Chapter 4

Effect of overexpression of *E. coli cs* gene on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030069

4.1 INTRODUCTION

Legumes are well known in their ability to enter into symbiotic associations with soil bacteria, which are collectively called rhizobia. This interaction leads to the formation of novel structures, the nodules, on the roots and, in certain cases, on the stems. The rhizobia inside the nodules are able to reduce atmospheric dinitrogen to ammonia, which is utilized by the plant for growth and development. The plant, in turn, provides the bacteria with a protective environment and carbon compounds necessary to generate the energy required to reduce atmospheric nitrogen. High concentrations of the dicarboxylic acids, malate and succinate, are found in the nodules (Streeter et al., 1987; Rosendahl et al., 1990; Fougere et al., 1991). These tricarboxylic acid (TCA) cycle intermediate compounds are believed to play an important role in determining the effectiveness of symbiosis.

Citrate synthase (CS) catalyzes the condensation of acetyl coenzyme A and oxaloacetate to produce citrate and is considered the limiting step in the Krebs cycle (Weitzman et al., 1976). Hence, it is the key enzyme governing the carbon flux into the TCA cycle which plays a dual function in the production of cellular energy and biosynthetic precursors under aerobic conditions and only latter under anaerobic conditions, respectively. CS is a non-redundant enzyme indispensable in the carbon metabolism under aerobic as well as anaerobic conditions (Park et al., 1994).

In organisms like *Bacillus subtilis* and *E. coli*, two *cs* genes are localized in the chromosome while in *Rhizobium tropici*, of the two *cs* genes one is chromosomally localized and another is found in the symbiotic plasmid (Patton et al., 1993; Jin and Sonenshein, 1994; Hernández-Lucas et al., 1995). The plasmid-borne gene (*pcsA*) has homology to the citrate synthase genes of proteobacteria, and mutants of *pcsA* show a 30 to 50% decrease in nodule number compared with the wild-type strain (Pardo et al., 1994).

Rhizobia with mutations in the genes encoding TCA cycle enzymes form nodules that are unable to fix nitrogen. *Sinorhizobium meliloti* mutants lacking isocitrate dehydrogenase initiate nodules on alfalfa that are ineffective in nitrogen fixation (McDermott et al., 1992). Similarly, rhizobia with mutations in genes encoding other TCA cycle enzymes, such as succinate dehydrogenase and α-ketoglutarate dehydrogenase, also induce ineffective nodules (Duncan et al., 1979; Gardiol et al., 1982; Walshaw et al., 1997). Mutations affecting bacterial surface components (particularly EPS and LPS), and mutations affecting TCA cycle enzymes and amino acid metabolism have been reported (McDermott and Khan 1992; Walshaw et al., 1997; Fraysse et al., 2003; Krishnan et al., 2003; Dymov et al. 2004). PQQ linked glucose dehydrogenase is required by *S. meliloti* for optimal nodulation efficiency and competitiveness on alfalfa roots (Bernardelli et al., 2008).

Sinorhizobium fredii USDA257 is a fast-growing bacterium that forms nitrogen-fixing nodules on soybeans and other legumes (Keyser et al., 1982; Heronet al., 1984; Pueppke et al., 1999). Most of the nodulation (*nod*) and nitrogen fixation (*nif*) genes of this strain are located on a *sym* plasmid. Even though this strain forms nitrogen-fixing nodules on soybeans, the effectiveness of nitrogen fixation is considerably less than the effectiveness of nitrogen fixation by the classical soybean symbiont, *B. japonicum USDA110* (DeTeau et al., 1986). Inactivation of the citrate synthase gene significantly reduced the ability of *S. fredii* USDA257 to initiate nodules on soybean. In addition, the citrate synthase mutant produced ineffective nodules on soybean, and the nodules had an aberrant ultrastructure. This confirmed that a functional citrate synthase gene is essential for efficient soybean nodulation and nitrogen fixation (Krishnan et al., 2003).

