2. REVIEW OF LITERATURE

During the late 19th and early 20th century, inorganic compounds containing nitrogen, potassium and phosphorus (NPK) were synthesized and used as fertilizers to increase crop productivity and to meet the ever rising demand for food due to the growing human population. Increased production cost and the hazardous nature of chemical fertilizers for the environment, have led to the resurgence of interest in the use of biofertilizers for enhanced environmental sustainability with low production cost and to achieve good crop yield.

Preparations of live microorganisms (bacteria, fungi) utilized for improving plant growth and crop productivity are generally referred as biofertilizers or microbial inoculants (Subba Rao and Dommergues 1998). *Rhizobium* spp. which fix nitrogen from the atmosphere and form root nodules on legumes, were the first biofertilizer identified and used commercially for legumes over 100 years (Kannaiyan 2002). Research in the field of biofertilizer has resulted in the development of different kinds of microbial inoculants like nitrogen-fixers, phosphate-solubilizers, vesicular-arbuscular mycorrhizae (VAM) and plant growth promoting rhizobacteria (PGPR). The carrier is an important component of bioinoculant technology which plays an important role in determining the quality and shelf life of the product.

A brief review of literature pertaining to the present research problem is presented under the following sub-headings:

1. The rhizosphere: a dynamic entity
2. Biofertilizers for sustainable agriculture
3. Importance of Nitrogen and Phosphorus
   1. Nitrogen-fixation and the nitrogen-cycle
   2. Phosphate solubilizing bacteria (PSB)
2.4 Plant growth-promoting rhizobacteria

2.4.1 Phytohormones

2.4.2 Importance of iron and siderophore production

2.5 Molecular characterization of bacteria

2.6 Technology of Bioinoculants production

2.6.1 Inoculant Formulation Technology

2.6.2 Liquid formulation

2.1 The rhizosphere: a dynamic entity

Below the soil surface, the rhizosphere is the crossroads of the soil habitat, a hub of biological, chemical, and physical activities surrounding the living infrastructure of plant roots. Complex fine-scale gradient of substrate availability, water potential, and redox state distinguish this habitat from bulk soil, and constrains the distribution and the activity of the tremendously diverse rhizosphere biota. The German agronomist Hiltner, first defined the rhizosphere, in 1904, as the effect of the roots of legumes on the surrounding soil, in terms of higher microbial activity because of the organic matter released by the roots. The rhizosphere is generally considered to be a narrow zone of soil subject to the influence of living roots, where root exudates stimulate or inhibit microbial populations and their activities (Brimecombe et al. 2007). Some authors estimated that plants release between 20-50% of their photosynthates through their roots (Bottner et al. 1999 and Buchenauer 1998). Plants select those bacteria which contribute most to their fitness by releasing organic compounds through exudates (Lynch 1990) and creating an environment where diversity is high (Lucas et al. 2001 and Marilley and Aragno 1999).

The bulk soil is generally a very poor, nutrient-diluted and therefore hostile environment in which nutrient bioavailability is often hampered by the soil biochemistry. Within this nutritional desert, the presence of plant roots provides the means for the formation of true oasis with flourishing microbial populations because all roots have the ability to actively secrete low- and high molecular-weight molecules into the rhizosphere. These include monomers such as glucose and amino acids, polymers such as polysaccharides and proteins, root debris and root border cells, root cap cells separated from the root apex
during root growth (Hawes and Lin 1990; Hawes et al. 2003). Plants invest a lot of energy in root exudation, which depends on light intensity, temperature, type of plant, nutritional state of plants, stress factors, microbial activity in the rhizosphere and type of soil (for example soil texture and thus mechanical impedance). The organic substances released from roots to the rhizosphere soil support higher microbial biomass and microbial activity in the rhizosphere than in the bulk soil. Not all compounds released from roots are organic, because roots can also release proton, oxygen and water. Root products can be classified according to their perceived function in excretions (CO$_2$, bicarbonate ions, H$^+$, electrons, ethylene, etc.) and secretions (mucilage, H$^+$, electrons, enzymes, siderophores, etc.) with the former being thought to facilitate internal metabolism and the latter external processes such as nutrient uptake (Uren 2007). Low-molecular-weight exudates can diffuse to a longer distance than high-molecular weight compounds, but they are more readily assimilated by soil microorganisms. Due to the deposition of highly bioavailable low-molecular weight carbon compounds by plant roots, rhizosphere microbial population is usually higher than the bulk soil (Anderson et al. 1993). This increased microbial activity in the zone of soil surrounding the root, has major implications on soil functions. Rhizosphere has been shown to influence carbon, nitrogen, phosphorus cycling (Blagodatsky and Richter 1998; Blagodatsky et al. 1998; Schilling et al. 1998), release of greenhouse gases (Paterson et al. 1997) and pollutant breakdown in soils (Crowley et al. 1996; Haby and Crowley 1996).

Root-microbe communication is another important process that characterizes the underground zone. Some compounds identified in root exudates that have been shown to play an important role in root-microbe interactions include flavonoids present in the root exudates of legumes that activate *Rhizobium meliloti* genes responsible for the nodulation process (Peters et al. 1986). Although the studies are not yet conclusive, these compounds may also be responsible for vesicular-arbuscular mycorrhiza colonization (Becard et al. 1992; Becard et al. 1995; Trieu et al. 1997). The unexplored chemodiversity of root exudates is an obvious place to search for novel biologically active compounds, including antimicrobials. For instance, Bais et al. (2002) identified rosmarinic acid in the root exudates of hairy root cultures of sweet basil (*Ocimum basilicum*) elicited by fungal cell wall extracts of *Phytophthora cinnamoni*. 
A complex web of interactions takes place between plant-microbe, and this may affect plant growth, directly or indirectly. A large number of macroscopic organisms and microscopic organisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they may influence the plant physiology to a greater extent, especially considering their competitiveness in root colonization (Antoun and Kloepper 2001). Plant species can be important in determining the structure of rhizosphere bacterial and fungal communities (Stephan et al. 2000), with both positive and negative effects on different microbial groups. Within plant species, microbial communities can be affected by plant genotype (Smith et al. 1999), plant nutrient status (Yang and Crowley 2000), pathogen infection (Yang et al. 2001), and mycorrhizal infection. Within root systems, microbial communities can even differ among root zones (Yang and Crowley 2000) and at different distances from the root surface to the place where soil grades into bulk soil (Marilley and Aragno 1999). The largest number of bacteria in the rhizosphere has been reported to occur in the zone of root elongation (Jaeger et al. 1999).

Pandey and Palni (2007) studied the rhizosphere effect exerted on the microbial communities by ten representative and important tree species of the Indian Himalayan region. The rhizosphere to soil (R:S) ratio was found to range from 0.2 to 3.5, 0.3 to 2.9, and 0.3 to 3.4, for bacteria, actinomycetes and fungi, respectively.

Swaby and Sperber (1958) reported that the population of phosphate solubilizing microorganisms, is more in the rhizosphere (20-40% of the total population) as compared to non-rhizosphere (10-15% of total population). Reyes and Valduz (2006) studied the biodiversity of phosphate-solubilizing microorganisms (PSM) isolated from rhizospheric and bulk soils of colonizer plants at an abandoned rock phosphate mine. Six efficient fungal strains and fifteen efficient strains of Gram-negative bacteria were isolated. A large number of PSM were found in the rhizosphere than in the bulk soil.

Neal et al. (1973) studied the rhizospheric and non-rhizospheric microbial diversity in different wheat cultivars. Results clearly showed that total bacterial (335.2 x 10^6 CFU/ml), cellulolytic (131.2 x 10^3 CFU/ml), pectinolytic (570.2 x 10^4 CFU/ml),
amyloytic (38.1 x 10^6 CFU/ml), ammonifying (116.1 x 10^6 CFU/ml) and nitrate reducing (3.8 x 10^5 CFU/ml) microbes were more in rhizospheric soil as compared to the non-rhizospheric soil.

