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RESEARCH ARTICLE

PHOSPHATE SOLUBILIZING BACTERIA IN AGRICULTURE BIOTECHNOLOGY: DIVERSITY, MECHANISM AND THEIR ROLE IN PLANT GROWTH AND CROP YIELD.

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Abstract

Phosphorus (P) is an important nutrient second only after nitrogen for plant growth and development. Its adequate amount is present in the soil but non-utilizable by the plants because it is complexed with inorganic metal ions or organic compounds. On an average in different soils its amount varies between 400-1200 mg kg^{-1}. To overcome the P nutrition requirement farmers use chemical fertilizers which are again ineffective due to the precipitation of a larger proportion of soluble P and also it raises the question of environmental consideration. In such situation, phosphate solubilizing bacteria (PSB) can bring out locked P into plant assimilable form in an eco-friendly fashion. PSB solubilize inorganic P primarily with the secretion of organic acids while organic P is mineralized by secreted phosphatases and phytase enzymes. Several PSB have been isolated, characterized and identified. Among these, the most powerful strains were denominated from the genus *Bacillus*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Acinetobacter*, Rhizobium and Serratia. However, the performance of such isolates is dependent on the soil physicochemical properties and climatic factors. Therefore, it is desirable to isolate, identify and characterize the efficient PSB in relation to soil physicochemical and environmental conditions for the development of soil specific bio-inoculants. Now a day's recombinant biotechnology is seen as a solution for any problem, only the major goal is the identification and isolation of a concerned gene. Thus, the best P solubilizing genes/ consortium of genes may be screened and stably incorporated into the genome of indigenous plant growth promoting bacteria which were adapted to a specific soil and climatic characteristic

Introduction:-

Phosphorus (P) is an essential macronutrient for plant growth and development. It accounts 0.2% of plant dry weight, limits the growth of plants and crop yield (Lin et al., 2006; Sharma et al., 2013). It is required for various metabolic processes, for example-synthesis of biomolecules, energy transfer reactions, photosynthesis, cell division, nutrient uptake, signaling and enzyme activity regulation by phosphorylation of serine, histidine, aspartate, threonine, or tyrosine residues (Raghothama, 1999). Due to integral component all the organisms have developed mechanisms for its assimilation from the environment. Plants absorb P as phosphate anions (HPO_4^{--} or $H_2PO_4^-$) from soil (Rodríguez and Fraga, 1999). However, these phosphate anions are highly reactive in the soil and their precipitation is soil pH dependent. In acidic soils phosphate anions get precipitated with free oxides and hydroxides of aluminium and iron, while in alkaline soils calcium is the main element involved in P fixation (Igual et al., 2001). Thus most soils are deficient in P availability worldwide and especially it is the limiting factor for plant growth and crop productivity in the tropical and subtropical regions (Raghothama, 1999; Richardson, 2001; Schneider et al., 2010). To overcome P limitation, farmers over use chemical fertilizers. P fertilizers are expensive and in developing

countries like India, they are either imported or manufactured from imported raw materials. P reserves in the world are also limited and gradually being depleted. In the recent past due to increase in their cost farmers are compelled to cut down the use of P fertilizers and it may lead to decrease in crop productivity (Sundara et al., 2002). In addition to above, approximately 70-90% of available P of chemical fertilizers precipitates soon after their application (Goldstein, 1986; Mikanová and Nováková, 2002; Chen et al., 2008). Therefore, over use of chemical fertilizers is further posing burden on the arable land and causes nutrient imbalance which is the point of environmental consideration (Achal et al., 2007; Sashidhar and Podile, 2010).

