesis and lowering the pH cause dissolution of P compounds (Banik and Dey, 1982; Kucey, 1988; Cunningham and Kuiack, 1992; Whitelaw, 2000; Pradhan and Sukla, 2005). The production of organic acid leads to acidification of microbial cells and their surroundings and, consequently, the release of P ions from the P mineral by H^+ substitution for Ca^{2+} (Goldstein, 1994). Organic acids produced by PSM were determined by methods such as high performance liquid chromatography (HPLC) and enzymatic methods (Whitelaw, 2000; Parks *et. al.*, 1990). Various organic acids are identified in the liquid cultures of PSM (Table 2.1), and can be associated with specific microbial groups, e.g., 2-ketogluconic acid and oxalic acid are commonly found in bacterial and fungal cultures, respectively. Gluconic, acetic and lactic acids have been observed from both types of microorganisms and gluconic acid seems to be the principal organic acid frequently found among PSM.

The impact of organic acid production on P solubilisation has been established for a while. Halder *et. al.*, (1990) reported that the amount of P solubilised by *R. leguminosarum* was nearly equivalent to the organic acid obtained from the culture. They also showed that the P release capacity was not an enzymatic process. Goldstein (1994) proposed that the direct periplasmic oxidation of glucose to gluconic acid, often as 2-ketogluconic acid, formed the metabolic basis of mineral P solubilisation in some Gram negative bacteria. Illmer and Schinner (1995) doubted the organic theory. They demonstrated that by using different concentrations of gluconic acid (0 to 5000 µM) tested at different pH values (pH 4 to 7), there was no effect of gluconic acid on Ca-P solubility at pH > 6. Although many researchers support the organic acid

theory, the amount of solubilised P is difficult to correlate with the organic acid measured in liquid culture (Illmer and Schinner, 1995).

Table 2.1. Organic acids accompanied with phosphate solubilisation

Organic acid	Microorganisms		Reference
	Fungi	Bacteria	
Oxalic	+		Cunningham and Kuiack, 1992
Citric	+		Cunningham and Kuiack, 1992
Lactic	+	+	Banik and Dey, 1982
Tartaric			Banik and Dey, 1982
Gluconic	+	+	Illmer and Schinner, 1995
2-ketogluconic		+	Halder *et. al.*, 1990
Acetic	+	+	Illmer and Schinner, 1995

+ Type of organic acid was observed from the culture solutions.

The acidification by H^+ excretion theory was introduced by Illmer and Schinner (1995) to explain Ca-P solubilisation accompanied by a decrease in pH. They investigated Ca-P solubilisation by *P. aurantiogriseum* and *Pseudomonas* sp. from a 23 forest soil containing Ca-P (hydroxyapatite and brushite). These authors observed that the P concentration in liquid broth increased with consumption of apatite and brushite, and that the P concentration also peaked at several points. Based on their results, they concluded that P concentration at the peaks might be due to the formation and secondary solubilisation of organic P compounds. They also inferred that the organic compounds were assimilated as nutrients by these two organisms when the liquid broth was low in inorganic substrates.

The H^+ release is thought to be associated with cation assimilation, such as ammonium ion (NH_4^+). H^+ excretion accompanying NH_4^+ assimilation is responsible for P solubilisation. Illmer and Schinner (1995) demonstrated that *P. aurantiogriseum* and *Pseudomonas* sp. solubilised hydroxyapatite and brushite effectively without contact between the cells and the substrates, and concurrently lowered the pH. They attributed the P mobilization to H^+ excretion at the cell surfaces. The excreted H^+ accompanying the decrease in pH acted as a solvent agent for P solubilisation (Illmer and Schinner, 1995). The NH_4^+-N had

the lowest pH value among different N sources and was the most effective on P solubilisation in liquid cultures by *P. bilaiae* (Cunningham and Kuiack, 1992).