Donate-Correa et al. (2005) isolated rhizobacteria with properties related to plant growth-promotion from the rhizosphere of the perennial legume *Chamaecytisus proliferus* sp. *proliferus* var. *palmensis* (tagasaste) growing in field conditions. The results showed that rhizosphere isolates formed a diverse community of mainly Gram-negative bacteria, with members of genera *Pseudomonas*, *Burkholderia* and *Sphingomonas* being predominant. A high level of selectivity was found in the rhizosphere environment as compared to the non-rhizosphere soil where Gram-positives were more abundant.

Coelho et al. (2009) assessed the molecular diversity and the quantity of the *nifH* gene sequences in the rhizospheres of two cultivars of sorghum, sown in Cerrado soil with contrasting levels of nitrogen fertilizer by using denaturing gradient gel electrophoresis (DGGE) and SYBR Green I quantitative real-time PCR (qPCR). Results of DGGE fingerprinting showed that for both cultivars, the presumptive nitrogen-fixing population in the rhizosphere was more diverse than in the bulk soil.

Baudoin et al. (2002) extracted bacterial communities from the bulk soil and adhering soil of three maize rhizosphere zones (ramification, root hair-elongation, apex), 2 and 4 weeks after planting. Biolog data showed that the functional abilities of bacterial communities from bulk and adhering soils were distinct after 2 weeks of plantation. The difference in responses between bulk and rhizospheric soil was more pronounced after 4 weeks of plantation, but rhizosphere samples showed more diversity. These results argue in favor of a greater influence of the maize rhizosphere environment on bacterial metabolic potentialities, mainly based on the developmental state of the plant.

Potential of methane production and trophic microbial activities at rhizospheric soil during rice cv. Supanburi 1 cultivation were determined by Chawanakul et al. (2009). They found that methane production was more in rhizospheric soil than non-rhizospheric soil, with the noticeable peaks during reproductive phase than vegetative phase.
2.2 Biofertilizers for sustainable agriculture

Sustainable farming systems strive to minimize the use of costly and environmental unfriendly synthetic pesticides/agrochemicals, and to optimize the use of alternative management strategies to improve soil fertility and to control soil-borne pathogens (Harrier and Watson 2004). A more sustainable agriculture i.e. ‘ecologically sound, economically viable, socially just and humane’ (Gips 1987) should aim to recycle minerals in the soil with no or few external inputs, maintain a high biodiversity in agro-ecosystem and have better exploitation of soil-plant-microbe interactions for plant nutrition and protection (Edwards et al. 1990). An answer to this is the biofertilizer, an environmental friendly fertilizer now used in many countries. During the last couple of decades, the use of biofertilizers-PGPR for sustainable agriculture has increased tremendously in various parts of the world. Vessey (2003) defined biofertilizer as a substance that contain living microorganisms which, when applied to seed, plant surfaces or soil, colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant. Various studies have demonstrated a positive influence of biofertilization on agronomically important crops (Asghar et al. 2002; Rodríguez Sr. 2006).

Biofertilizers contribute to the nutrition of plants through a variety of mechanisms including direct effects on nutrient availability (e.g. N$_2$-fixation by diazotrophs and P-mobilization by many microorganisms), enhancement of root growth (i.e. through plant growth promoting rhizobacteria, or PGPR), as antagonists of root pathogens (Raaijmakers et al. 2009) or as saphrophytes that decompose soil detritus and subsequently increase nutrient availability through mineralization and microbial turnover.

2.3 Importance of Nitrogen and Phosphorus

Nitrogen is one of the most common nutrients required for plant growth and productivity as it forms an integral part of proteins, nucleic acids and other essential biomolecules (Bockman 1997). More than 78 % of nitrogen is present in the atmosphere, but this is unavailable to plants. It needs to be converted into ammonia, a form available to plants and other eukaryotes. Atmospheric nitrogen is converted into forms utilized by plants by three different processes a) conversion of atmospheric nitrogen into oxides of
nitrogen in the atmosphere b) industrial nitrogen fixation uses catalysts and high temperature (300-500°C) to convert nitrogen to ammonia and c) biological nitrogen fixation involves the conversion of nitrogen to ammonia by microorganisms using a complex enzyme system identified as nitrogenase (Kim and Rees 1994). Biological nitrogen fixation fixes about 60% of the earth’s available nitrogen and represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha et al. 1997).

2.3.1 Nitrogen-fixation and the nitrogen-cycle

Biological nitrogen (N) fixation is the process of the conversion of atmospheric di-nitrogen (N₂) to the non-gaseous N compound ammonium (NH₄⁺). N-fixation is not exclusively a biological process. Natural abiotic N-fixation, which can be mediated by lightning or fires, oxidizes N₂ to nitrate (NO₃⁻). The NO₃⁻ produced in this way can be washed out from the atmosphere with precipitation and is thus deposited in terrestrial ecosystems. Plants, animals and many prokaryotes depend on the availability of fixed N for their N supply. Since N is an essential nutrient for all living organisms, N-fixation is a key process of the N-cycle (Delwiche 1981; Postgate 1982; Zehr et al. 1996). Biological nitrogen-fixation (BNF) involves the enzymatic reduction of N₂ to ammonia (NH₃), which in solution is present mostly as ammonium (NH₄⁺) which represents the starting molecule for the biosynthesis of amino-acids and other N-containing biomolecules. According to current knowledge, only prokaryotes (members of the domains Archaea and Bacteria) are capable of performing BNF. All eukaryotes (including higher plants and animals) naturally depend on the BNF activity of the N-fixing prokaryotes (diazotrophs) for their N supply.

The availability of fixed N within an ecosystem depends not only on N-inputs and outputs, but also on the mineralization (ammonification) of the N bound in biomass and organic material. In most terrestrial systems, plant uptake is a major sink for mineral N (Paul and Clark 1996; Lin et al. 2000). Both NH₄⁺ and NO₃⁻ are taken up by plants, although many plants prefer the uptake of one over the other (Marschner 1995). In most soils the cation NH₄⁺ is relatively immobile and can be bound to cation exchange sites or irreversibly fixed in interlayer sites of three-layer clay minerals (Stevenson 1986). Under
aerobic conditions, \( \text{NH}_4^+ \) rapidly transformed to \( \text{NO}_3^- \) by nitrifying microorganisms. \( \text{NO}_3^- \) is much more mobile in soil in comparison to \( \text{NH}_4^+ \) and can percolate into the groundwater (\( \text{NO}_3^- \) leaching) if it is not taken up by plants or microorganisms. Nitrate accumulation in groundwater poses a great health risk as water is used for drinking purposes (Vitousek et al. 1997). Denitrification, a process of anaerobic respiration that transforms \( \text{NO}_3^- \) to \( \text{N}_2 \), completes the N-cycle by returning N to the atmosphere (Stevenson 1986).

Biological nitrogen-fixation is a complex process that involves a number of functional and regulatory gene products (Triplett et al. 1989). The actual reduction of \( \text{N}_2 \) is performed by the nitrogenase protein complex, which consists of two metalloproteins: the nitrogenase, or nitrogenase molybdenum-iron protein (MoFe protein), and the nitrogenase reductase or nitrogenase iron protein (Fe protein).