P is one of the least available nutrients in the soil, accounts only 0.1% availability to that of its total pool present in the agricultural soils (Illmer and Schinner, 1995). In most soils the concentration of available P in soil solution is several orders of magnitude less compared to plant tissues. In many soils it is present in the range of 1 µM, however, optimum crop production can be achieved at approximately 30 µM (Sashidhar and Podile, 2010). A survey of Indian soils reveals that 98% of these require P fertilization either through chemical or biological fertilizers (Tilak et al., 2005). In spite of the fact that most soils are deficient in plant available P, soils contain a huge amount of total P (Richardson, 2001) which is locked with iron, aluminium and calcium through charge related associations. Total P content in agricultural soils varies between 400-1200 mg kg^{-1} and its cycle in the biosphere can be described as open or sedimentary due to no interchange with the atmosphere (Rodríguez and Fraga, 1999). A considerable amount (approximately 10 kg ha^{-1} $year^{-1}$) of the above mentioned total P has accumulated as a consequence of regular chemical fertilizer use (McLaughlin et al., 1988; Richardson, 2001). Organic forms contribute 30-50% of that total P present in agricultural lands. Organic P in the soil is derived from decaying plant, microorganism and animal materials (Behera et al., 2013). Inositol phosphate alone constitutes 50% of the organic P pool. It is synthesized by microorganisms and plants and is the most stable form of organic P. Other forms of organic P in the soil are present as phosphomonoesters, phosphodiesters and phosphotriesters as a component of phospholipids, nucleic acids, xenobiotic phosphonates, which are used as pesticides and other biochemicals. Most of these organic P compounds are high molecular weight materials, which could not be assimilated. Therefore, they must be degraded before utilization by cells (Rodríguez and Fraga, 1999). The largest reserves of P are rock deposits such as primary apatite and other primary minerals formed during geological age. India alone accounts for the rock deposits of approximately 40-260 million tons which may serve the cheapest source of P fertilization (Roychoudhary and Kaushik, 1989; FAI, 2002). Rocks can be dissolved by the acids produced as metabolic byproducts of the microorganisms and the process could be beneficial to plants and microbes both. Biological weathering by microorganisms and the roots of plants play an important role in the supply of locked nutrients for plant growth and productivity (Chang and Li, 1998). The use of rock phosphate in association with microorganism has become a valid alternative to expensive chemical fertilizers (Sahu and Jana, 2000). Seed or soil inoculation with phosphate solubilizing bacteria (PSB) is known to mobilize the precipitated P resulting in higher crop yield. Thus, rock phosphate in combination with PSB will serve a cheap source for P fertilizer production in *in-vitro* and also PSB inoculation in soil could improve the availability of poorly soluble or insoluble inorganic and organic forms of P. PSB occur in soil but their numbers are not high enough to compete with bacteria commonly established in the rhizosphere. Therefore, crop yield improvement requires inoculation of target microorganism at much higher concentration than those found in the soil. The diversity and richness of established PSB varies from soil to soil depending upon the physicochemical properties. P solubilizing ability of PSB also varies from organism to organism and even in different strains of the same species. Thus, their biodiversity assessment along with soil physicochemical variables and screening of better strains is necessary for developing a soil specific PSB biofertilizer. Finally, the isolated PSB must be evaluated for their crop yield enhancement potential. Therefore, this review paper presents a brief account of PSB isolation and characterization, P solubilization mechanism, biodiversity assessment and their role in crop growth enhancement.

Isolation and characterization of PSB:-

PSB are present in every ecological niche. However, their numbers are substantially high in the rhizospheric soil, rhizoplane, phyllosphere and phosphate rock deposit areas. Thus potential PSB can be isolated from rhizospheric and insoluble P rich soils/ rock deposits. First time PSB isolation was reported on Pikovskaya's agar medium (Pikovskaya, 1948). Pikovskaya's agar medium contains tricalcium phosphate as a source of insoluble P and uses selective pressure for isolation of PSB. In the presence of tricalcium phosphate media plates are opaque. Thus bacterial isolates solubilizing insoluble P display clear halos around their colonies (Fig. 1). The visual observation and measurement of clear halo diameter allows semi-quantitative evaluation of PSB. However, it is not universally true because some PSB may produce metabolites due to which medium remains opaque eg-oxalic acid secreted by PSB forms insoluble calcium oxalate crystal. Therefore, bromo phenol blue dye based mediums have been devised

exploiting the mechanism of P solubilization through organic acid secretion or proton excretion (Gupta et al., 1994; Mehta et al., 2001). As the pH of the medium declines around colonies, the color of bromophenol blue changes to yellow. Mehta et al. (2001) designed NBRIP-BPB medium and claimed it as one of the efficient method for qualitative screening of PSB. The above discussed methods solely rely on acidification of the medium. However, pH decrease is not always in the same magnitude as P solubilization by microorganism (Gulati et al., 2008; Kumar et al., 2010; Pei-Xiang et al., 2012). In addition, PSB follow different mechanisms of P solubilization (Sharma et al., 2013). Hence these common tests fail to detect the really best solubilizer. Thus, the colonies grown on the above given mediums must be evaluated for quantitative P solubilization.

