**Summary**

Phosphate solubilizing bacteria release P from inorganic complexes by the secretion of wide range of low molecular weight organic acids. P solubilization potential of these bacteria varies based on the amount and nature of organic acid produced. Citric, oxalic, and gluconic acids are the product of central carbon metabolism. *Rhizobium* strains show myriads of catabolic diversity in their carbon metabolism. The amount of the organic acid secretion may differ between members of the same genus and sometimes between strains of the same species. Moreover, organic acid secretion in the rhizosphere depends not only on the metabolic potential of the organism but also on the plant physiology as complex set of interactions mediated many compounds including the root exudates as the major C source. The nature of root exudates varies from plant to plant and composed of a complex mixture of several nutrients in low amount. Therefore, it is difficult to predict the organic acid secretion by rhizobacteria which may also vary with plant physiology. Hence, to develop an efficient P biofertilizer with the potential plant growth promoting properties for multiple host systems and diverse eco habitats, the present study was an effort to understand the following aspects: (i) to develop a potential genetic modification strategy to increase the MPS ability of nitrogen fixing *Rhizobium* ; (ii) to understand *in vitro* metabolic effects of genetic modification on different strains of *Rhizobium* and (iii) to determine the consistency and performance of genetically modified strains in alkaline vertisol soils.

Chapter 1 describes about *Rhizobium* as a PGPR ,nitrogen fixing microorganism and P solubilizer. It also describes about central carbon metabolism and its use for metabolic engineering. Chapter 2 dealt with various materials and methods used in the study.

The distribution of glucose between two catabolic pathways: GDH mediated extracellular direct oxidation pathway and intracellular phosphorylative pathway involving active glucose uptake followed by the action of glucokinase and G-6-PDH are responsible for MPS ability of *Rhizobium*. Higher amount of total glucose utilized and less glucose consumption in *B. japonicum and M. loti* strain compared to the control correlated with enhanced gluconic acid production and MPS ability (**Table 1 and 2**).

Chapter 3 demonstrates the construction of *B. japonicum and M. loti* strain harboring *S. elongatus* PCC 6301 *ppc* gene under *lac* promoter in broad host range plasmid pUCPM18 containing tetracycline resistance. The overexpression of *ppc* gene resulted in increased PPC activity with a simultaneous increase in gluconic acid (~15 mM) and citric acid (~7 mM) secretion as compared to the control and wild type. The MPS ability in terms of P solubilization index in PVK agar medium and red zone acidification on TRP plate was in the order of WT≤pAB4<pAB3 clearly indicating the enhanced effect of *ppc* gene overexpression in terms of citric acid secretion(**Table 3**).

Chapter 4-5-6 demonstrates the construction of *B. japonicum and M. loti* strain harboring *E. coli* NADH insensitive *cs* gene , *E. coli* NADH insensitive *cs* gene and *S. typhimurium* sodium dependent citrate transporter (*yc*) operon under *lac* promoter respectively in broad host range plasmid pUCPM18 containing kanamycin resistance. The overexpression of *cs*, NADH insensitive *cs* and *yc* operon resulted in increased CS activity with a simultaneous increase in citric acid (~7 mM,~8 mM and ~10 mM,respectively) and gluconic acid (~9 mM,~25 mM and ~26 mM, respectively) secretion as compared to the vector control. The overexpression in terms of citric acid produced was in the order of WT≤pAB8 <pAB7<pJNK3<pJNK4 clearly indicating the enhanced effect of both NADH insensitive citrate synthase and citrate transporter over wild type citrate synthase(**Table 3**).

The MPS ability in terms of P solubilization index in PVK agar medium and red zone acidification on TRP plate was in the order of WT≤pAB8<pAB7< pJNK3<pJNK4 clearly indicating the enhanced effect of both NADH insensitive citrate synthase and citrate transporter over wild type citrate synthase in terms of citric acid secretion.

The main objective of the present study was to design a stable and broad host range expression system for enhancing citric acid secretion which could provide MPS ability in *Rhizobium* strains. Considering the stability of the established genetic modification and to test the efficacy in multiple host system, the 7th Chapter describes the effect of *yc* operon integration into the genome of *B. japonicum, M. loti* and *S. fredii*

strain. All these strains are known for nitrogen fixation and other PGPR activities. *B. japonicum, M. loti* and *S. fredii* strains are reported to possess poor P solubilizing ability. A systematic study was carried out to determine the effects of genomic integration in TRP broth medium and compare with the wild type and plasmid transformants of *yc* operon. MPS phenotype and Pi release of the genomic integrants of all strains decreased than the plasmid bearing strains (**Table 2**).

Chapter 8 describes the effect of genomic integration on growth promotion ability of mung bean plants in alkaline vertisol soil without supplementation of rock phosphate in green house conditions. Genome integrant of *S. fredii* NGR234 released 0.56 mM P released 0.56 mM P from TRP medium as compared to 0.05 mM P of native strain. Inoculation of *S. fredii* NGR 234 genome integrant to mung bean plants increased the N and P levels in soil. Mung bean plants inoculated with genome integrant increased nodule number, bigger nodules, bacteroids number and nitrogenase activity of nodules. These plants had more biomass and pods coupled with bigger pods. All parameters showed a consistent increase in growth compared to control. Improvement in plant growth appears to be due not only to increased availability of P but also to decreased oxidative stress.