The Fe protein is responsible for shuttling electrons to the MoFe protein using at least two MgATP per electron (Halbleib et al. 2000). The molybdenum-iron-sulfur-homocitrate clusters of the MoFe protein are the actual sites of binding and reduction of the substrate \( \text{N}_2 \), and other alternative substrates, such as acetylene, protons and many others (Postgate 1982). Thus, various scientists used acetylene reduction assay (ARA) as indirect method to study the efficiency of nitrogenase enzyme.

A study was undertaken by Islam et al. (2010) to determine the free living culturable diazotrophic bacteria of paddy soils in a long-term fertilizer management experiment. Out of 165 distinct bacterial morphotypes observed during the isolation process, only 32 were positive for acetylene reduction assay (ARA). The ARA activity of the isolates ranged from 1.8 to 2,844.7 nmol ethylene h\(^{-1}\) mg protein\(^{-1}\). Chowdhury et al. (2007) isolated diazotrophic bacterial isolates from the surface-sterilized roots of \textit{Lasiurus sindicus} and showed predominance of Gram-negative bacteria, and studied nitrogen fixing capacity by acetylene reduction assay.

Park et al. (2005) isolated free-living nitrogen fixing bacteria from rhizosphere of seven different plants namely sesame, maize, wheat, soybean, lettuce, pepper and rice grown in Chungbuk Province, Korea. Five isolates with nitrogenase activity of more than 150 nmol h\(^{-1}\) mg\(^{-1}\) protein were identified by acetylene reduction assay. Similarly, other
workers also used ARA for evaluation of nitrogen fixing potential of diazotrophic isolates (Pal et al. 2001; Martinez et al. 2003; Donate-Correa et al. 2005; Piao et al. 2005; Mehnaz et al. 2007; Egamberdiyeva 2007; Hsu and Buckley 2009).

The nitrogenase complex is very sensitive to oxygen (Postgate 1982) and prokaryotes have evolved various strategies to deal with this problem (Marchal and Vanderleyden 2000). They reduce or eliminate nitrogenase production and activity at high oxygen partial pressures (Kullik et al. 1991; Klein et al. 1996; Marchal and Vanderleyden 2000), produce heterocyst (Cyanobacteria), slime production (e.g. Derxia), possess high respiration rates, have leghemoglobin production and alginate capsules (Paul and Clark 1996, Marchal and Vanderleyden 2000; Oelze 2000; Sabra et al. 2000). However, many diazotrophs are adapted to microaerobic or anaerobic niches, thus avoiding or reducing the need for oxygen protection mechanisms (Hill 1992; Paul and Clark. 1996).

Biological nitrogen fixation plays an important role in nitrogen availability, but it is an intensive energy input process which is tightly regulated on several levels and by different factors. There is profound (and still rapidly increasing) understanding of the mechanisms involved in it. However, there is only limited information available on the performance of these mechanisms under different environmental conditions, for example in soil, or their impact on the development of complex free-living diazotroph communities.

2.3.2 Phosphate solubilizing bacteria (PSB)

Next to nitrogen, phosphorus (P) is the most important nutrient required by plants and other organisms. Phosphorus is a constituent of nucleic acid, phospholipids, phytin, co-enzymes, phosphorylated sugars and nucleotide, and is involved in many essential processes like cell division, photosynthesis, sugar breakdown, transfer of energy and nutrient uptake (Sanyal and De Datta 1991; Kapoor 1995). Phosphorus has been reported to stimulate root growth (Hayman 1975) and is essential in seed germination, and occurs in large quantity in plant seed and fruit. It is well established that every aspect of the process of formation of the N₂ fixing nodule is limited by the availability of P, and legumes show a high positive response to P-supplementation (Deng et al. 1998).
Phosphorus constitutes about 0.2 per cent of plant dry weight (Shenoy and Kalagudi 2002). An adequate supply of phosphorus in the early stages of plant growth is important for laying down primordia for the reproductive parts of plants (Gaur 1990). The high energy phosphate bonds play a vital role in respiratory and photosynthetic processes. Deficiency of this mineral nutrient leads to stunted plant growth, usually deep green colour, tilted petioles and leaflets, crinkly foliage and failure of leaflets to expand normally (Gaur 1990; Grewal and Sud 1990; Sharma and Parmar 1997).

Theoretical estimates have suggested that the accumulated phosphorus (P), in agricultural soils due to fixation, is sufficient to sustain maximum crop yields world-wide for about 100 years (Goldstein et al. 1993). Although P is abundant in soils in both inorganic (originating mainly from applied P fertilizer) and organic forms (derived from microorganisms, animals and plants), it is still one of the major plant growth limiting nutrients (Paul and Clark 1989). On average, most nutrients in the soil solution are present in millimolar amounts, but phosphorus is present only in micromolar or lesser quantities (Ozanne 1980). These low levels of P are due to the high reactivity of soluble P with calcium (Ca), iron (Fe) or aluminium (Al), which leads to P precipitation. Inorganic P in acidic soils is associated with Al and Fe compounds whereas, calcium phosphate is the predominant form of inorganic phosphates in calcareous soils.

Organic P may also make up a large fraction of soluble P, as much as 50% in soils with high organic matter content (Barber 1984). Phytate, a hexaphosphate salt of inositol, is the major form of P in organic matter, contributing between 50 to 80% of the total organic P (Alexander 1977). Although microorganisms are known to produce phytases that can hydrolyze phytate which tends to accumulate in virgin soils as it becomes insoluble as a result of forming complex molecules with Fe, Al and Ca (Alexander 1977). Phospholipids and nucleic acids form a mother pool of labile P in soil that is easily available to most of the organisms present (Molla and Chowdary 1984).

To circumvent the problem of P deficiency, the addition of phosphatic fertilizers has become a common practice in modern agriculture. The production of chemical phosphate fertilizers is a highly energy-intensive process, requiring energy worth US$4 billion per annum in order to meet the global needs (Goldstein et al. 1993). The situation
is further compounded by the fact that almost 75-90% of added P fertilizer, is precipitated by Fe, Al and Ca complexes present in the soils, creating a demand for suitable alternatives to mobilize this fixed fraction of the important bioelement (Stevenson 1986). Soil microorganisms are able to mobilize insoluble mineral phosphate in a sustainable and eco-friendly manner.

The involvement of microorganisms in solubilization of inorganic phosphates was known as early as 1903 (Kucey et al. 1989). It is estimated that P-solubilizing microorganisms may constitute 20 to 40% of the culturable population of soil, and out of which, a significant proportion is in the rhizosphere soil (Kucey 1983; Chabot et al. 1993). Most PSB are isolated from the rhizosphere of various plants, and are known to be metabolically more active than those isolated from sources other than rhizosphere (Baya et al. 1981). Important phosphate solubilizing microorganisms (PSMs) including bacteria and fungi have been well reviewed (Rodríguez and Fraga 1999). In general, P solubilizing bacteria commonly outnumber P solubilizing fungi by 2-150 folds (Kucey 1983; Kucey et al. 1989). However, fungal isolates exhibit greater P-solubilizing ability than bacteria in both liquid and solid media (Kucey 1983). In addition, the P-solubilizing ability in bacteria may be lost upon repeated sub-culturing, but no such loss has been observed in case of P-solubilizing fungi (Kucey 1983). The majority of the phosphate solubilizing microorganisms (PSMs) mobilize Ca-P complexes and only a few can solubilize Fe-P and Al-P complexes (Kucey et al. 1989).

