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 (GADH) 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.

Herbaspirillum seropidecae Z67, nitrogen fixing endophyte, significantly promotes the growth of cereals and economically important crops. *Herbaspirillum* does not secrete organic acids; therefore this bacterium was genetically modified for the secretion of organic acids to solubilize mineral phosphate. Organic acids determine the extent of phosphate solubilization and citrate synthase (CS) activity is a key enzyme of tricarboxylic acid cycle. Plasmids pAB7, pJNK3 and pJNK4 containing *E. coli cs*, NADH insensitive *cs* (*cs**) and *cs** along with *Salmonella typhimurium* Na^+ dependent citrate transporter (*citC*) genes were constructed under constitutive *lac* promoter in broad host range plasmid pUCPM18-Km^r. The plasmid transformants of *H. seropidecae* Z67 were obtained by electroporation. *H. seropidecae* Z67 (pAB7) and (pJNK3) transformants increased CS activity but citric acid secretion was not significant. *Hs* (pJNK3) secreted 45 mM acetic acid while *Hs* (pJNK4) secreted 2.7mM and 51 mM citric and acetic acids, respectively. *Hs* (pJNK3) and (pJNK4) transformants, released ~80 µM and ~110 µM amount of P, respectively, in buffered medium in both aerobic and microaerobic conditions. These transformants also showed better growth and colonization parameters. Upon inoculation to rice plants (Gujarat – 17), increase of Fresh weight, Dry weight N, P and K content was observed.

H. seropidecae Z67, an endophytic diazotroph of cereals, encodes glucose dehydrogenase (GDH) apoprotein but does not possess pyrroloquinoline quinone (*pqq*) genes required for its activity. *pqqE* of *Erwinia herbicola* (pJNK1), two different *pqq* gene clusters of *Pseudomonas fluorescens* B16 (pOK53) and *Acinetobacter calcoaceticus* (pSS2) were over expressed in *H. seropidecae* Z67. Transformants (pJNK1, pSS2 and pOK53) secreted 0.011 µM, 1.1 µM and 2.55 µM PQQ, respectively. GDH activity in the *Hs* (pJNK1) was 14.98 U GDH while *Hs* (pSS2) and *Hs* (pOK53) 87.50 U and 86.34 U, respectively, and secreted *Hs* (pJNK1) 4.26 mM, *Hs* (pSS2) 32.00 mM and *Hs* (pOK53)

33.46 mM of gluconic acid. Transformants of *pqqE* (pJNK1) did not show P solubilization phenotype, while *Hs* (pSS2) and *Hs* (pOK53) transformants showed P solubilization phenotype on HEPES agar plate and released 125.47 µM and 168.07 µM P, respectively, in minimal medium containing 50 mM glucose under aerobic condition. *Hs* (pSS2) and *Hs* (pOK53) could also solubilise rock phosphate in minimal medium containing in a mixture of 25 mM glucose and 25mM xylose. Under N free HRP minimal medium, *Hs* (pSS2) and *Hs* (pOK53) released P up to 130.19 µM and 172.04 µM from rock phosphate. Additionally, *H. seropidecae* Z67 *pqq* gene cluster transformants had enhanced growth in nitrogen free medium, biofilm formation, exopolysaccharide (EPS) secretion while IAA production was inhibited. Thus, *pqq* gene cluster but not *pqqE* is necessary for phosphate solubilization and *Hs* transformants can solubilize rock phosphate even under nitrogen fixing conditions.