In conclusion, the present study illustrates a novel genetic engineering approach of enhancing citric acid secretion and MPS ability by genomic integration of NADH insensitive *E. coli cs* gene along with *S. typhimurium* sodium dependent citrate transporter operon constructed under *lac* promoter in *B. japonicum, M. loti* and *S. fredii* strains. Genomic integration appears to be a better strategy than plasmid based expression which creates a milestone for getting stable expression system in metabolic engineering studies. Further, genomic integration of yc operon led to enhanced plant growth promotion under P limitation and without supplementation of external rock phosphate by *Rhizobium* spp. Among *B. japonicum, M. loti* and *S. fredii* strains tested, the overall performance of *S. fredii* genomic integrants was found to be the better in terms of MPS ability in laboratory condition and so was further used for growth promotion of mung bean in green house.

Future prospective will be to test the efficacy of these strains in field condition with multiple host plant under different environmental condition to determine their consistency of P supplementation and other plant growth promotion abilities in agricultural field conditions.

**Table 1: Comparative growth and physiological effects of overexpression of *ppc*, cs, NADH insensitive *cs*, NADH insensitive *cs* along with *citC* transporter genes in *B. japonicum* USDA110, *M.loti* MAFF030669 and *S.fredii* NGR 234**

| *Rhizobium* Strain | Specific Growth Rate (fold) | Glucose Utilized mM | Glucose Consumed mM (fold) | Biomass yield (fold) | Specific glucose Utilization rate (fold) |
|---|---|---|---|---|---|
| *Bj* (pAB4) | 0.26 | - | 36.5 | 1.7 | 0.15 |
| *Bj* (pAB8) | 0.23 | - | 37.1 | 1.6 | 0.17 |
| *Bj* (pAB3) | 0.53 (2.1) | No change | 29.6 (1.2) | 2.2 (1.3) | 0.11 (1.4) |
| *Bj* (pAB7) | 0.33 (1.5) | No change | 36.4 (1.4) | 2.2 (1.4) | 0.12 (1.4) |
| *Bj* (pJNK3) | 0.26 (1.1) | No change | 19.6 (1.9) | 2.5 (1.6) | 0.10 (1.7) |
| *Bj* (pJNK4) | 0.34 (1.5) | No change | 19.6 (1.9) | 2.5 (1.6) | 0.10 (1.7) |
| *Bj* (Native) | 0.18 | - | 38.23 | 1.78 | 0.14 |
| *Bj* ..IYCV | 0.32 (1.7) | No change | 33.1 (1.2) | 2.7 (1.5) | 0.09 (1.5) |
| *Ml* (pAB4) | 0.29 | No change | 36.1 | 1.6 | 0.15 |
| *Ml* (pAB8) | 0.26 | No change | 37.1 | 1.06 | 0.24 |
| *Ml* (pAB3) | 0.48( 1.7) | No change | 31.6 (1.1) | 1.9(1.2) | 0.13 (1.2) |
| *Ml* (pAB7) | 0.33 (1.3) | No change | 36.2(1.1) | 1.08 | 0.23 |
| *Ml* (pJNK3) | 0.38(1.5) | No change | 20.9(1.8) | 1.7 (1.6) | 0.14 (1.7) |
| *Ml* (pJNK4) | 0.32(1.3) | No change | 18.5(2.0) | 2.4 (2.2) | 0.28 (2.2) |
| *Ml* (Native) | 0.221 | - | 37.07 | 1.36 | 0.19 |
| *Ml..IYCV* | 0.38(1.7) | No change | 33.6(1.1) | 2.8 (2.0) | 0.09 (2.1) |
| *Sf* (Native) | 0.26 | No change | 37.2 | 1.85 | 0.15 |
| *Sf*..IYCV | 0.57(2.2) | No change | 26.8(1.4) | 3.9 (2.1) | 0.08(1.9) |

**Table 2 Comparative effects of overexpression of *ppc*, cs, NADH insensitive *cs*, NADH insensitive cs and citC transporter genes on enzyme activities in *B. japonicum* USDA110, *M.loti* MAFF030669 and *S.fredii* NGR 234.**