Phosphorus biofertilizers can help in increasing the availability of fixed phosphates for plant growth by solubilization (Goldstein 1986; Kucey et al. 1989). PSMs also exhibit other traits beneficial to plants, such as production of phytohormones, antibiotics, siderophores, vitamins, antifungal substances and hydrogen cyanide (Kloeper et al. 1989; Rodriguez and Fraga 1999). In addition to being better scavengers of soluble P, the microorganisms involved in P solubilization can also enhance plant growth by increasing the efficiency of biological nitrogen fixation and enhancing the availability of trace elements such as Fe, Zn, etc. (Kucey et al. 1989; Rodriguez and Fraga 1999).
At molecular level, the precise mechanism used by different PSMs is still not properly understood (Rodriguez et al. 2006). Nevertheless, it is generally believed that the production of organic acids leads to drop in pH, which is the main driving force for mobilization of mineral phosphates (Illmer et al. 1995; Goldstein 1996; Rodriguez and Fraga 1999). Apart from that, Goldstein (1996) proposed a direct oxidation of glucose to gluconic acid (GA) as a major mechanism for mineral phosphate solubilization (MPS) in Gram-negative bacteria. As a result of acidification of the surrounding medium, soluble orthophosphate ions ($H_2PO_4^{−1}$ and $HPO_4^{−2}$) can be readily released. The PSMs produce a range of low molecular weight organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate, etc. (Goldstein 1986; Kim et al. 1998). More precisely, the organic acids secreted can either directly dissolve the mineral phosphate as a result of anion exchange of $PO_4^{−3}$ by acid anion or can chelate both Fe and Al ions associated with phosphate (Moghimi et al. 1978). Strong support for the existence of this mechanism has been provided by the fact that addition of NaOH generally abolishes the P-solubilization process, indicating that pH reduction of the system is probably responsible for the P-solubilizing ability of these organisms.

However, acidification does not seem to be the only mechanism of P-solubilization, as the ability to reduce the pH in some cases does not correlate with the ability to solubilize mineral phosphates (Subba Rao 1982). For instance, a genomic DNA fragment from Enterobacter agglomerans showed mineral phosphate solubilization activity in E. coli JM109, although the pH of the medium was not altered (Kim et al. 1997). Similarly, Kucey (1988) has demonstrated that the chelating property of the organic acids is also important, as it has been shown that the addition of 0.05M ethylene diamine tetraacetic acid (EDTA) to the medium has the same solubilizing effect as inoculation with a phosphate solubilizing organism. In addition, under some circumstances phosphate solubilization has been observed at only slightly acidic or alkaline pH values (Altomare et al. 1999). On the other hand, mineral phosphate solubilization has been reported in the absence of detectable chelating agents or organic acids, merely by acidifying the medium (Illmer et al. 1995). Overall, the exact mechanism(s) utilized by PSMs is/are yet to be pin pointed (Rodriguez et al. 2006).
Microorganisms also rely on various forms of enzymes (Garcia et al. 1992; Rodriguez et al. 2006) in order to mobilize organic phosphate sources. These include: (1) non-specific phosphatases which dephosphorylate phospho-ester or phosphoanhydride bonds in organic matter; (2) phytases, which result in release of P from phytic acid; and (3) phosphonatases and C-P lyases which perform the C-P cleavage in organophosphonates. The main activity apparently corresponds to the work of acid phosphatases and phytases due to the presence of their substrates in soil in less quantity.

Biological mechanisms of microbial induced phosphate solubilization represent innovative solution for making P from the unavailable P. PSM’s have the potential to enhance dissolution of indigenous sources of rock phosphorus and transform them into more agronomically effective P-fertilizers.

2.4 Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria, isolated from the rhizosphere, which, when applied to seeds or crops, enhance the growth of the plant or reduce the damage from soil-borne plant pathogens (Kloepper et al. 1980). It has been estimated that more than 100 million tonnes of nitrogen, potash and phosphate-chemical fertilizers have been used annually in order to increase plant yield (Glick et al. 1999). The negative effect of chemical fertilizers on the global environment, and the cost associated with production, has led to research with the objective of replacing chemical fertilizers with bacterial inoculants.

Bacteria referred to as PGPR, are found in the rhizosphere of the roots of many different plants (Kloepper et al. 1989). The effect of PGPR on agricultural crops has been investigated and published by various authors in the last two decades (Bashan and Holguin 1998; Enebak et al. 1998). Salamone (2000) reported the growth-promoting effect of *P. fluorescens* strain G20-18 on wheat and radish plants by production of cytokinin phytohormones. As the effect of PGPR on plants was demonstrated, the concept of PGPR began to gain importance and a large number of bacterial strains were isolated, screened (Chanway and Holl 1993; Cattelan et al. 1999; Bertand et al. 2001) and evaluated for plant growth promotion (Lifshitz et al. 1987; Chanway et al. 1989; Abbas and Okon 1993; Glick et al. 1997; Zhang et al. 1997; Bashan 1998; Mayak et al. 1999; Salamone 2000; Bent et al. 2001).
Rhizosphere bacteria promote plant growth and yield either directly or indirectly (Kloepper et al. 1989; Glick 1995). The direct mechanisms of plant growth promotion may involve the synthesis of substances by the bacterium or facilitation of the uptake of nutrients from the environment (Glick et al. 1999). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of plant pathogens by production of inhibitory substances or by increasing the natural resistance of the host (Handelsman and Stabb 1996; Nehl et al. 1996; Cartieaux et al. 2003).

The direct growth promoting mechanisms are i) nitrogen fixation ii) solubilization of phosphorus iii) sequestering of iron by production of siderophores iv) production of phytohormones such as auxins, cytokinins, gibberellins and v) lowering of ethylene concentration (Kloepper et al. 1989; Glick 1995; Glick et al. 1999). For example strain GR12-2, P. putida, isolated from the rhizosphere of plants growing in the Canadian High Arctic, was found to promote growth of canola cv. Tobin by fixing nitrogen and enhancing the uptake of phosphate under gnotobiotic conditions (Lifshitz et al. 1986; Lifshitz et al. 1987), by synthesizing siderophores that can solubilize and sequester iron from the soil and supply it to the plants, by production of the phytohormone IAA and by lowering of ethylene concentration via production of the enzyme ACC deaminase.

The indirect mechanisms of plant growth promotion by PGPR include i) antibiotic production ii) depletion of iron from the rhizosphere iii) synthesis of antifungal metabolites iv) production of fungal cell wall lysing enzymes v) competition for sites on roots and vi) induced systemic resistance (Weller and Cook 1986; Kloepper et al. 1988; Dunne et al. 1993; Liu et al. 1995; Glick et al. 1999).

2.4.1 Phytohormones

One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones (Glick 1995). Frankenberger and Arshad (1995) have discussed in detail the role of auxins, cytokinins, gibberellins, ethylene and abscisic acids (ABA) which, when applied to plants, help in increasing plant yield and growth. Microbial production of individual phytohormones such as auxins and cytokinins has been reviewed by various authors over the last 20 years (Pilet et al. 1979; Hartmann et al. 1983; Fallik and Okon 1989; Barbieri and Galli 1993; Patten and Glick 1996; Patten and Glick 2002).
Phytohormones play an important role as regulators of growth and development of plants. According to the conventional classification, there are five groups of phytohormones: auxins, gibberellins, cytokinins, ethylene, and abscisic acid. Phytohormones contribute to the coordination of diverse physiological processes in plants, including the regulation of quiescence and seed germination, root formation, florescence, branching and tillering, and fruit ripening. They increase plant resistance to environmental factors and induce or suppress the expression of genes, and the synthesis of enzymes, pigments, and metabolites.

The ability to form plant hormones is believed to be a major property of rhizospheric, epiphytic, and symbiotic bacteria that stimulate and facilitate plant growth (Tien et al. 1979; White 1987; Cacciari et al. 1989; Kameneva and Muronets 1999; Suzuki et al. 2003).