However, despite its key position, no direct correlation has been demonstrated between CS activity and bacterial citric acid accumulation. Genetic modifications leading to citric acid accumulation in bacteria include isocitrate dehydrogenase (ICDH) mutation in *E. coli* (K and B strains) and *Bacillus subtilis* (in early stationary phase) as well as aconitase mutation in *Streptomyces coelicolor* (Lakshmi and Helling, 1976; Matsuno et al., 1999; Viollier et al., 2001; Aoshima et al., 2003; Kabir and Shimizu, 2004).

Most phosphate-solubilizing bacteria (PSB) solubilize mineral phosphates by secreting a variety of organic acids, principally gluconic acid. However, the nature and amount of organic acids limit the efficacy of PSB in soils and in field conditions (Kucey et al., 1989; Gyaneshwar et al., 2002; Srivastava et al., 2006; Khan et al., 2007). Organic acids at concentrations ranging from 10 to 100 mM are required to release phosphate from alkaline soils, citric acid being the most effective (Gyaneshwar et al., 1998; Srivastava et al., 2006). Of the known PSB, several strains of *Bacillus sp.* and *Citrobacter koseri* have been reported to secrete citric acid along with various other organic acids (Gyaneshwar et al., 1998). Apart from mineral phosphate solubilization, citric acid secretion by PSB could also implicated in mediating aluminium tolerance in *P. fluorescens* ATCC 13525 (Mailloux et al., 2008), and as a siderophore in *Bradyrhizobium* and *Pseudomonas aeruginosa* (Guerinot et al., 1990; Carson et al., 1992; Marshall et al., 2009).

4.1.1: Biochemical basis of citric acid accumulation in bacteria

Citric acid is an industrially important metabolic product and hence physiological and biochemical conditions allowing citrate accumulation have been extensively studied and reviewed (Berovic and Legisa, 2007; Legisa and Mattey 2007; Papagianni, 2007). Microbial citric acid production and secretion are distinct yet interdependent processes and its biochemical basis is not very clear. Several mutants of coryneform bacteria like *Corynebacterium*, *Arthrobacter* and *Brevibacterium* produce citric acid from n-paraffin and related substrates (Rohr et al., 1996). Many other bacteria mainly including *Bacillus* sp., *Bradyrhizobium* strain and *Citrobacter koseri* are known to secrete low levels of citric acid but the biochemical basis of its formation is not well understood (Carson et al., 1992; Gyaneshwar et al., 1998; Khan et al., 2006).

4.1.2: Genetic manipulations for citric acid overproduction

Amongst bacteria, *E. coli* K and B isocitrate dehydrogenase (*icd*) mutants accumulated high levels of citrate when grown on glucose with a concomitant increase in CS activity up to more than 2 fold (Lakshmi and Helling, 1976; Aoshima et al., 2003).

Similarly, *B. subtilis icd* mutant in early stationary phase accumulated ~15 fold higher intracellular citrate levels as compared to the wild type (Matsuno et al., 1999). Metabolic studies on citric acid producing fungi, yeasts and *E. coli* demonstrated that high citric acid yields could be attained on glucose and depending on the host metabolism; glucose transport, flux though catabolic pathways and the regulatory mechanisms influenced by intracellular metabolite pools appear to facilitate the citrate accumulation. However, role of CS in citrate accumulation is unclear.

4.1.3: Effects of *cs* gene overexpression in *E. coli* and other microorganisms

Despite the key position of CS, less information is available regarding the effects of *cs* gene manipulations on cellular metabolism and their role in citric acid overproduction. *E. coli* lacking functional *cs* gene failed to utilize glucose unless supplemented with glutamate (or other TCA cycle intermediates) and had reduced growth as compared to the wild type (Gruer et al., 1997; Vandedrinck et al., 2001; De Maeseneire et al., 2006). On the other hand, *cs* gene overexpression or under expression in *E. coli* had no effect on growth on glucose while on acetate as sole carbon source; CS levels strongly affected the growth rate (Walsh and Koshland, 1985a; Vandedrinck et al., 2001). *glt*A gene overexpression in *E. coli* increased the maximum cell dry weight by 23% and reduced acetate secretion (De Maeseneire et al., 2006).