Phosphorus is an essential nutrient for plants, but is often not available due to its fixation in soil, Phosphate solubilising Bacteria (PSB) solubilise insoluble phosphate and make it available to the plants (Bhattacharya and Jain, 2000). Indian soils on an average contain 0.05% phosphorus that constitutes 0.2% of plant dry weight. Even applied phosphorus combines with metal ions PSB are required for its release (Bagyaraj and Verma, 1995, Schachtman *et. al.,* 1998). Some bacterial species have mineralization and solubilisation potential for organic and inorganic phosphorus, respectively (Hilda and Fraga, 2000; Khiari and Parent, 2005). PSB secrete organic acids and enzymes that act on insoluble form, thus, providing phosphorus to plants. PSB also produce amino acids, vitamins and growth promoting substances (Gonzalez *et. al.*, 1983; Zimmer *et. al.*, 1998), which promote plants growth. Phosphorus solubilising 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 solubilisation takes place through various microbial processes / mechanisms including organic acid production and proton extrusion (Surange *et. al.*, 1995; Dutton and Evans, 1996; Nahas, 1996). General sketch of P solubilisation in soil is shown in Figure 2.2. A wide range of microbial P solubilisation mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey, 1982). Phosphorus solubilisation is carried out by a large number of saprophytic bacteria and fungi acting on sparingly soluble soil phosphates, mainly by chelation-mediated mechanisms (Whitelaw, 2000). Inorganic P is solubilised by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, Ca) and decrease the pH in basic soils (Kpomblekou and Tabatabai, 1994; Stevenson, 2005). The PSB dissolve the soil P 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_2/CO_2) exchanges. Phosphorus solubilisation ability of PSB has direct correlation with pH of the medium.

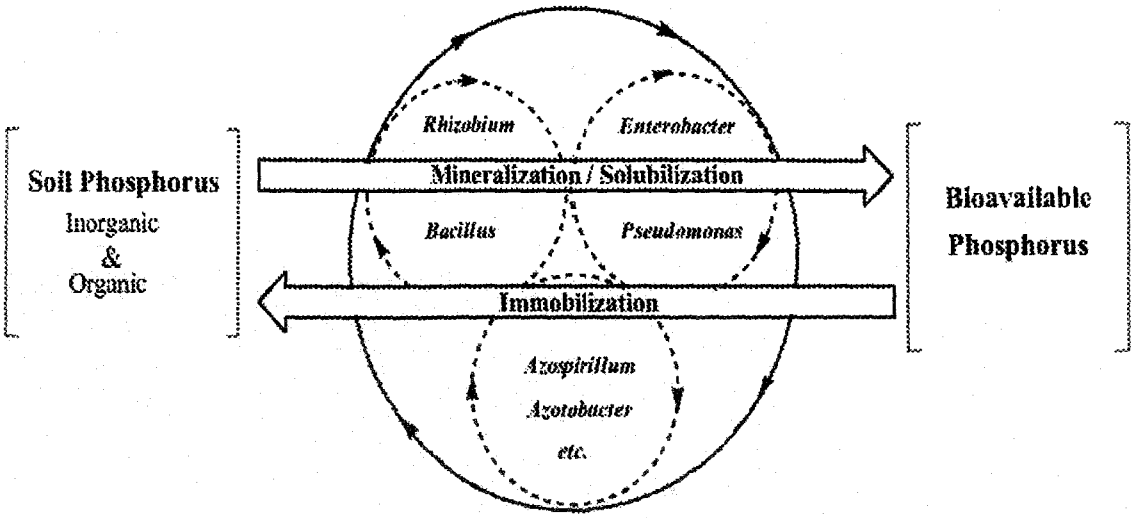

Figure 2.2. Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (reproduced from Kim *et. al.*, 1998)

Release of root exudates such as organic ligands can also alter the concentration of P in the soil solution (Hinsinger, 2001). Organic acids produced by PSB solubilise insoluble phosphates by lowering the pH, chelation of cations and competing with phosphate for adsorption sites in the soil (Nahas, 1996). Inorganic acids e.g. hydrochloric acid can also solubilise phosphate but they are less effective compared to organic acids at the same pH (Kim *et. al.*, 1997b). In certain cases phosphate solubilisation is induced by phosphate starvation (Gyaneshwar *et. al.*, 1999).

Phosphate solubilising microorganisms convert insoluble phosphates into soluble forms generally through the process of acidification, chelation and exchange reactions. Thus such microorganisms may not only compensate for higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to soil (Pradhan and Sukla, 2005).