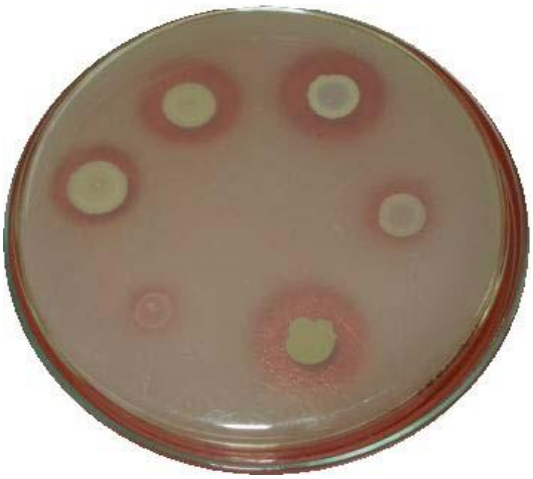

Fig. 1: PSB isolate showing clear halo zone around their colonies on Pikovskaya's agar medium.

Mechanism of P solubilization:-

PSB employ different mechanisms of P solubilization based on the type of insoluble P source. Both inorganic and organic forms of insoluble P are present in the soil. Thus, solubilization mechanisms can be put into two categories i.e. inorganic and organic P solubilization.

Inorganic P solubilization:-

Phosphate rock deposits are about 260 million tons in India and inorganic forms contribute more than 50% insoluble P in the soil. The principal mechanism of inorganic phosphate solubilization is the synthesis and secretion of low molecular weight organic acids (Sperber, 1957; Goldstein, 1995; Buch et al. 2008). The production of these organic acids results in the acidification of microbial cells and its surroundings (Rodríguez and Fraga, 1999; Chen et al., 2006; Lin et al., 2006). Subsequently ionization of the acid takes place and either proton produced becoming responsible for P release from mineral phosphate by proton substitution for Ca, Al and Fe or carboxylic anions chelate cations and release phosphate anions. Further, production of organic acids by PSB has also been well documented (Rodríguez and Fraga, 1999; Hwangbo et al., 2003; Chen et al., 2006; Lin et al., 2006; Park et al., 2009; Kumar and Rai., 2015)**.** Among the various organic acids produced, gluconic and keto-gluconic acids are the principal components for P solubilization. Production of these acids was reported from the strains of *Pseudomonas* (Park et al., 2009), *Enterobacter* (Hwangbo et al., 2003; Kumar et al. 2015) and *Burkholderia* (Lin et al., 2006). Other organic acids produced by PSB are citric, lactic, isovaleric, isobutyric, malonic, oxalic, glycolic, tartaric, pyruvic and succinic. Experimental evidence in support of the role of organic acids in mineral P solubilization was provided by Halder et al. (1990). Organic acids isolated from the culture of *Rhizobium leguminosarum* solubilized nearly equal amount of P as was solubilized by the culture. Furthermore, treatment of the culture filtrates from several rhizobial strains with pepsin or acetone (protein precipitation) had not affected the phosphate solubilizing activity of the filtrates, while addition of sodium hydroxide lost the P solubilization activity. Based on such studies cloning and characterization of the genes involved in organic acid production has been carried out (Rodríguez et al., 2006) and it was concluded that genes directly or indirectly involved in organic acid synthesis or regulation of the expression of organic acid synthesizing genes are responsible for mineral P solubilization (Babu-Khan et al., 1995; Buch et al., 2010). Some researchers believe that the protons extruded to the outer surface with the help of proton translocation ATPase play an important role in P mineralization as well as there is an exchange of protons for cation uptake (Illmer and Schinner, 1995). Likewise, Lin et al. (2006) also demonstrated *in-vitro* that the protons of the

