SUMMARY

Phosphorus deficiency is a major problem for plant growth and grain yield. This problem persists in whole world over the century but very little progress has been done towards meeting the demand of food supply required into next 50 years. Multiple factors are responsible for this problem including depletion of natural rock phosphate (RP) ore reserve, pollution generated during the chemical fertilizers production; expensive chemical fertilizers along with its strong refixation ability of free P in the soil limit the use of chemical fertilizers. Disadvantage of chemical fertilizers can be overcome by applying biofertilizers in the field. Various phosphate solubilizing microorganisms (PSMs), including fungi and bacteria, have been isolated from different soil and environmental conditions. In soil, PSBs dominated over PS fungi in numbers which account 1- 50 and 0.5% of total population respectively. PSB isolated from rhizosphere are more metabolically active compared to bulk soil. However, drastic variations were observed in the performance of PSMs under laboratory and field condition as a result of variations in soil, environment and plant rhizosphere. Alternative method to select better PSMs involves isolation of organisms mimicking natural conditions such as buffering of alkaline vertisols, salinity and high or temperatures, nutrient availability and colonization ability. PSMs are integral part of soil and control the soil fertility throughout biogeochemical cycle.

Organic acids secreted by PSMs play a major role in P solubilization which chelate mineral ions and drop pH. Most of the PSMs secrete gluconic acid or 2-ketogluconic acid in the periplasm of Gram negative bacteria *via* direct oxidation pathway. In direct oxidation pathway glucose gets converted into gluconic acid by glucose dehydrogenase (GDH) while gluconate converted into 2-ketogluconic acid by gluconate dehydrogenase (GAD) enzymes. However, in plant rhizosphere where PSBs colonize, glucose might not available in sufficient amount to

solubilize bound P. Root exudates are major carbon and energy sources for rhizobacteria and are known to contain high amount of sucrose and fructose along with small molecular weight organic acids. PSMs ability to use multiple substrates for organic acid production will be more effective in field conditions. Most of the rhizobacteria produce gluconic acid is required in high amount but 2-ketogluconic acid is stronger acid than gluconic acid. Most of the PSMs do not possess GAD required for the conversion of gluconic acid into 2-ketogluconic acid. Rhizobacteria having the ability to use multiple carbon sources along with 2-ketogluconic acid secretion may perform better in field condition.

Organic bound phosphates constitute 50-70% of total bound phosphate of soils. Most plants produce phytase but they are not able to mineralize the bound phytates. Although many rhizobacteria secrete phytase, several factors prevent their application for plant growth promotion. Phytate remain recalcitrant in soil due to precipitation and adsorption on soil particle which are resistant to solubilize by three major classes of phytaes *viz* histidine acid phosphatases (HAPs), purple acid phosphatases (PAPs) or β-propellor phytases (BPPs). Addition of gluconic acid, citric acid and malic acid in surrounding stimulate the phytate mineralization. Hence organic acid secreting rhizobacteria which express phytase could efficiently mineralize the phytate and release P in the surrounding.

Availability of oxygen plays an important role in the colonization and survival of PSMs as oxygen levels modulate the energy metabolism. Oxygen and cognate oxygen radicals can affect bacterial cell either by altering the function or biosynthesis of specific proteins mediated by global transcription regulation. *Vitreoscilla* hemoglobin (VHb) encoded by *vgb* gene gets expressed when dissolved oxygen is less than 2% air saturation. VHb protein known to

significantly increases energy metabolism, protein biosynthesis, metabolite production and prevents from oxidative and nitrasive stress under hypoxic conditions. Hence present study deals with genetic manipulation of *E. asburiae* PSI3 for enhance P nutrition.

E. asburiae PSI3 exhibits MPS ability on Tris buffered rock phosphate minimal medium by secretion of high amount of organic acids in TRP medium by phosphate starvation inducible GDH enzyme on glucose or mixture of sugars. Incorporation of *gad* operon of *Pseudomonas putida* KT 2440 in *E. asburiae* PSI3 resulted in 438U of enzyme activity and secretion of 21.65 mM gluconic and 11.63 mM 2-ketogluconic acid in the medium. Pi release from rock phosphate (RP) in the medium was 0.84 mM in presence of 100mM Tris pH 8.0 and 45mM glucose while wild type could only solubilize RP in presence 75mM glucose. Improvement in the phosphate solubilization was found in Mung bean (*Vigna radiata*) plant inoculation study which showed improvement in plant dry shoot weight, shoot/root dry weight and P content as compared to wild type vector control.

Incorporation of periplasmic invertase (*invB*) of *Zymomonas mobilis* and periplasmic invertase (*Suc*2) of *S. cerevisiae* showed 438 mU and 29mU enzyme activity, respectively. Periplasmic localization of invertase in *E. asburiae* PSI3 resulted in 18.65 mM gluconic acid and 0.180mM P in the medium when 75mM sucrose and 75 mM Tris was used. Expression of *suc*2 showed much better MPS phenotype by producing 22 mM gluconic acid and 0.438 mM P in presence of 75mM Tris and 50 mM sucrose. MPS phenotype was further improved when mixture of 50mM sucrose and 25 mM glucose in 100 mM Tris buffer was used by secretion of 34 mM gluconic acid and resulted in 0.479 mM P release into the medium. Thus, invertase incorporation in *E. asburiae* PSI3 improved MPS ability by producing gluconic acid on sucrose.

Aspergillus fumigatus phyatse (*phyA*) gene and *Citrobacter braakii* phytase (*appA*) gene were incorporated in *E. asburiae* PSI3 for phytase mineralization. Expression of both genes under *tac* promoter resulted in 1.7 and 1.5U of enzyme activity, respectively. In 100mM Tris buffered Ca-phytate medium, *E. asburiae* PSI3 (*phyA*) produced 43 mM gluconic acid and released 3.93 mM P in the medium on 75mM glucose while *E. asburiae* PSI3 (*appA*) produced 47 mM gluconic acid and released 4.46 mM P in the medium.

Incorporation of *vgb* gene in *E. asburiae* PSI3 increased GDH activity (by ~3 fold), growth and MPS ability in TRP medium. *vgb* expression reduced the glucose requirement for MPS ability to 60 mM in 100 mM Tris buffer medium by secretion of 38 mM gluconic acid and released of 0.53 mM P. When *vgb* gene was coexpressed with *appA* no significant increase was found in phytase activity but improved the phytase mineralization. In 100mM Tris buffer and 60 mM glucose, *E. asburiae* PSI3 (*appA, vgb*) secreted 39.7 mm gluconic acid and released 3.1 mM P into the medium. Coexpression of *appA, vgb* and *Uinv* genes further improved phytase mineralization in presence of sucrose and mixture of sugars. In presence of 50 mM sucrose and 75 mM Tris, *E. asburiae* PSI3 (*appA, vgb, Uinv*) produced 21 mM gluconic acid and released 2.8 mM P in the medium. In mixture of sugars, the amount of each of the sugar required for P release was reduced to 10 mM from 15 mM. In presence of 100 mM Tris and 10 mM mixture of each sugar resulted drop in pH up to 4.0 and released 3.3 mM P in the medium.

Overall, genetic manipulation with a set of genes consisting of *appA, vgb, Uinv* and *gad* enabled *E. asburiae* PSI3 to significantly improved qualitative as well as quantitative phosphate solubilization and mineralization leading to much improved P mobilization. These results could

significantly improve the efficacy of *E. asburiae* PSI3 in field conditions. Additionally, similar genetic manipulations could incorporate very good PSM ability in plant growth promoting rhizobacteria (PGPR).