P-solubilising bacteria release bound phosphate by secreting a number of organic acids although it is not the only way by which P is solubilised (DeFreitas *et. al.*, 1997; Kim *et. al.* 1997b). Banik and Dey (1983) and Asea *et. al.*, (1988) detected organic acids in culture solutions of PSM but did not show any correlation between the solubilisation of P and amount of organic acids produced by PSM.

It is believed that microbial mediated solubilisation of insoluble phosphates in soil is through the release of microbial metabolites in addition to organic acids (Gyaneshwar *et. al.*, 1998; Carrillo *et. al.*, 2002; Rodriguez *et. al.*, 2004). However in addition to acid production, other mechanisms can cause phosphate solubilisation (Nautiyal *et. al.*, 2000). Phosphate solubilisation has been reported to depend on the structural complexity and particle size of phosphates and the quantity of organic acid secreted by microbes (Gaur, 1990).

The overall results of the study indicate that acid production was not the only reason for phosphate release into the medium. This finding was in agreement with data obtained earlier (Abd Alla, 1994; Whitelaw, 2000). It is generally accepted that the mechanism of mineral phosphate solubilisation by PSB strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; Kim *et. al.*, 1997a), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994). However, P-solubilisation is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et. al.*, 1998). There is experimental evidence to support the role of organic acids in mineral phosphate solubilisation (Halder *et. al.*, 1990).

Alternative possibilities other than organic acids for mineral phosphate solubilisation have been proposed based on the lack of a linear correlation between pH and the amount of solubilised P [Ehrlich, 1990; Thomas, 1985; Asea *et. al.*, 1988]. In addition, no significant amounts of organic acid production could be detected from a phosphate solubiliser fungus, *Penicillium* sp. (Illmer & Schinner, 1992). Studies have shown that the release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase could constitute alternative ways for solubilisation of mineral phosphates. Other mechanisms have been considered, such as the production of chelating substances by microorganisms (Sperber, 1958b; Duff & Webley, 1959) as well as the production of inorganic acids, such as sulphidric (Sperber, 1958b; Rudolfs, 1922), nitric, and carbonic acid (Vazquez, 1996). However, the effectiveness of these processes has been questioned and their contribution to P release in soil appears to be negligible (Rudolfs, 1922; Vazquez, 1996).

2.6. Genetics and Molecular Biology of Microbial Phosphate Solubilisation Activity

To scavenge Pi from organic phosphate, bacteria have developed an elaborate system that mineralizes the organic P into via enzymes – alkaline and acid phosphates. The regulation of this phenomenon has been extensively studied. Several genes are induced under phosphate starvation and constitute the *Pho* regulation. A number of genes are involved including *PhoA*, the gene that codes for alkaline phosphatase. The *Pho* regulon is activated by positive activator, *Pho* B (Torriaini & Ludtke, 1985). The *Pho* B binds to the *Pho* box, which is sequence shared by regulatory region of *Pho* A, *Pho* B, *Pho* T and *Pst* S and activates from the *Pst* B promoter (Makino *et. al.*, 1989). *Pho* R protein regulates the *Pho* regulon negatively with excess phosphate and positively with limited phosphate. *Pho* M is postulated to inhibit the *Pho* R product, into an inactive form, *Pho* R^m. Pho U exhibits a negative control in the presence of Pi. The *pst-pho* U region appears to be an operon with a transcription attenuator between *Pho* S and *Pho* T (Wanner 1987). Extensive studies have been done on this system and this review does not permit going into elaborate details. But what is interesting is that, the externally added Pi represses this system indicating physiological regulation. Similarly, repression of MPS trait was noticed in the presence of increasing levels of Pi in the medium indicating the existence of regulatory controls common to *Pho* regulon and *mps* genes (Bagyaraj, *et. al.*, 2000). 20mM of the Pi completely inhibited MPS activity by *Erwinia herbicola* (Goldstein 1986). Similarly, it was found that externally added K_2HPO_4 inhibited the MPS activity of *Pseudomonas Psd* 201 (Krishnaraj, 1996) and also by diverse isolates of Gram negative bacteria (Santhi, 1998).