organic acids are involved in P solubilization. However, effective buffer systems are present in the soil. Therefore, carboxylic anions formed by dissociation of organic acids and other high molecular weight organic substances may probably have the highest efficiency under soil conditions. The carboxyl and hydroxyl groups of organic acids compete with cations (Ca, Al and Fe), thus make chelate complexes with metal ions and convert the insoluble P into soluble (Kpomblekou-A and Tabatabai, 1994). The organic acids secreted in the medium can be measured by using high performance liquid chromatography apparatus (Park et al., 2009; Kumar and Rai, 2015). Additionally, inorganic P solubilization occurs as a consequence of nitrogen assimilation (nitrate formation), carbon dioxide evolution and sulphur oxidation. These processes led to the production of nitric, carbonic and sulphuric acid (Sperber, 1958). Such acids were secreted by *Nitrosomonas* and *Thiobacillus* for P mobilization (Sharma et al., 2013). However, acidification could not be presumed the sole mechanism of inorganic P solubilization because the extent of soluble P released and pH drop are not always correlated (Gulati et al., 2008; Pei-Xiang et al., 2012). *Trichoderma harzianum* Rifai has not produced any known organic acid but solubilized P by chelating and reducing molecules (Altomare et al., 1999). Additionally, siderophores and exopolysaccharides synthesized by PSB bring out locked P into soluble form probably by charge related interactions (Yi et al., 2008; Sharma et al., 2013). Although, their role and mechanism need to be understand. Thus, the organic acids as well as chelating and reducing molecules produced by PSB are responsible for inorganic P solubilization. As can be seen from the above account, majority of the studies are relying on the organic acid concept of P solubilization and these organic acids were utilized as an alternate source of energy by PSB resulting in the increased biomass yield (Buch et al., 2010; Kumar and Rai, 2015).

Organic P solubilization:-

Solubilization of organic P is also known as mineralization of organic P, which occurs on the expense of plant, animal and microbial residues. Mineralization of organic P is carried out with the help of different enzymes. In this process mainly three categories of enzymes are involved i.e. phosphatases, phytases and phosphonatases. Phosphatases hydrolyze organic phospho-ester and phosphoanyhydride bonds, and classified as acid (pH < 6) or alkaline (pH > 7) phosphatase depending on their pH optima (Behera et al., 2013). Their dominance is determined by soil pH, in acidic soils acid phosphatases play major role while in neutral to alkaline soils alkaline phosphates are prevalent (Rodríguez and Fraga, 1999; Sharma et al., 2013). Several genes encoding for acid and alkaline phosphatases with broad substrate specificity have been cloned and characterized (Rosollini et al., 1998; Li et al., 2007; Nilgiriwala et al., 2008). Interest in these enzymes has increased from last decade due to their suggested biotechnological potential (Rodríguez et al., 2006). A newer approach is the integration of best screened gene into the plant growth promoting bacterial chromosome to obtain a promising PSB strain without the risk of horizontal gene transfer (Behera et al., 2013).

Phytate is the major source of inositol phosphate and accounts more than 50% organic P form in the soil (Rodríguez et al., 2006). It is synthesized by microorganisms, plant seeds and pollen grains (Rodríguez and Fraga, 1999). Phytases release utilizable P from inositol phosphate component of phytate. Initially phytases were used to improve animal nutrition; however, the current approach may be the use of phytase secreting PSB to improve the plant growth and development (Richardson and Simpson, 2011). The above approach is based on the concept that *Arabidopsis* plants genetically engineered with phytase gene from *Aspergillus niger* were competent to acquire P from phytate. The growth and P content of the plant was comparable to those plants supplied with soluble P.

Phosphonatases and C-P lyases release P by hydrolyzing C-P bond of oraganophosphonates (Rodríguez et al., 2006). However, they would not be major contributors in soil solution P due to scarce availability of their substrates.

PSB biodiversity:-

Numerous PSB have been isolated and characterized from diverse habitats to solubilize tricalcium phosphate, dicalcium phosphate, hydroxyapatite, rock phosphate and organic phosphates (Goldstein, 1986; Illmer and Schinner, 1995; Kim et al., 1998; Mikanová and Nováková, 2002; Chung et al., 2005; Chen et al., 2006; Pérez et al., 2007; Delvasto et al., 2008; Castagno et al., 2011; Azziz et al., 2012; Pei-Xiang et al., 2012; Acevedo et al., 2014; Taktek et al., 2105). These PSB include strains of *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Arthrobacter*, *Serratia*, *Pantoea*, *Bacillus*, *Achromobacter*, *Burkholderia*, *Rhizobium*, *Bradirhizobium*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Ervinia*, *Ralstonia*, *Brevibacterium*, *Sarcina*, *Staphylococcus*, *Stenotrophomonas*, *Xanthomonas*, *Rhodococcus*, *Delftia*, *Chryseobacterium*, *Phyllobacterium*, *Microbacterium*, *Rhizobium*, *Lecleria*, *Klebsiella*, *Cedecea*, *Raoultella*, *Sphingomonas*, *Streptomyces* and *Gordonia*. Among these bacteria *Enterobacter*, *Pantoea*, *Bacillus*, *Pseudomonas*, *Serratia* and *Acinetobacter* strains are most effective P solubilizers and also their population is much higher in different soils. Occurrence and isolation of PSB from different soil types of Indian

continent were also reported. Specifically these were isolated from alkaline soils (Johri et al., 1999; Nautiyal et al., 2000), seawater and marine sediments (Ayyakkannu and Chadramohan, 1971; De Souza et al., 2000), composts and macrofauna (Hameeda et al., 2006), cold Himalyan region (Katiyar and Goel, 2003; Gulati et al., 2008), rice and banana rhizosphere (Kumar et al., 2010; Naik et al., 2008), Most of the isolates from the above different environments were identified as *Pseudomonas*, *Bacillus*, *Serratia*, *Aerococcus* and *Enterobacter*.