P-solubilization was improved by overexpression of PEP carboxylase (*ppc*) and citrate synthase (*cs*) genes in fluorescent pseudomonads. CS activity was directly correlated with citrate accumulation. Remarkably, only ~2-fold increase in CS activity in *Pf* (pAB7) elevated extracellular and intracellular citric acid levels by about 15- and 2-fold, respectively, which suggested that CS activity was probably limiting for citrate accumulation in *P. fluorescens* ATCC 13525 utilizing glucose (**Fig.4.1, 4.2, 4.3;** Buch et al., 2009).

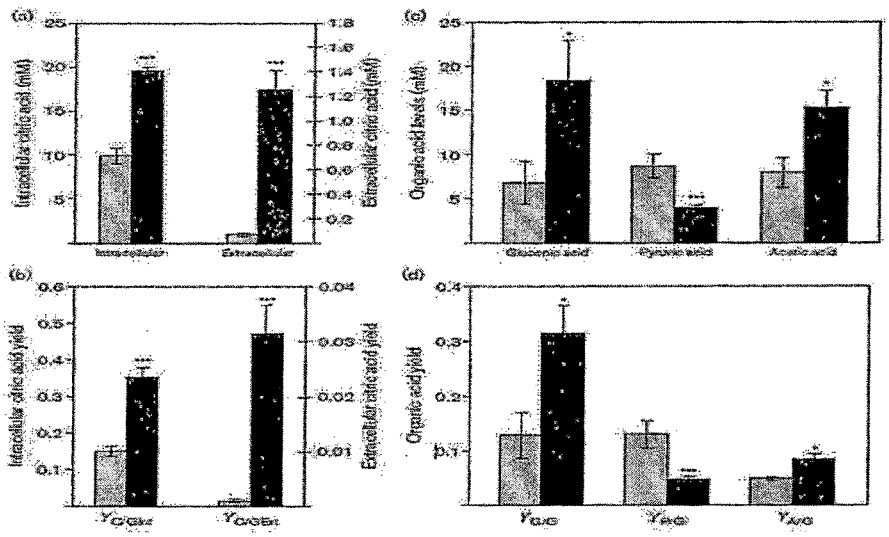


Fig. 4.1: Effect of *E. coli gltA* gene overexpression on organic acid secretion by *P. fluorescens* ATCC 13525. Yields are expressed as g citric or other organic acid (g glucose)$^{-1}$ (g dcw)$^{-1}$ (Buch et al., 2009).

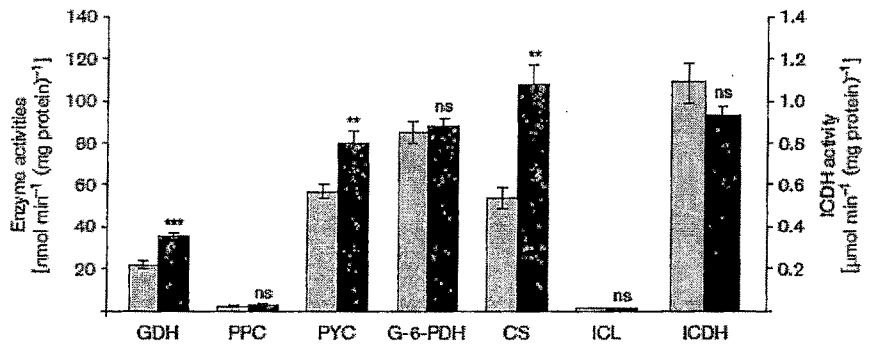


Fig. 4.2: Activities of key carbon utilization enzymes in *P. fluorescens* ATCC 13525 overexpressing *E. coli gltA* gene (Buch et al., 2009).