2.7. Plant growth promoting substances by phosphate solubilising microorganisms

The potential use of P solubilising microorganisms as inoculants with rock phosphates to increase P availability to plants has been studied intensively (Kucey, 1988; Illmer and Schinner, 1995; Sanders, 2003). Phosphate solubilising microorganisms have an important contribution to overall plant P nutrition and growth, and have increased yields of many crops (Rodriguez and Fraga, 1999; Whitelaw, 2000; Leggett *et. al.*, 2001). Indirect growth promotion

by PSM is achieved by reducing pathogen infection via the antibiotic or siderophores which are synthesized and supplied by the bacteria (Antoun *et. al.*, 1998; Rosas *et. al.*, 2006). A rhizospheric bacterium *Pseudomonas fluorescens*, solubilises P, and produces antibiotics such as pyoluteorin (Trujilo *et. al.*, 2003). *Pseudomonas putida* produced siderophore equivalent to 13 µmol benzoic acid/mL (Pandey *et. al.*, 2006). A very small percentage of *R. leguminosarum* produced hydrogen cyanide and cyanogens. Hydrogen cyanide produced by *Pseudomonas* was used as a biological control of black root rot of tobacco (Antoun *et. al.*, 1998). Direct growth promotion includes fixing N_2 (*Rhizobium* biological N_2 fixation), increasing root surface area (mycorrhizal associations), enhancing root systems by branching roots and stimulating root hair development (phytohormones stimulation), and solubilising inorganic phosphate (*Penicillium* fungi) [Rodriguez and Fraga, 1999; Richardson, 2003]. *P. bilaiae*, the active organism in the JumpStart ® was initially selected to solubilise phosphate. *P. bilaiae* also promotes root growth and enhances root hair production (Gulden and Vessey, 2000). Two strains of *Rhizobium leguminosarum* bv. *phaseoli* stimulated root colonization on maize and lettuce in soils which had different P availability and also increased P concentration significantly (Chabot *et. al.*, 1996a). Phosphate solubilising rhizobacteria enhanced the growth and yield of canola (DeFreitas *et. al.*, 1997).

IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots (Okon and Kapulnik, 1986). Microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells (Frankenberger and Arshad 1991; Benizri *et. al.*, 1998).

One of the most commonly reported direct plant growth promotion mechanism by bacteria is the production of plant growth substances such as auxins, gibberellins (Holl *et. al.*, 1988; Chanway, 2002).

Therefore, the production of IAA, in root exudates, by the still-living cells of *Pseudomonas fluorescens* can result from utilisation of tryptophan released by dying cells. In contrast, amino acids like asparagine, alanine and lysine, known to be present in root maize exudates, can stimulate the activity of enzyme like tryptophan aminotransferase (Martens and Frankenberger, 1994). Moreover, sugars, present in exudates, could be used as C-source by bacteria; Leinhos (1994) has shown that those compounds had an effect not only on

plant growth but also on auxin production by *Pseudomonas* sp. The bacterium-fungus association increases this effect.

The purpose of applying biofertilisers is to bring beneficial organisms in contact with radical and seminal roots during and immediately after seed germination. Successful inoculation can be achieved by direct inoculation of the seed, which is most convenient, easy, economic, and the most effective method of inoculation (Kaiser, 1990). The rate of inoculant application depends primarily, among other factors, on the microbial population in carrier-based inoculum, seed rate and seed size.

2.8. Effect of PSM on crop production

Since, phosphate solubilising micro-organisms, (PSM) proportion in natural microbial population is not more than 1%, hence it is a common practice in several Russian States, European and Asian countries to inoculate soil with PSM to increase P concentration in the soil solution (Taha *et. al.*, 1969).

PSB secrete organic acids and enzymes that act on insoluble phosphates and convert it into soluble form, thus, providing phosphorus to plants. PSB also produce amino acids, vitamins and growth promoting substances (Gonzalez *et. al.*, 1983; Zimmer *et. al.*, 1988), which promote plants growth. Increased growth and yield of oats, coffee, tea, banana, mustard, maize, rice, sorghum, barley, chickpea, soyabean, groundnut, sugarbeeet, cabbage and tomato to the extent of 10-20% have been reported by using of PSB (Saxena and Sharma, 2003; Saifudheen and Ponmurugan, 2003; Ponmurugan and Gopi, 2006).