**Auxins**

Auxins were discovered early in the twentieth century as plant growth regulators and the most common and naturally occurring auxin with broad physiological effects is Indole-3-acetic acid (IAA). IAA known to be involved in root initiation, cell division, and cell enlargement and the capacity to synthesize this phytohormone is widely spread among PGPR (Salisbury and Ross 1992). It has been estimated that 80% of bacteria isolated from the rhizosphere can produce the phytohormone IAA (Patten and Glick, 1996).

In plant cells, IAA is largely formed by *de novo* synthesis from tryptophan, which undergoes either oxidative deamination or decarboxylation. In microorganisms various known pathways of IAA biosynthesis are also related to tryptophan metabolism (Costacurta and Vanderleyden 1995; Patten and Glick 1996; Kameneva and Muronets 1999). (i) IAA formation via indole-3-pyruvic acid and indole-3-acetic aldehyde is found in the majority of microorganisms studied, such as *Erwinia herbicola*, Agrobacterium, *Pseudomonas*, Bradyrhizobium, Azospirillum, Rhizobium, Klebsiella, and Enterobacter (Tien et al. 1979; Mordukhova et al. 1991; Koga et al. 1994; Brandl and Lindow 1996; Muronets et al. 1997; Manulis et al. 1998; Kameneva and Muronets 1999); methyllobacteria (Ivanova et al. 2001); Nostoc sp. (Sergeeva et al. 2002); Saccharomyces
uvarum, Fusarium, Rhizoctonia, and Colletotrichum (Thakur and Vyas 1983; Furukawa et al. 1996; Chung et al. 2003). (ii) The conversion of tryptophan into indole-3-acetic aldehyde may involve an alternative pathway in which tryptamine is formed (Kameneva and Muronets 1999). This pathway is believed to operate in pseudomonades and azospirilla (Mordukhova et al. 1991). (iii) IAA biosynthesis via indole-3-acetamide formation takes place in phytopathogenic bacteria Agrobacterium tumefaciens, Pseudomonas syringae (Costacurta and Vanderleyden 1995), and E. herbicola (Manulis et al. 1998); certain streptomycetes (Manulis et al. 1998); saprophytic pseudomonades Pseudomonas putida and P. fluorescens (Mordukhova et al. 1991); methyllobacteria Methylobacterium mesophilicum and Aminobacter aminovorans (Trotsenko et al. 2001); and phytopathogenic fungi of the genus Colletotrichum (Chung et al. 2003; Maor et al. 2004). (iv) IAA biosynthesis that involves tryptophan conversion into indole-3-acetonitrile is found in plants, Alcaligenes fecalis (Kameneva and Muronets 1999) and possibly in cyanobacterium Synechocystis sp. (Sergeeva et al. 2002). (v) The tryptophan-independent pathway, more common in plants, is also found in microorganisms azospirilla (Costacurta and Vanderleyden 1995) and cyanobacteria (Sergeeva et al. 2002). However, the contribution of this pathway to IAA biosynthesis is insignificant, and the mechanisms are largely unknown.

Many bacteria and fungi are capable of synthesizing auxins using several pathways (Patten and Glick 1996; Costacurta and Vanderleyden 1995; Muronets et al. 1997; Kameneva and Muronets 1999; Sergeeva et al. 2002; Chung et al. 2003), which increases the potential for forming associations with plants. Moreover, epiphytic and rhizospheric microflora of plants are of utmost importance in the conversion of tryptophan (which is present in plant exudates) into IAA. Omission of tryptophan from the culture medium decreases the level of IAA synthesis by the culture’s microorganisms.

The most efficient auxin producers are found among bacterial and fungal inhabitants of plant rhizosphere and phyllosphere (Libbert and Risch 1969; Gunasekaran and Weber 1972; Manka 1980; Thakur and Vyas 1983; Mordukhova et al. 1991; Weger et al. 1995; Sergeeva et al. 2002; Tsavkelova et al. 2005). The amount of the auxins formed depends on the composition of the medium and the conditions of culturing i.e. temperature, aeration, etc. (Mordukhova et al. 1991; Olyunina and Shabaev 1996;

Genes responsible for IAA synthesis in bacteria may have plasmid or chromosomal localization (Mordukhova et al. 1991; Muronets et al. 1997). As a rule, pathogenic bacteria contain Ti plasmids that control the formation of the phytohormone, whereas in saprophytic microorganisms, auxin biosynthesis is governed by chromosomal genes (Costacurta and Vanderleyden 1995; Nizan et al. 1997; Kameneva and Muronets 1999). Differences in the production of IAA among bacterial strains can be attributed to the various biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes to convert active free IAA into conjugated forms. It is also dependent on environmental conditions (Patten and Glick 1996).

Tien et al. (1979) demonstrated that production of indole acetic acid and indole lactic acid by *Azospirillum brasilense* Sp13t SR2 increased with increasing concentrations of tryptophan (1 - 100 μg/ml). However, the production of indole acetic acid and indole-3-butyric acid by cultures of *A. brasilense* in the absence of tryptophan was also identified by Fallik and Okon (1989). Synthesis of IAA by *Rhizobium* spp. in presence and absence of tryptophan has also been demonstrated (Kittel et al. 1989). Plant growth-promoting rhizobacteria strain G20-18 and two mutants produced IAA in pure culture (Salamone 2000). Bent et al. (2001) reported that the production of indole compounds by three different strains, *Paenibacillus polymyxa* L6, *P. polymyxa* Pw-2, and *Pseudomonas fluorescens* M20 increased in concentration with increasing concentrations of tryptophan (0-200 mg/ml) at different intervals. Reports by Asghar et al. (2002) showed that PGPR strains produced 24.6 μg/ml of auxins in the presence of the precursor L-tryptophan in the medium, which was 184-fold more than that without L-tryptophan.

Xie et al. (1996) generated a mutant strain of *P. putida* GR12-2 that produced four times more indole acetic acid than the wild type in the presence of 0.1 mg/ml L-tryptophan. Patten and Glick (2002) showed IAA production by the wild-type and an IAA-deficient mutant of *P. putida* GR12-2 in the presence of varying concentrations of
tryptophan. The levels of IAA secreted by the wild type ranged from 0.5 – 32.7 μg/ml, while for the mutant it ranged from 0.5 – 2 μg/ml. Similar results were obtained by other workers (Dey et al. 2004; Zaidi et al. 2006; Roesch et al. 2007; Tsavkelova et al. 2007; Egamberdiyeva 2007; Ashrafuzzaman et al. 2009; Khan and Doty 2009; Abbas-Zadeh et al. 2010).

2.4.2 Importance of iron and siderophore production

Iron is a growth-limiting factor for the majority of microorganisms (Archibald 1983). Some notable exceptions are Lactobacilli, Legionella, Neisseria, and the fungus Sacchoromyces cervisiae (Neilands et al. 1987). For example, Lactobacilli have no heme enzymes and they use the cobalt form of ribonucleotide reductase, so do not require iron (Wayne and Neilands 1975).

Being a component of cell, iron deficiency can cause growth inhibition, decrease in RNA/DNA synthesis, inhibition of sporulation and can also change cell morphology. Iron is also required in metabolic processes such as TCA cycle, electron transport chain, oxidative phosphorylation and photosynthesis (Leong 1986). It is also known to regulate the biosynthesis of porphyrins, toxins, vitamins, antibiotics, cytochromes, pigments, siderophores, aromatic compounds and DNA/RNA (Chincholkar et al. 2000). Ferrous and ferric iron can act catalytically to generate hydroxyl radicals that are most potent known oxidizing agents (Guerinot and Yi 1994).