| | GDH activity* (fold) | PPC activity* (fold) | PYC activity* (fold) | CS activity* (fold) | ICDH Activity* (fold) | G-6-PDH activity* (fold) |
|---|---|---|---|---|---|---|
| *Bj* (pAB4) | 14.86 | 4.98 | 18.39 | 18.72 | 13.17 | 14.77 |
| *Bj* (pAB8) | 12.95 | 5.23 | 18.46 | 18.64 | 13.51 | 18.38 |
| *Bj* (pAB3) | 20.2(1.4) | 45.6(9.1) | 25.6 (1.4) | 19.9 | 24.1(1.8) | 26.5(1.8) |
| *Bj* (pAB7) | 18.9(1.5) | 5.57 | 36.9 (2.0) | 56.7(3.0) | 28.0(2.1) | 18.9 |
| *Bj* (pJNK3) | 19.3(1.5) | 5.7 | 41.8(2.3) | 62.4(3.4) | 26.5(1.9) | 28.2(1.5) |
| *Bj* (pJNK4) | 18.4(1.4) | 7.3(1.4) | 40.3(2.2) | 72.3(3.9) | 25.7(1.9) | 33.5(1.8) |
| *Bj* | 12.54 | 3.29 | 16.29 | 17.85 | 11.96 | 14.89 |
| *Bj* ..IYCV | 24.5(2.0) | 5.6(1.7) | 42.7(2.6) | 47.1(2.6) | 18.0(1.5) | 23.3(1.6) |
| *Ml* (pAB4) | 16.67 | 5.82 | 13.97 | 14.54 | 14.29 | 14.9 |
| *Ml* (pAB8) | 15.8 | 5.2 | 15.43 | 13.89 | 18.29 | 19.31 |
| *Ml* (pAB3) | 23.9(1.4) | 35.6(6.1) | 16.4(1.2) | 17.8 | 16.8(1.2) | 29.9(2) |
| *Ml* (pAB7) | 19.0(1.2) | 6.2(1.2) | 37.0(2.4) | 42.1(3.0) | 22.3(1.2) | 21.4(1.1) |
| *Ml* (pJNK3) | 20.9(1.3) | 6.6 | 49.4(3.2) | 67.0(4.8) | 27.9(1.5) | 26.5(1.4) |
| *Ml* (pJNK4) | 18.5(1.2) | 7.0(1.3) | 50.3(3.3) | 64.6(4.7) | 26.5(1.5) | 23.8(1.2) |
| *Ml* | 14.9 | 4.09 | 15.87 | 12.86 | 13.95 | 14.81 |
| *Ml..IYCV* | 19.5(1.3) | 5.3(1.3) | 40.3(2.5) | 54.8(4.3) | 25.5(1.8) | 21.1(1.4) |
| *Sf* | 14.36 | 5.03 | 17.5 | 16.0 | 17.26 | 16.7 |
| *Sf*..IYCV | 30.3(2.1) | 5.5 (1.1) | 34.3(2.0) | 34.5(2.1) | 27.6(1.6) | 24.6(1.5) |

***All enzyme activities are expressed as nmoles/mg protein**

**Table 3 Comparative effects of overexpression of *ppc*, cs, NADH insensitive *cs*, NADH insensitive cs and citC transporter genes on organic acid production and P solubilization in *B. japonicum* USDA110, *M.loti* MAFF030669 and *S.fredii* NGR 234.**

| *Rhizobium* Strain | Gluconic acid mM (fold) | Citric acid mM (fold) intracellular | Citric acid mM (fold) extracellular | P release mM (fold) |
|---|---|---|---|---|
| *Bj* (pAB4) | 7.3 | 0.8 | 0.76 | 0.078 |
| *Bj* (pAB8) | 6.6 | 0.7 | 0.73 | 0.050 |
| *Bj* (pAB3) | 16.3 (2.2) | 0.8 | 7.1 (9.3) | 0.58(7.4) |
| *Bj* (pAB7) | 9.5 (1.4) | 1.1 | 7.3 (9.9) | 0.37 (7.4) |
| *Bj* (pJNK3) | 26.3 (4.0) | 0.7 | 7.9 (10.8) | 0.65(13.0) |
| *Bj* (pJNK4) | 26.8 (4.10 | 0.8 | 10.3 (14) | 0.73 (14.6) |
| *Bj* | 6.2 | 0.8 | 0.73 | 0.05 |
| *Bj* ..IYCV | 13.0 (2.0) | 0.8 | 6.9 (9.4) | 0.59(12.04) |
| *Ml* (pAB4) | 6.9 | 0.8 | 0.76 | 0.057 |
| *Ml* (pAB8) | 6.7 | 1.0 | 0.76 | 0.043 |
| *Ml* (pAB3) | 14.3 (2.0) | 1.2 | 6.8 (8.9) | 0.45 (7.9) |
| *Ml* (pAB7) | 9.9 (1.5) | 0.9 | 6.0 (7.9) | 0.36 (8.3) |
| *Ml* (pJNK3) | 25.0 (3.7) | 0.8 | 0.6 (4.9) | 0.55 (12.9) |
| *Ml* (pJNK4) | 27.6 (4.1) | 0.8 | 10.8 (14.2) | 0.73 (17.0) |
| *Ml* | 6.53 | 0.8 | 0.73 | 0.058 |
| *Ml..IYCV* | 12.4 (1.9) | 1.1 | 7.0 (9.6) | 0.50 (8.6) |
| *Sf* | 6.76 | 1.5 | 0.76 | 0.05 |
| *Sf*..IYCV | 19.2(2.8) | 1.1 | 6.0 (7.9) | 0.56 (11.2) |