Very little percentage of the applied phosphorus is available to plants, making continuous application necessary (Abd Alla, 1994). However, phosphorus deficiencies are widespread on soil throughout the world and phosphorus fertilisers represent major cost for agricultural production.

Soil microorganisms play an important role in making P available to plants by mineralizing organic P in soil and by solubilising precipitated phosphates, the latter are called Phosphate Solubilising Bacteria (PSB) and have been isolated from many soils (Pal, 1998; Chung *et. al.*, 2005; Chen *et. al.*, 2006). Inoculation of these microorganisms improved growth and increased the

yield and P uptake in a variety of crop plants (Jisha and Algawadi, 1996; DeFreitas *et. al.*, 1997; Kumar *et. al.*, 2001; Zaidi *et. al.*, 2003; Hameeda *et. al.*, 2006).

PSB application has promoted P-uptake as well as the yields in several crops (Tomar, 1998; Khalid *et. al.*, 2004). They are capable of producing phytohormones and growth promoting substances. The production of Indole Acetic Acid (IAA), gibberellins and cytokinins by PSB has been reported earlier by several workers (Barea *et. al.*, 1976; Sattar and Gaur, 1987; Khalid *et. al.*, 2004; Kuklinsky-Sobral *et. al.*, 2004).

In some studies the analysis of variance showed no significant effect of phosphorus fertilizers, bacterial strains and mycorrhiza treatment and their interaction effects on plant height. It seems that phosphorus does not play an important role in enhancement of plant height. The application of chemical phosphorus fertilizer and phosphate solubilising microorganisms did not have any significant effect to increase the plant height (Mehrvarz *et. al.*, 2008).

Many researchers have reported an increase in P uptake and seed yields, due to PSB inoculation of wheat, barley, mungbean, chickpea and maize genotypes (Singh and Kapoor, 1999; Ramirez *et. al.*, 2001). Kumar *et. al.*, (1999) reported a significant increase in sorghum plant height by inoculation of different bacterial strains. Similarly Algawadi (1996) reported an increase in size of ear head and number of spikelets per ear of sorghum by the co-inoculation of *Trichoderma harzianum* and *Pseudomonas striata.*

Further, the use of rock phosphate as a phosphate fertilizer and its solubilisation by microbes (Kang *et al.*, 2002), through the production of organic acids (Maliha *et. al.*, 2004), have become a valid alternative to chemical fertilizers. Rock phosphate is widely distributed throughout the world, both geographically and geologically (Zapata and Roy, 2004). In conjugation with phosphate solubilising microorganisms (PSM), rock phosphate provides a cheap source of P fertiliser for crop production. In this regard, several studies have conclusively shown that PSM solubilises the fixed soil P and applied phosphates, resulting in higher crop yields (Zaidi 1999; Gull *et. al.*, 2004). The alternative approach is to use these PSM along with other beneficial rhizospheric microflora to enhance crop productivity. In this context, the simultaneous application of *Rhizobium* and PS microorganisms (Perveen *et. al.*,

2002) and PSM and arbuscular mycorrhizal (AM) fungi (Zaidi *et. al.*, 2003) has been shown to stimulate plant growth more than inoculation of each microorganism alone in certain situations when the soil is P deficient. AM fungi, on the other hand, encourage the plant roots to rapidly absorb solubilised P.

Application of PSB fertilizer for soybean production at the rate of 100 kg/ha can save 60 kg P_2O_5 /ha equal to 375 kg SSP /ha by farmers' fertilizer level equally to reduce 600,000 VND/ha in terms of phosphorus supply with 1,600VND/kg SSP, 25.000VND/kg PSB fertilizer, recommended dose as 100 kg /ha (Son *et. al.*, 2006).

It is also noted that not all laboratory or field trials have offered positive results. For example, an inoculant using *Bacillus megaterium* var. *phosphoricum*, was applied successfully in the former Soviet Union and India but it did not show the same efficiency in soils in the United States (Smith *et. al.*, 1962). Undoubtedly, the efficiency of the inoculation varies with the soil type, specific cultivar, and other parameters. The P content of the soil is probably one of the crucial factors in determining the effectiveness of the product.