Although iron is present in abundance, it is unavailable due to its presence as insoluble iron oxyhydroxide polymers under aerobic conditions at biological pH. Ferric iron’s (Fe^{3+}) solubility under these conditions is 10^{-17} M, whereas cytoplasmic iron concentrations are approximately 10^{-7} M in metabolically active microbes (Ishimaru 1993). This difference in concentration illustrates that uptake by diffusion is not an option for these microbes. High affinity iron transport systems in general are made up of several components, including siderophores, outer membrane receptor proteins, periplasmic binding proteins, ATP-dependent ABC-type transporters, and the TonB-ExbB-ExbD protein complex, each is vital to the success of transport system.
Siderophores

Lankford coined the term siderophore in 1973 to describe low molecular weight molecules that bind ferric iron with an extremely high affinity (Lankford 1973). Term siderophore was derived from a Greek term meaning ‘iron carrier’ (Ishimaru 1993). This is an appropriate term because the siderophore binds iron with an extremely high affinity and is specifically recognized by a corresponding outer membrane receptor protein, which in turn actively transports the complex into the periplasm of the cell. The molecular weights of siderophores range from approximately 600 to 1500 daltons, and because passive diffusion does not occur for molecules greater than 600 daltons, siderophores must be actively transported (Ishimaru 1993). Siderophores are classified on the basis of the chemical functional groups they use to chelate iron. Ferric iron is chelated by up to six atoms (mostly oxygen) in high spin form, resulting in an exchangeable and thermodynamically stable pseudo-octahedral coordination complex. Many bacteria and fungi are capable of producing more than one type of siderophore or have more than one iron-uptake system to take up insoluble iron (Neilands 1981).

Siderophore production by microorganisms is highly regulated by iron availability in the surrounding environment. Carson et al. (1992), reported that *Rhizobium leguminosarum* bv, viciae MNF 710, an isolate from wild vetch in Japan excretes an hydroxamate siderophore when grown in medium containing low (0.5 µM) amount of added iron. Aronson and Boyer (1992) conducted experiments under both iron-replete and iron limited culture conditions on *Frankia* strain 52065, and concluded that *Frankia* produced a hydroxamate siderophore in response to iron limitation in culture. Korat et al. (2001) in a study conducted on the detection and chemical characterization of siderophores produced by certain fungi, showed that out of 18 fungi examined, all except *Rhizopus sp.* and *Aspergillus flavus* produced siderophores as evidenced by FeCl₃ test and CAS assay. Sharma and Johri (2003) studied the production and regulation of siderophore by fluorescent *Pseudomonas* strain GRP3A. Among various media tested, standard succinate medium (SSM) promoted maximum siderophore production of 56.59 mg l⁻¹. In deferrated SSM, siderophore level was quantified to be 68.74 mg l⁻¹. Supplementation with iron (FeCl₃) resulted in decreased siderophore levels depending on concentration. Strain GRP3A showed plant growth promotion under iron limited conditions.
Kumar and Dube (1993) isolated Fluorescent *Pseudomonas* sp. RBT13 from tomato rhizoplane and examined them for siderophore production. Isolates produced siderophore in succinate medium deficient in iron, and the production was highest in 48 hrs old culture at pH 7.5. Iron was found to suppress siderophore production. Gupta *et al.* (2001) reported that *P. aeruginosa* (GRCI) isolated from potato rhizosphere which grew better on succinate medium than tryptic soy medium, produced hydroxamate type siderophore in Fe-deficient succinate medium. In a study conducted by Mahmoud and Abd-Alla *et al.* (2001), it was found that out of eighty one isolates of rhizospheric micro flora, 42 were strongly or moderately positive on chrome azurol assay. Terano *et al.* (2002) found that *P. fluorescens* isolated from rhizosphere of barley produced siderophore under iron limiting conditions. Its chemical structure was identified as pyochelin on the basis of 1H and 13C NMR data of stable methyl ester derivative. Similar results were obtained by other workers (Rajkumar and Freitas 2008; Ma *et al.* 2010; Jha *et al.* 2010; Abbas-Zadeh *et al.* 2010).

**Importance of microbial siderophore**

Microbial siderophore may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron-uptake system (Kloepper *et al.* 1980; Marek-Kozaczuk *et al.* 1996). The existence of siderophore-producing microorganisms in the rhizosphere had led to the hypothesis, that without the capability of using Fe chelated by siderophores, all plants would become Fe deficient (Kloepper *et al.* 1980).

Marschner and Romheld (1994) reported that plants may utilize siderophores synthesized by microorganisms colonizing the rhizosphere; that may be a source of soluble iron for the host plant. Different plants have demonstrated the ability to use radio-labelled microbial siderophores as a sole source of iron (Bar-Ness *et al.* 1991; Wang *et al.* 1993). Growth of cucumber in the presence of microbial siderophores resulted in increased plant biomass and chlorophyll content (Ismande 1998). Tests conducted in nutrient solutions indicate that both FeFOB and FePSB act as moderately effective Fe source to both dicots and monocots (Bar-Ness *et al.* 1991).
Siderophores produced by several fluorescent *Pseudomonas* spp, play a role in the biological control of plant pathogens and in plant growth promotion through competition for Fe (Vandenbergh *et al.* 1983; Schippers *et al.* 1987). It has been shown that in peanut plants grown in an iron deficient soil which has been amended with Fe\(^{3+}\)-pseudobactin, the proportion of fluorescent pseudomonads in the rhizosphere increases (Jurkevitch *et al.* 1988). Carrillo and Vazquez (1992) have reported that strains of *Rhizobium phaseoli* and *P. fluorescens* showed inhibitory activity against phytopathogenic bacterial strains and this was mediated by competition for iron through excretion of siderophores. Manwar *et al.* (2000) investigated *in vitro* suppression of plant pathogens by siderophores of fluorescent pseudomonads designated as SC1 and SC4, isolated from soil. They found that siderophores produced by these organisms exerted killing effect on plant deleterious fungi. At the same time, it promoted growth of the test plant. Arora *et al.* (2001) isolated two siderophile producing strains of root-nodulating bacterium, *Rhizobium meliloti* from the medicinal plant *Mucuna pruriens*. The two isolates (RMP3 and RMP5) were able to inhibit a widely occurring plant pathogen, *Macrophomina phaseolina* that causes charcoal rot in groundnut. These two rhizobial strains showed orange and yellow orange coloured halo around the colonies, on CAS reagent overlaid on yeast extract mannitol agar. Suryakala *et al.* (2004) isolated plant growth promoting rhizobacteria belonging to fluorescent pseudomonads from the rhizosphere of different crops and found that isolates belonging to *Pseudomonas fluorescens* produced siderophores when grown in casamino acid medium under iron deficiency. These siderophores were antagonistic to fungal pathogens like *Fusarium oxysporum*, *Alternaria sp.* and *Colletotrichum capsicii*.

Biocontrol action of siderophores is useful since many crops including aromatic plants are affected by plant pathogens. *Fusarium* spp. are found to cause root rot during south west monsoon season in Geranium (Kumar *et al.* 1997). The roots, leaves and stems of geranium are affected by *Fusarium oxysporum* and *Bortryodiplodia theobromae* which cause wilting and death of Geranium plants (Sarwar 1973). The addition of a siderophore-producing *Pseudomonas putida*, converted a *Fusarium*-conducive soil into a *Fusarium*-suppressive soil for the growth of three different plants (Scher and Baker 1982).
2.4 Molecular characterization of bacteria

Characterization of bacteria is an important component of ecological studies and has often been carried by phenotypic methods based upon carbohydrate metabolism and enzyme production. Polyacrylamide gel electrophoresis of total proteins, intrinsic antibiotic resistance, bacteriophage sensitivity and fluorescent antibody techniques also play important role in identification of bacteria. All intracellular and extracellular proteins (enzymes) which form the basis for these analyses are encoded by DNA. Therefore, the genotypic methods are of equal interest for such studies. The method of determination of base sequences of certain key nucleic acids, including 5S rRNA, 16S rRNA and 23S rRNA sequences, are important tools to study bacterial genomes. All these approaches are highly specific and are used to establish relationship among the tested strains.

