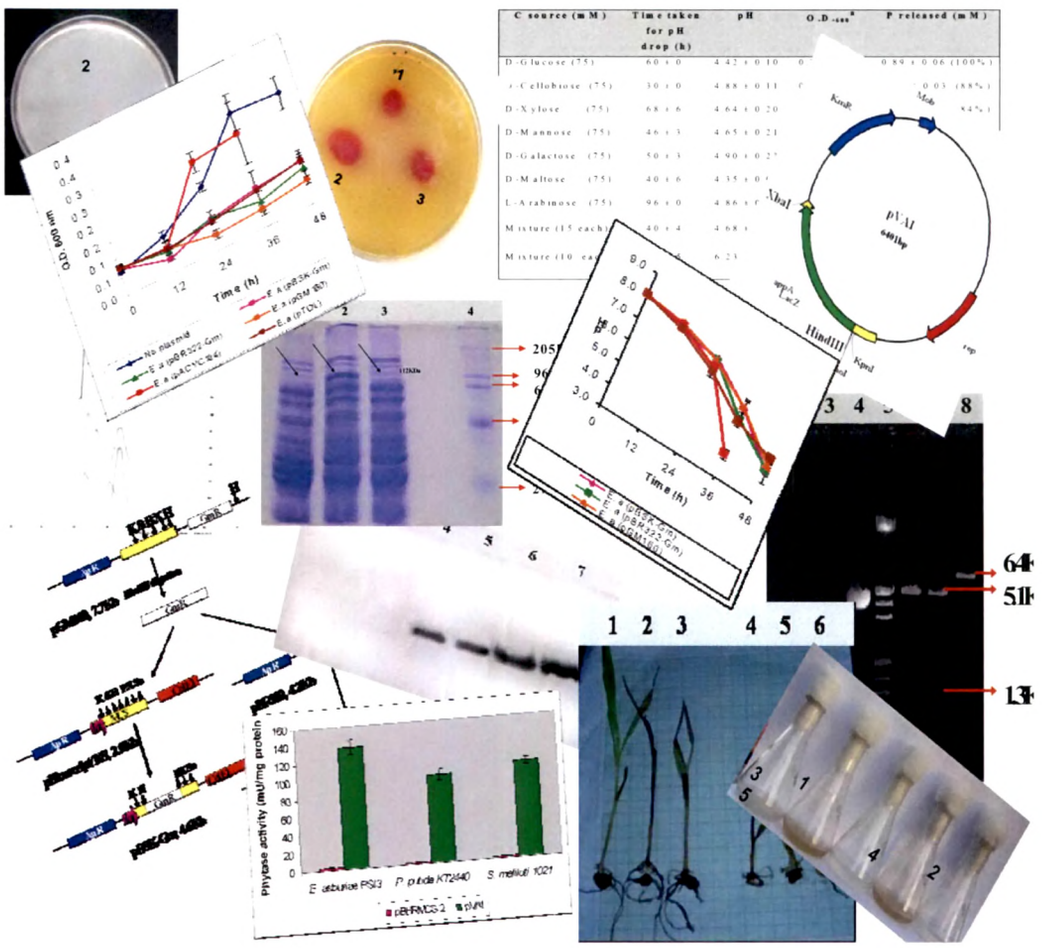
SUMMARY



Phosphate which is abundant in soil due to excessive use of chemical fertilizer remains unavailable to the plants due to insoluble complexes formed with Ca, Al and Fe present in soil. Similar fate happens to organic phosphate which accounts for up to 60% of total P. This excess of chemical fertilizers has reduced the health and fertility of soil. This excess P in soil also causes environmental hazards such as eutrophication, and hypoxia of lakes and marine estuaries. Along with environmental hazards and soil health, economical burden has also led the agronomists to find an alternate for chemical fertilizers which are environmental friendly, economical and can sustain agriculture for long time. Biofertiliser, such as P- solubilising microorganisms (PSMs), was found to be an ideal alternative to chemical fertilizers. The efficacy of these PSMs in soil depends upon abiotic factors such as substrate availability especially ability to use wide range of available substrates which are present in micro molar range, colonization ability, buffering capacity of alkaline vertisols, rapid (re)fixation of soluble P in alfisols and metabolic load caused by plasmids. Biotic factors include competition with other microorganisms, colonization ability etc.

Mechanism and efficacy of PSMs is determined is the ability to secrete organic acids. P release from the insoluble P sources depends upon acidification in case of Ca phosphate and chelation in case of Al and Fe phosphate. Therefore, quantity and quality of organic acid secreted is an important criterion for the efficacy of P solubilisation. Molecular mechanism for P solubilisation by rhizobacteria studied till date is attributed to secretion of gluconic acid by direct oxidation pathway catalysed by glucose dehydrogenase with (pyrroloquinoline quinone) PQQ as cofactor. P solubilisation ability was incorporated by genetic modification of microorganisms either directly by over expressing *pqq* synthase gene or indirectly by screening of genomic DNA library from *Synechocystis* PCC 6803 in *E. coli.*

Chapter 2 focuses on P solubilisation ability of *Enterobacter asburiae* PSI3 a rhizospheric isolate of *Cajanus cajan* on various monosaccharides and disaccharides GDH sugars alone and in mixture. *E. asburiae* PSI3 solubilizes mineral phosphates by the action of a phosphate starvation-inducible GDH (EC 1.1.5.2). The GDH activity of this isolate shows broad substrate range, being able to act on mono and disaccharides. *E asburiae* PSI3 was proficient at bringing about a drop in pH and

solubilization of RP with the use of 75 mM of each of the GDH substrate sugars tested as the sole C source. It liberated amounts of P ranging from 450 µM on arabinose to 890 µM on glucose. When grown on a mixture of 7 GDH substrates at concentrations of 15 mM each, the bacterium solubilized RP equivalent to 46% of the value when 75 mM glucose was the C source. HPLC analysis of the culture supernatant under these conditions showed that the acidification of the media is primarily due to the production of organic acids.