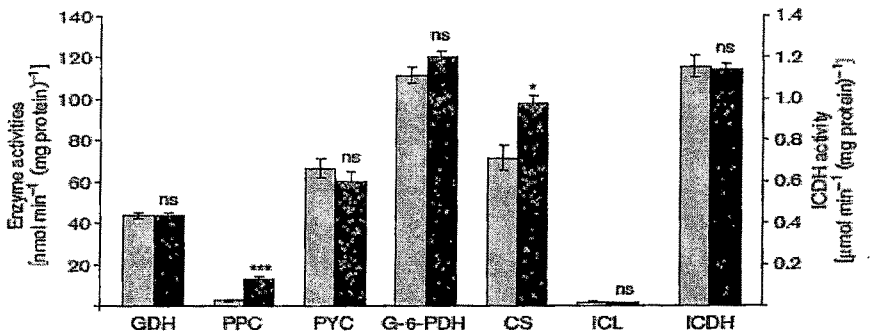


Fig.4.3: Activities of key enzymes of carbon utilization in *P. fluorescens* 13525 co-expressing the *ppc* and *gltA* genes (Buch et al., 2009).

Enhanced phosphate solubilization by *Pf* (pAB7) suggested that increasing CS activity could be an interesting strategy in developing efficient phosphate-solubilizing *P. fluorescens* (Buch et al., 2009).

4.1.4: Rationale for *cs* gene overexpression in *Rhizobium* spp.

Glucose metabolism in *E. coli* and *Bacillus* respectively, occurs via traditional EMP pathway whereas *Rhizobium* utilizes glucose by ED pathway (Keele et al., 1970; Stowers et al., 1984). In addition, glucose flux through TCA cycle in *Rhizobium* is high resulting in lower acetate overflow (Fuhrer et al., 2005). Increase in CS activity was postulated to be a better strategy for citric acid production in *E. coli* rather than isocitrate dehydrogenase (*icd*) mutation which reduces biomass and growth (Aoshima et al., 2003). Overexpression of *E. coli cs* gene in *P. fluorescens* ATCC 13525 yielded millimolar levels of intracellular and extracellular citric acid (Buch et al., 2009). The amount of citric acid produced by *P. fluorescens* overexpressing *E. coli cs* gene was similar to that secreted by the phosphate solubilizing *Bacillus coagulans* and *Citrobacter koseri* on glucose (Gyaneshwar et al., 1998). However, the levels were insufficient for releasing P from soils (Gyaneshwar et al., 1998; Srivastava et al., 2006). With a view to increase the flux through the *anaplerotic node* for increasing oxaloacetate levels, *ppc* gene of

Synechococcus elongatus was over-expressed in fluorescent pseudomonads. *ppc* overexpression enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increased secretion of gluconic, pyruvic and acetic acids while citric acid secretion was not increased. (Buch et al., 2010). When *ppc* and *gltA* genes were overexpressed, the biomass yield was increased but citric acid secretion was not observed (Buch et al., 2009).

To increase the flux through the anaplerotic node for increasing oxaloacetate levels, phosphoenolpyruvate carboxylase (*ppc*) gene of *Synechococcus elongatus* was over-expressed in *Rhizobium* strains. *ppc* gene overexpression enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increased gluconic and citric acids secretion but this high citric acid was due to increased activities of both PYC and PPC. So in this chapter the experiment was designed to increase the citric acid secretion by increasing CS activity. The present work describes the effect of overexpression of NADH sensitive *cs* gene of *E. coli* on glucose metabolism and its role in altering the citrate levels in *B. japonicum* USDA110 and *M. loti* MAFF030669.

4.2 EXPERIMENTAL DESIGN

The experimental plan of work includes the following-

4.2.1: Bacterial strains and plasmids used in this study

Table 4.1: List of bacterial strains used. Detailed characteristics of these strains and plasmids are given in Section 2.1. Parent strains and the transformants of *E. coli* S17.1 and *Rhizobium* were respectively grown at 37°C and 30°C with ampicillin and kanamycin as and when required, at final concentrations varying for rich and minimal media as described in Section 2.2.