Molecular approaches for the detection and characterization of microbes have resulted in dramatic change in our understanding of microbial diversity. It is now recognized that approximately 99 per cent of the microbes in the environment can not be cultivated (Amann et al. 1995). However, nucleic acid based approaches for the characterization of microbes have provided new information inspite of their own limitations.

Within the last two decades, availability of molecular methods and their use in microbial ecology has allowed scientists to address many of the questions, but at the same time new challenges have become apparent. The rRNA approach opened the window to the enormous diversity of natural microbial communities (Alef 1995; Rotthauwe et al. 1997). The introduction of the polymerase chain reaction (PCR) into molecular biology represented another milestone, allowing for the specific detection and investigation of even minor traces of genetic material. Torsvik (1980) reported the first extraction of DNA from soil that was suitable for downstream applications. This method created the opportunity to adapt or specifically develop an arsenal of molecular methods for soil microbial purposes. A continuously increasing number of studies have sought to explore the diversity of soil microbial communities with the help of molecular methods, most prominently using the rRNA genes as genetic markers (Alef 1995; Rotthauwe et al. 1997).
Phylogenetic comparison based on conserved part of genome, has been shown to be much stable than classification solely based on phenotypic traits and other features (Dubnau et al. 1965; Woese 1987; Clarridge 2004). Hence the use of rRNA molecules was advocated for making phylogenetic comparisons (Woese et al. 1985; Woese 1987). All the three kinds of rRNA molecules i.e., 5S, 16S, 23S and spacers between these can be used for phylogenetic analyses, but the small and large size of 5S rRNA (120 bp) and 23S rRNA (3300 bp) have restricted their use. 16S rRNA gene (1550 bp) is the most commonly used marker that has revolutionized the field of microbial systematic (Woese et al. 1985; Amann et al. 1995; Mora and Amann 2001). Widespread use of 16S rRNA gene sequences in bacterial taxonomy started after the pioneering work of Woese in 1987. This gene has universal distribution, highly conserved nature, fundamental role of ribosome in protein synthesis, no horizontal gene transfer and its slow rate of evolution which represents an appropriate level of variation between organisms, thus this gene is used for inferring the phylogenetic relationship among bacteria (Stackebrandt and Goebel 1994; Mora and Amann 2001; Clarridge 2004). The 16S rRNA molecule comprises of variable and conserved regions, and universal primers for amplification of full 16S rRNA gene are usually chosen from conserved region while the variable region is used for comparative taxonomy. The arrangement of the currently valid taxa and even more the taxonomic outline as proposed in the recent edition of Bergey's Manual of Systematic Bacteriology (Garrity 2005), are based upon small subunit rRNA phylogeny for the most part.

Igual et al. (2001) used various molecular techniques including 16S rRNA sequencing for the characterization of phosphate solubilizing bacteria. They used bacterial universal primers i.e. fD1 and rD1 for amplification of 16S rRNA gene, which yield 1.5 kb band. Tejera et al. (2005) isolated Azotobacter and Azospirillum from the sugarcane rhizosphere of four different agricultural locations in spain, and molecularly identified these isolates by restriction fragment analysis of PCR amplified 16S rDNA (Amplified rDNA restriction analysis). They used primers 41f and 1488r for Azotobacter spp. and fD1 and rD1 for Azospirillum spp. Bhatia et al. (2008) studied the diversity of 76 strains of Azotobacter spp. isolated from cotton-wheat cropping systems. They screened out 20 efficient isolates and biochemically characterize them for various
PGPR’s activities and sugar utilization by BIOLOG. These isolates also molecularly characterized by using bacterial universal primers fD1 and rD1 for amplification of 16S rRNA gene and clustered *Azotobacter* into four different clusters. Similarly, other workers used bacterial universal primers for 16S rRNA gene for identification and phylogenetic studies of bacteria (Yang *et al.* 2001; Pinto *et al.* 2007; Khan and Doty 2009; Han *et al.* 2009; Islam *et al.* 2010).

As sequencing of rRNA genes has become a common tool in microbial investigations, a considerable volume of sequence data of rRNA genes from diverse bacteria has been deposited in public data bank (NCBI, EMBL) enabling the phylogenetic affiliation of bacteria. This line of work has considerably extended knowledge of microbial diversity, community structure and community response to environmental conditions.

2.6 Technology of Bioinoculants Production

Development of a successful inoculant involves several critical elements such as strain selection, selection of a carrier, mass multiplication, formulation of the inoculant, and packaging and marketing. Stringent quality assurance at various steps of production ensures the production of consistently high quality inoculants. In general, shortly after the bacteria are introduced into the soil, the bacterial population declines progressively (van Elsas *et al.* 1986; Bashan and Levanony 1988). This phenomenon may prevent the build-up of a sufficiently large microbial population in the rhizosphere to obtain the intended plant response. The key obstacle is that the soil is a heterogeneous and unpredictable environment, even on small scale (van Elsas and van Overbeek 1993). The inoculated bacteria must compete with the often better adapted native microflora. A major role of inoculant formulation is to provide a more suitable microenvironment (even temporarily) to prevent the rapid decline of introduced bacteria in the soil. Although much of it is known about the survival of bacteria within the protective environment of an inoculant carrier, little is known about the stresses that bacteria must endure upon transfer to the competitive and often harsh soil environment (van Elsas and Heijnen 1990; Rodriguez-Navarro *et al.* 1991; Heijnen *et al.* 1992). Inoculants have to be designed to provide a dependable source of beneficial bacteria that survive in the soil and become available to the plant.
The manufacturing of bioinoculants requires four major steps (a) *Selection of efficient strain*, (b) *Mass culture*, (c) *Carrier materials and their processing* and (d) *Packaging*, which are to be followed sternly to ensure the production of a quality product (Gosal *et al.* 2001).

2.6.1 **Inoculant Formulation Technology**

Formulation is a crucial aspect for producing inoculants containing an effective bacterial strain that can determine the success or failure of a biological agent (Bashan 1998). Formulation typically consists of establishing the active ingredient i.e., microorganism (s) in a suitable carrier together with additives that aid in the stabilization and protection of the microbial cells during storage and transport, and at the target site. Whether a product is new or improved, it is imperative that the formulation be stable during production, distribution, storage, and transportation. The formulation should also be easy to use, protects the desired organism from harmful environmental factors, and maintain or enhance activity of the organism in the field (Jones and Burges 1998). Another important consideration is the cost-effectiveness of the formulation. Therefore, several critical factors including user preference have to be considered before delivery of the final product.

To facilitate introduction of high cell numbers and increase survival of microorganisms in soil, different formulations using carrier materials have been used. The issue of quality inoculant production depends on use of good carrier material in biofertilizer production unit (Bisoyi 2004). The carrier is the delivery vehicle of live microorganisms from the factory to the field; however, no universal carrier or formulation is available for the release of microorganisms into soil (Trevors *et al.* 1992). Carrier materials may act to enhance survival of inocula by providing microorganisms with a protective environment in order to escape unfavourable conditions in soil. The reasons for a decrease in inoculum population in soil over time include insufficient nutrients available for maintenance and replication, and suboptimal environmental conditions, such as pH, ionic strength, temperature etc (van Elsas and van Overbeek 1993). Predation by bacteriovorus microorganisms, such as protozoa, and competition with indigenous bacteria can also decrease inoculum concentration.
To be successful, a carrier material must enhance survival of inocula during storage and after introduction into soil. The carrier must display two fundamental properties i.e. it must support the growth of the target organism and maintain a desired population of inoculant over an acceptable time period. To achieve these goals, a carrier must also display high water holding capacity and retention characteristics, display chemical and physical uniformity and be non-toxic to inoculant strains and environmentally safe (Stephens and Rask 2000).