Pyrroloquinoline quinone (PQQ) and 2-ketogluconic acid determine mineral phosphate solubilization in bacteria. *H. seropedicae* Z67 significantly improves N status of cereals. Two plasmids containing 5.1 kb *pqq* gene cluster of *Acinetobacter calcoaceticus* alone (pJNK5) and with *Pseudomonas putida* KT2440 *gad* operon (pJNK6) were constructed in pUCPM18Gm^r under *Plac* promoter and transformed in *H. seropedicae* Z67. *Hs* (pJNK5) and *Hs* (pJNK6) secreted ~1.15 µM and ~1.17 µM PQQ in medium, had ~221.66 U and ~234.33 U GDH activity, respectively while *Hs* (pJNK6) showed ~414.00 U of GADH activity. *Hs* (pJNK5) and *Hs* (pJNK6) secreted ~23.47mM and ~3.79 mM of gluconic acid, respectively, while *Hs* (pJNK6) ~15.83 mM of 2-ketogluconic acid. Under aerobic condition, *Hs* (pJNK5) and *Hs* (pJNK6) solubilized ~239.66 µM and ~ 457.66 µM P on HEPES rock phosphate and, 76.66 µM and 222.66 µM K HRPF (feldspar), respectively, minimal medium containing 50 mM glucose. Under N free HRP and HRPF minimal medium, similar results of P and K solubilization were obtained. These transformants also showed increase in Fresh weight, Dry weight N, P, K content upon inoculation to rice plants (Gujarat – 17). Rice plants contained ~0.73 ng/g PQQ levels. Thus, these genetic modifications can significantly enhance the efficacy of *H. seropedicae* Z67 in promoting the growth of rice plants in field conditions.

The pOK53 plasmid containing *pqq* gene cluster of *P. fluorescens* B16, pSS2 plasmid containing *pqq* gene cluster of *A. calcoaceticus* and *pqq E* gene of *Erwinia herbicola* were transformed in *H. seropedicae* Z67 by electroporation,. Native *H. seropedicace* Z67 did not show any fluorescence, while *Hs* (pOK53) and *Hs* (pSS2) had showed good fluorescence equivalent to *E. coli* containing pUC18*gfp*. The fluorescence was attributed to ~1.15 μM and ~1.17 μM amount of PQQ secreted by *Hs* transformants (pJNK5) and *Hs* (pJNK6), respectively, in M9 minimal medium.

H. seropedicae Z67 does not produce any antibacterial compounds. However, *H. seropedicae* Z67 harboring *pqq* gene clusters demonstrated antimicrobial activity against pathogenic bacteria and fungi. Antifungal activity was found against *Fusorium oxosporium, Botrytis cinerea, Magnaporthe grisea* and *Rhizoctonia solani.* In contrast to antibacterial activity, *pqqE* has shown zone of inhibition against pathogenic fungi. *H. seropedicae Z67* containing *pqq* gene clusters and extract from the transformants showed antimicrobial activity against *Xanthomonas oryzae, Serratia, Shingella* and *Salmonela typhimurium* while *H. seropedicae* containing *pqqE* has not shown any zone of inhibition against any pathogenic bacteria, intrestingely wild type *Enterobacter asburiae* PSI3 is PQQ producer, inhibited Pathogenic *Xhanthomonas* growth, while mix inoculum of *H. seropedicae Z67* and *E. asburiae* PSI3 showed antibacterial activity.

PQQ is known to confer tolerance to Cd and *Hs* transformants secreting PQQ, *Hs* (pJNK5) and *Hs* (pJNK6), could grow and tolerate $CdCl_2$ up to 100 μM while native strain could tolerate only 40 μM $CdCl_2$. Under hydroponic condition, rice seedling treated with 10 μM Cd showed growth inhibition on root and shoot length by ~70% and ~80%, respectively, when compared with control seedlings while PQQ treated seedlings with 10 μM Cd had root and shoot length of rice similar to that of untreated seedlings. Cd treated rice seedlings exhibited a significant change in the activities of CAT and SOD. CAT activity of control seedlings was 18 U while seedling treated with 10 μM Cd. When PQQ supplemented with 10 μM Cd, SOD activity was significantly reduced by ~2.3 folds as compared to 10 μM Cd treated seedlings.

In conclusion, present study demonstrated incorporation of PQQ and 2-ketogluconic acid secretion into *H. seropedicae* Z67. Native apoprotein of glucose dehydrogenase (GDH) enzyme present in *H. seropedicae* Z67 was functionally active and sufficient for high gluconic acid synthesis. 2-ketogluconic acid was secreted up to 15 mM which was sufficient for solubilizing rock phosphate and feldspar in buffered medium. The PQQ secreted by the transformants enabled to improve Cd tolerance to rice plants by decrease the oxidative damage. Thus, *H. seropedicae* Z67 transformants developed in this study could be useful in helping the rice plants by increasing P, N and K status, conferring Cd tolerance and protect against plant bacterial and fungal pathogens which may significantly enhance the efficacy of this bacteria in field conditions.