Chapter 3 focuses on metabolic load imparted by plasmids on P solubilisation ability of *E. asburiae* PSI3. Effect of the metabolic load caused by the presence of plasmids on the mineral phosphate solubilizing (MPS) ability of *E. asburiae* PSI3 was monitored with four artificial and one native plasmids, varying in size, nature of the replicon, copy number and antibiotic resistance genes. Presence of pBSK-Gm, pBR322-Gm, pGM160 and pTOL*gfp*mut3b plasmids resulted in diminished inherent MPS phenotype of *E. asburiae* PSI3 on RP under buffered conditions. But, the transformant bearing pACYC184 released P from RP under same conditions. However, when limited (0.1mM) P was supplemented with RP, plasmid load was more pronounced with pGM160 and pTOL*gfp*mut3b plasmids which are supported from specific growth rate, gluconic acid yield and the time for acidification. *E. asburiae* PSI3 harboring pBSK-Gm showed similar acidification time to pBR322-Gm but delayed compared to pACYC184 and also released lower levels of P. The load was diminished when phosphate sufficient condition (10mM) was provided. However, pACYC184 plasmid was found to be unstable in the absence of chloramphenicol selection.

Chapter 4 focuses on the overexpression of *Escherchia coli appA* gene in different rhizobacteria namely *E. asburiae* PSI3, *Pseudomonas putida* and *Sinorhizobium meliloti* 1021. Effect of acidification on phytate utilization by these transformants and growth promotion of maize by *S. meliloti* transformant was investigated. *appA* gene was amplified from *E. coli* and cloned under broad host range plasmid under the control of *lac* promoter which is strong and constitutive in *Pseudomonas* and *Rhizobium* while it is inducible in *E. asburiae* PSI3. AppA protein was detected in the periplasm of all transformants by western blot using rabbit anti-AppA antibody. Transformants had increase in phytase activity by ~ 40-50 fold

compared to control. All the transformants *E. asburiae* PSI3, *P. putida* KT2440 and *S. meliloti* harboring *appA* could drop the pH below 5.0; therefore, release P under unbuffered media containing CaP or NaP as sole P source. Transformants of *E. asburiae* PSI3 could drop the pH below 5.0 buffered media containing CaP or NaP. *P. putida* KT2440 *appA* transformants could not drop the pH and therefore did not release P from CaP containing buffered medium. However *appA* transformants could drop the pH and release P in buffered medium containing NaP. *S. meliloti appA* transformants could neither drop pH of the medium nor release P in buffered medium containing CaP or NaP. *S. m* (pVA1) was able to release P from the NaP under sterile M.S. synthetic medium which promoted maize plant growth.

Chapter 5 focuses on the effect of heterologous overexpression of citrate synthase (*cs*) and phosphoenol pyruvate carboxylase (*ppc*) genes in *E. asburiae* PSI3. *cs* from *E. coli* and *ppc* from *Synechococcus elongatus* PCC 6301 were cloned under *tac* promoter in pTTQ18 vector. Over expression of *cs* in *E. asburiae* PSI3 resulted in ~1.5 and 2.5 fold increase in CS activity on glycerol and fructose containing minimal media. However, citrate was not detected when cs transformants were grown on either fructose or glycerol but specific growth rate was increased ~1.2 fold in case glycerol grown cells whereas there was no change in fructose grown cells. Approximately 2.4 mM (0.14 g L^{-1}) and 2.6 mM (0.15 g L^{-1}) acetate was produced in wild type grown on glycerol and fructose respectively. When grown on fructose, ~5 and ~2 fold increase in acetate levels were found in *E. asburiae* PSI3 bearing control plasmid and plasmid bearing citrate synthase gene. Very low concentration of acetate was detected with cells containing control plasmid grown on glycerol.

Plasmid pVSppc could complement *E. coli ppc* mutant (JWK3928). Over expression of *ppc* increased PPC activity by ~4.6 and ~2.2 fold higher compared to control when grown on fructose and glycerol, respectively. Over expression of *ppc* resulted in decrease in the acetate secretion by ~3.7 fold and increase in the specific growth rate by~1.6 fold on fructose grown cells. On glycerol, there is no change in the specific growth rate, however, specific growth rate was high ~2 fold on fructose compared to *cs* over expressed cells *E. a* (pJE2) which shows ~1.3 fold higher acetate production. Overexpression pf *ppc* resulted in secretion of low concentration 2.3mM (0.27 g L^{-1}) of succinic acid.

Double transformants *E. a* (pVSppc, pJE2) showed ~2.2 fold decrease in acetate level compared to control on fructose grown cells, which was ~1.5 and ~1.1 fold higher compared to single *ppc* and *cs* transformants. Low concentration of acetate (~1mM) was secreted by *E. asburiae* PSI3 bearing control plasmids grown on glycerol similar to single transformants. ~1.8mM (0.21 g L^{-1}) of succinic acid was secreted in double transformants which was ~1.3 fold lower compared to single transformant.

In conclusion, the present study demonstrates the significance of utilisation of variety of carbon sources, nature and amount of organic acid secretion on phytate utilization and metabolic load caused by plasmids on the efficacy of P solubilisation. Genetic modification by heterologous overexpression of genes could help in enhancing the P solubilisation and phytate utilization ability.