Peat is the most suitable carrier material used in India and worldwide. Most of the production units in developed countries are using peat. Peat of a good quality (more than 75% carbon) is a rare commodity in India. The main limitation of peat is its variability in composition (Trevors et al. 1992) and possible production of toxic products during sterilization (Roughly and Vincent 1967; Strijdom and van Rensburg 1981). Finally, peat formulations are prone to contamination that can reduce the shelf life of the inoculants (van Elsas and Heijnen 1990; Fages 1992; Olsen et al. 1994 a, b). Alternative carriers, both natural and derived, have been investigated that include mineral soils (Chao and Alexander 1984), plant byproducts (Sparrow and Ham 1983; Arora et al. 2008), various clays (Graham-Weiss et al. 1987), charcoal (Kremer and Peterson 1983; Sparrow and Ham 1983), lignite, a range of coals (Paczkowski and Berryhill 1979), composted plant materials (Kostov and Lynch 1998), animal manure, perlite, rock phosphate, talc, entrapped alginate beads (Bashan 1986; Trivedi and Pandey 2008), Polyacrylamide gels (Dommergues et al. 1979) and mixture of xanthan and gum (Jung et al. 1982). Often, the ability of an alternative carrier or entrapment methodology, to sustain organism growth is marginal, while cost and consistency of supply, and quality in many instances, work against more widespread adoption of these alternative materials.

2.6.2 Liquid Formulation

To overcome the problems faced with solid carrier based formulations, there is need to develop new inoculant formulations which would ensure longer survival, no contamination, ease of applicability. In recent years, many of the formulations of the liquid based inoculants are introduced which have been shown to tolerate adverse environmental conditions in a better way and are free from other problems that are encountered with solid carrier based preparations.
Liquid bioinoculants are special formulations containing not only the desired microorganisms and their nutrients, but also, special cell protectants or substances that encourage the longer shelf life and tolerance to adverse conditions (Vora et al. 2008). Researchers have shown that the performances of liquid rhizobial formulations are comparable to that of peat-based products under field conditions (Hynes et al. 1995; Hynes et al. 2001). Liquid formulations typically are aqueous-, oil-, or polymer-based products. Polysaccharides such as gums, carboxymethylcellulose and polyalcohol derivatives are frequently used to alter the fluid properties of liquid formulations (Paau 1988). Several liquid formulations (Table 3.1) available today sustain high viable microbial counts for extended periods of time. Studies have shown that it is possible to make rhizobia survive in a liquid medium for more than six months with the help of cell protectants (Hedge 2002) such as trehalose, polyvinylpyrrolidone, etc. A liquid inoculant formulation with good field performance characteristics that uses low cost materials and are easily attainable by small producers, could overcome many problems associated with processing solid carriers (Singleton et al. 2002). Liquid inoculants has got wider scope, particularly in India where high cost is involved in using carriers for its transportation, pulverization, neutralization, sterilization etc. (Gupta 2005).

Table 2.1 Various liquid carriers used in the bioinoculant formulations

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Liquid carriers</th>
<th>References</th>
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<tbody>
<tr>
<td>1.</td>
<td>Synthetic medium</td>
<td>Vendan and Thangaraju (2006); Albareda et al. (2008)</td>
</tr>
<tr>
<td>2.</td>
<td>Sludge</td>
<td>Rebah et al. (2002); Rebah et al. (2007)</td>
</tr>
<tr>
<td>4.</td>
<td>Paneer-Whey</td>
<td>Bissonnette et al. (1986); Estrella et al. (2004); Pandey and Maheshwari (2007)</td>
</tr>
<tr>
<td>5.</td>
<td>Malt sprouts</td>
<td>Boiardi and Ertola (1985)</td>
</tr>
<tr>
<td>7.</td>
<td>Vegetable oil</td>
<td>Kremer and Peterson, (1982); Hoben et al. (1991)</td>
</tr>
</tbody>
</table>
Characteristics of a good liquid inoculant include non toxicity, low cost, readily available uniform materials, adaptable to normal cell culture conditions, amenable to nutrient supplements, rapid release of microorganisms in the soil, supports their growth and survival and is easily manageable in the mixing and packaging operation (Smith 1992; Singleton et al. 2002).

Many research investigations were made to evaluate the superiority of liquid formulations over the solid carrier based formulations. Vendan and Thangaraju (2006) developed liquid formulation of *Azospirillum* by using various cell protectants (trehalose, polyvinylpyrrolidone and glycerol) which resulted in increased shelf life by maintaining population level at $10^8$ CFU/ml. Singleton et al. (2002) developed liquid formulation of *Rhizobium* by adding various additives in the yeast extract mannitol media and claimed cell numbers of $1 \times 10^{10}$ cells/ml in the liquid inoculant. Pandey and Maheshwari (2007) evaluated different carriers for the formulation of *Burkholderia* spp. strain MSSP and found that paneer whey and wheat bran were found out to be efficient carriers in maintaining higher population.

A field experiment was carried out to evaluate and compare the response of pigeonpea to liquid and solid carrier based *Rhizobium* inoculant. The results showed the presence of higher number of nodules in case of liquid based inoculant treatments as compared to the solid carrier based inoculants (Yadav et al. 2006). Similar results were also reported by other workers like Ali et al. (2005); Kupper et al. (2006) and Ramarethinam and Krishnan (2007). Hynes et al. (1995) in their experiment found that liquid bioinoculants yielded equal or better nodulation than that observed with peat inoculant in pea plants. In an experiment conducted with *Rhizobium* inoculants, liquid inoculant was found equally effective in comparison to solid carrier based inoculant (Gupta 2005).

Beside this, cyst based bioinoculant formulations have twin advantages of longer shelf life and tolerance to adverse or harmful environmental conditions. Vendan and Thangaraju (2007) developed cyst based liquid formulation of *Azospirillum* using glycerol as protective agent which maintains population at $10^8$ CFU/ml up to 420 days. Inamdar et al. (2000) reported that cyst based inoculant of *Azotobacter* maintained viable
counts above $10^8$ cysts/ml for more than four years. The cyst formation and its tolerance against stress conditions by *Azotobacter* was reported earlier (Vela and Gagle 1969; Sadoff 1975). It was observed that the cyst cells of *Azospirillum* are resistant to desiccation from a few hours to one month (Lamm and Neyra 1981). Similar desiccation tolerance experiment was conducted by Sadasivan and Neyra (1987) with cyst form of *Azospirillum* cells. Galinski and Herzog (1990) found various osmotolerant solutes during stress conditions in microbes. The various cell protectants used in liquid formulation enhance cell tolerance to dessication, osmotic pressure, temperature stress and stabilize both enzymes and cell membranes (Streeter 1985; Andre et al. 1988; Vendan and Thangaraju 2007), which are helpful in ensuring longer shelf life and better adaptability to survive in the harsh conditions in the soil environment.

So from the various above cited studies, it can be concluded that biofertilizers are environment friendly, low cost agricultural inputs that have an important role to play in improving nutrient supply to crops. Plant Growth Promoting microorganisms have the potential to increase the availability of primary nutrients and other growth inducing factors to the plants. To achieve this, it is imperative that efficient organisms adapted to the local conditions are required to be isolated for making region specific preparations. Carrier is the most important component of biofertilizer technology, and the selection of an economically viable and easily available carrier, capable of maintaining high viable count is an important area of research.