2.9. Interaction of PSB with other Microorganisms

Symbiotic relationship between PSB and plants is synergistic in nature as bacteria provide soluble phosphate and plants supply root borne carbon compounds (mainly sugars), that can be metabolized for bacterial growth (Perez *et. al.*, 2007). The PSM along with other beneficial rhizospheric microflora enhance crop production. Simultaneous application of *Rhizobium* with PSM (Perveen *et. al.*, 2002) or arbuscular mycorrhizae (AM) fungi (Zaidi *et. al.*, 2003) has been shown to stimulate plant growth more than with their sole inoculation in certain situations when the soil is P deficient. Synergistic interactions on plant growth have been observed by coinoculation of PSB with N_2 fixers such as *Azospirillum* (Belimov *et. al.*, 1999) and *Azotobacter* (Kundu and Gaur, 1984), or with vesicular arbuscular mycorrhizae (Kim *et. al.*, 1998).

Tomar (1998) applied different combinations of *Azotobacter*, vesicular-arbuscular mycorrhizae (VAM), phosphorus solubilising bacteria (PSB) and NPK fertilizers in wheat. They reported that yield was 2.63 tones ha^{-1} in control, 3.41

tones ha^{-1} with NPK only and the highest (3.80 tones ha^{-1}) with NPK+VAM+PSB.

2.10. Assessment of Phosphate Solubilisation by Microorganisms

2.10.1 Assessing techniques

There are two main techniques used for evaluating P solubilisation by microorganisms. One uses a precipitated phosphate agar plate assay and the other uses a liquid media/culture broth. Precipitated phosphate agar assays are used widely in the initial selection for P solubilising microorganisms (Pikovskaya, 1948; Halder *et. al.,* 1991; Abd-Alla 1994; Wenzel *et. al.,* (1994). Microorganisms capable of solubilising phosphate minerals are grown on an agar medium with insoluble-phosphates (such as $Ca_3(PO_4)_2$) as the only P source and produce a visible clear zone around their colonies. The production of a clear/halo zone on the plate is due to the excretion of organic acids into the surrounding medium (Pikovskaya, 1948). To improve the clarity of the clear/halo zone, dyes such as bromophenol blue and alizarin red S are often used in the agar media (Cunningham and Kuiack, 1992; Gupta *et. al.,* 1994). The precipitated phosphate agar assay is a fast and easy-to-use method. It can be used to screen large numbers of isolates quickly and simultaneously. Despite the popularity of the precipitated phosphate agar assay, reliability concerns have been raised because many isolates did not produce a halo zone on the agar plates, but could solubilise various types of insoluble inorganic phosphates in liquid media (Louw and Webley, 1959; Gupta *et. al.,* 1994; Nautiyal, 1999). Moreover, correlations between the size of clear zones on the plates of precipitated phosphate agar and the more quantitative data of P solubilisation in the liquid media vary from study to study (Gupta *et. al.,* 1994; Nautiyal *et. al,* 1999; Whitelaw, 2000).

In contrast to the precipitated phosphate agar plate assays, a direct measurement of phosphate solubilisation in liquid media is considered more accurate (Nautiyal, 1999; Bhadauria *et. al.,* 2000; Sangeeta and Nautiyal, 2001). The liquid media/culture technique measures P released into the liquid from the initial insoluble phosphate substrate used. The rate of P solubilisation is typically estimated by subtracting the final culture solution P from the un-

inoculated control of P substrate (Rodriguez and Fraga, 1999). Unfortunately, the liquid media method is labour intensive and time consuming.