PSB diversity studies rely on traditional culture techniques due to the lack of any culture independent technique. Thus, much of the PSB diversity remains unexplored. However, the isolated PSB can be sorted at the genus, species and strain level by using staircase electrophoresis of low molecular weight RNA molecules (5.8S rRNA, 5S rRNA and tRNA), two primers RAPD fingerprinting (TP-RAPD), REP-PCR (Repetitive Extragenic Palindromic elements), ERIC-PCR (Enterobacterial Repetitive Intergenic consensus) and BOX-PCR (Igual et al., 2001). TP-RAPD fingerprinting is an appropriate technique for grouping PSB at genus and species level (Rivas et al., 2001). The unique TP-RAPD profiles represent distinct operational taxonomic units and can be used to compute Shannon diversity and evenness indexes (Azziz et al., 2012). The representative strains of unique TP-RAPD profiles were selected and identified by 16S rRNA gene sequence comparison.

PSB in plant growth promotion and crop yield:-

PSB enhance plant growth and crop production by direct and indirect mechanism. The direct growth promotion can be due to P solubilization, N-fixation, phytohormone production, and thus making the availability of nutrients. Indirect growth promotion involves decrease or prevention of deleterious effects of pathogenic microorganisms by synthesis of antiobiotics or siderophores (Rodríguez and Fraga, 1999; Sharma et al., 2103). The biofertilizer ability of different PSB was evaluated to enhance crop productivity in the different regions of the world. Growth and crop production of mung bean was increased with the inoculation of *Bacillus circulans* and cold tolerant mutants of *Pseudomonas fluorescens* (Singh and Kapoor, 1998; Katiyar and Goel, 2003), common bean with *Burkholderia cepacia* (Peix et al., 2001), green gram, Indian mustard and canola with *Bacillus* (de Freitas et al., 1997; Zaidi and Khan, 2006; Zaidi et al., 2006), cabbage with *Pseudomonas* (Poonguzhali et al., 2008), potato with *Pseudomonas*, *Stenotrophomonas*, *Arthrobacter*, *Microbacterium* and *Pantoea* (Bharadwaj et al., 2008; Malboobi et al., 2009; Babu et al., 2015), alfalfa and soybean legumes (Li et al., 2008; Rosas et al., 2006; Wasule et al., 2007), pepper and cucumber with the mixture of PSB and potassium solubilizing strains (Han et al., 2006). Enhanced production of peanut (Dey et al., 2004), chickpea (Zaidi et al., 2003), radish (Antoun et al., 1998), maize (Hameeda et al., 2008; Kaur and Reddy, 2014), rice (Vasudevan et al., 2002; da Costa et al., 2015), tomato (Ghosh et al., 2015) and sugarcane (Sundara et al., 2002) has also been demonstrated with the use of different PSB as biofertilizer.

Conclusion:-

Although, adequate amount of P is present in the soil, its availability to the plants is limited and thus becomes a limiting factor for crop production. Applied chemical P fertilizers are ineffective and their prices increase at enormous rate. PSB bring out the locked P into plant utilizable form through solubilization and mineralization of inorganic and organic phosphate compounds in an eco-friendly manner. Inorganic P is solubilized with the secretion of low molecular weight organic acids (lowering soil pH), while mineralization of organic P is brought out mainly by acid phosphatases and phytases. Soil physicochemical characteristics and environmental variables severely hamper the establishment and performance of PSB. Therefore, one approach may be to identify and characterize the efficient PSB in relation to physicochemical and environmental conditions for the development of soil specific bio-inoculants. Another approach involves the integration of best screened P solubilizing gene/ consortium of genes into the chromosome of native plant growth promoting bacteria which were adapted to a specific soil and climatic characteristics. In addition to above, development of molecular biology tools for monitoring the activity of microbial population, gene expression and regulation in soil environment is an important task of research.

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