2.10.2. Media composition for isolation of phosphate solubilising microorganisms

Solubilisation efficacy of microorganisms is influenced greatly by media composition, especially the N and C sources, and the buffering capability of the medium used (Cunningham and Kuiack, 1992; Whitelaw, 2000; Sangeeta and Nautiyal, 2001; Pradhan and Sukla, 2005). The impact of medium composition was often studied in liquid (Table 2.2). P solubilised and released from various Ca-P compounds by PSM varied greatly with growth media and incubation times (Table 2.2). *P. radicum* released more P in the presence of NH_4^+-N compared to NO_3^- -N (Whitelaw *et. al.*, 1999). A 27.1% reduction in P released in bacterial culture solution occurred when KNO_3 was used as a sole source of N compared to $(NH_4)_2SO_4$ (Nautiyal, 1999). *Aspergillus* sp. also preferred NH_4^+-N among NH_4^+-N, NO_3^--N, urea and casein as different N source (Pradhan and Sukla, 2005). *P. bilaiae* however released more P from insoluble Ca-P in culture solution with NO_3^--N and sucrose as the C source (Cunningham and Kuiack, 1992). Furthermore, the concentration of NH_4^+-N also affects the amount of P solubilisation; higher concentrations promote P solubilisation (Nautiyal, 1999). It was also suggested that with 2.5g $(NH_4)_2SO_4$/l, P solubilisation by *Pseudomonas* sp. was promoted (Nautiyal, 1999).

The identity of the C source is considered the most influential factor for acid production. Sugars in media are converted by enzymes into intermediate metabolites including organic acids. Enzyme systems vary from microorganism to microorganism. Hence, the metabolic pathway and the types of organic acids produced by microorganisms are either a result of the regular metabolic routes or the type of sugar used (Nahas, 2007). Glucose and maltose decreased culture solution pH and resulted in the highest P solubilisation, whereas minimal pH change and P solubilisation occurred in the absence of a C source (Pradhan and Sukla, 2005). Nautiyal (1999) also found that not only was glucose necessary, but its concentration was important for bacterial P solubilisation in liquid. Soluble P concentration increased with an increase in glucose (Nautiyal, 1999). *P. radicum* favoured higher sucrose concentration (e.g., 30g/L) for P solubilisation (Whitelaw *et. al.*, 1999).

Table 2.2. Soluble phosphate released from various calcium phosphate compound by PSM in culture solution

Microorganism		Soluble P (mg L^{-1})			Reference
Fungi	Bacteria	$CaHPO_4$	$Ca_3(PO4)_2$	$Ca_5(PO_4)_3.OH$	
P. bilaiae		837			Cunningham and Kuiack, 1992
P. radicum		475			Whitelaw *et. al.*, 1999
P. radicum			360		Whitelaw *et. al.*, 1999
P. radicum		186			Whitelaw *et. al.*, 1999
P. radicum			213		Whitelaw *et. al.*, 1999
Aspergillus sp.			480		Pradhan and Sukla, 2005
Penicillium sp.			275		Pradhan and Sukla, 2005
	Pseudomonas sp.		30		Illmer and Schinner, 1995
	Pseudomonas sp.		8		Nautiyal, 1999
	Pseudomonas sp.		35		Nautiyal, 1999
	Bacillus sp.		8		Nautiyal, 1999
	Pseudomonas sp.		26		Nautiyal, 1999
	Pseudomonas sp.		90		Nautiyal, 1999
	Bacillus sp.		21		Nautiyal, 1999
	Bacillus sp.		268		Alikhani *et.* al.,2006
	Bacillus sp.			7.5-20	DeFreitas *et. al.*,1997
	Pseudomonas sp.		52		Illmer and Schinner, 1992
	Pseudomonas striata		156		Rodriguez and Fraga, 1999
	Pseudomonas striata			22	Halder *et. al.*,1993
	R. leguminosarum			356	Halder *et. al.*,1993
	R. leguminosarum		88-197		Alikhani *et. al.*, 2006
	R. melilot			165	Halder *et. al.*,1993

In soil, high C concentration in the rhizosphere supports and enhances microbial P solubilisation activities (Lynch and Whipper, 1990), while decomposition of plant residues replenishes the C source. The solubilisation of two types of rock P increased significantly during decomposition of wheat straws and cattle urine (Singh and Amberger, 1991). In addition to the C and N source, certain mineral elements are important; K and Mg concentration is critical for optimal P solubilisation by soil bacteria (Nautiyal, 1999). Phosphate solubilisation is a result of microbial activity under different growth conditions.