Bacterial strains	Characteristics	Source/Reference
	***E. coli* strains**	
E. coli JM101	F′ *traD36 proA+B+ lacIq* Δ*(lacZ) M15/*Δ*(lac-proAB) glnV thi*	Sambrook and Russell, 2001
E. coli S17.1	*thi pro hsdR recA RP4-2* (Tet::Mu) (Km::Tn7); Tmpr	Simon et al., 1983
S 17.1 (pAB7) *E. coli*	S 17.1 with pAB7 plasmid; Amp^r, Km^r	Buch et al, 2008
S 17.1 (pAB8) *E. coli*	S 17.1 with pAB8 plasmid; Amp^r, Km^r	Buch et al, 2008
	***Rhizobium* strains**	
Bradyrhizobium japonicum USDA110	NC_004463.1	NCBI
Mesorhizobium loti MAFF030669	NC_002678.2	NCBI
Bj (pAB7)	*B. japonicum USDA110* with pAB7 plasmid; Ap^r, Km^r (*cs* wild type)	This study
Bj (pAB8)	*B. japonicum USDA110* with pAB8 plasmid; Ap^r, Km^r (control vector)	This study
Ml (pAB7)	*M. loti MAFF030669* with pAB7 plasmid; Ap^r, Km^r (*cs* wild type)	This study
Ml (pAB8)	*M. loti MAFF030669* with pAB8 plasmid; Ap^r, Km^r (control vector)	This study

pUCPM18 plasmid containing kanamycin resistance gene named as pAB8 and pUCPM18 plasmid containing wild type *E. coli* citrate synthase gene, Km^r named as pAB7 were used in this chapter (**Fig. 4.4**; Buch et al., 2008).

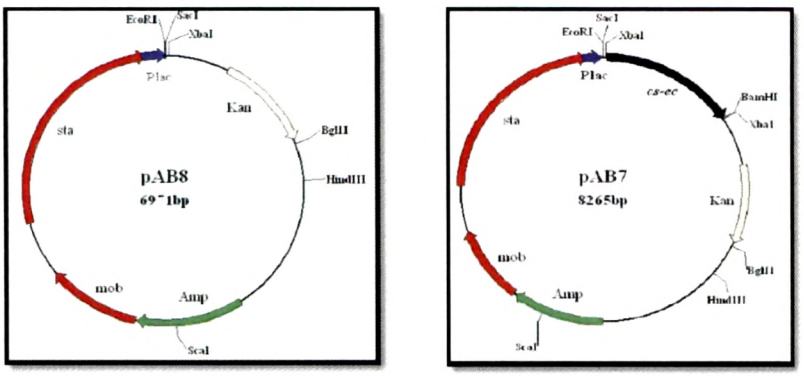


Fig. 4.4: Restriction maps of the plasmids used in this chapter (Buch et al., 2008).

4.2.2: Development of *B. japonicum* USDA110 and *M. loti* MAFF030669 harboring *E. coli cs* gene

The recombinant plasmids pAB7 and pAB8 were transformed in *B. japonicum* USDA110 and *M. loti* MAFF030669 by electroporation (Section 2.4.2). The transformants were selected on kanamycin selection plates and were confirmed by restriction endonuclease digestion. (Section 2.3).

4.2.3: Effect of *E. coli cs* gene expression on the physiology and glucose metabolism of *B. japonicum* USDA110 and *M. loti* MAFF030669.

B. japonicum USDA110 and *M. loti* MAFF030669 *cs* transformants were subjected to physiological experiments involving growth and organic acid production profiles on TRP medium with 50 mM glucose as carbon source. The samples withdrawn at regular interval were analyzed for $O.D._{600}$nm, pH, and extracellular glucose (Section 2.9). Stationary phase culture harvested at the time of pH drop was subjected for organic acid estimation (Section 2.9.3; 2.11). The physiological parameters were calculated as in section 2.9.3. The enzyme assays were performed as described in Section 2.10, with PPC, PYC, G-6-PDH and GDH being assayed in mid-log to late-log phase cultures while CS, ICL and ICDH being assayed in the stationary phase cells.

4.3: RESULTS

4.3.1: Heterologous overexpression of *E. coli cs* gene in *B. japonicum* USDA110 and *M. loti* MAFF030669.

The plasmids incorporated in *B. japonicum* USDA110 and *M. loti* MAFF030669 transformants were isolated from the transformants and were confirmed based on restriction digestion pattern (**Fig. 4.5, 4.6**) before studying the effect of overexpression of *E. coli cs* gene.

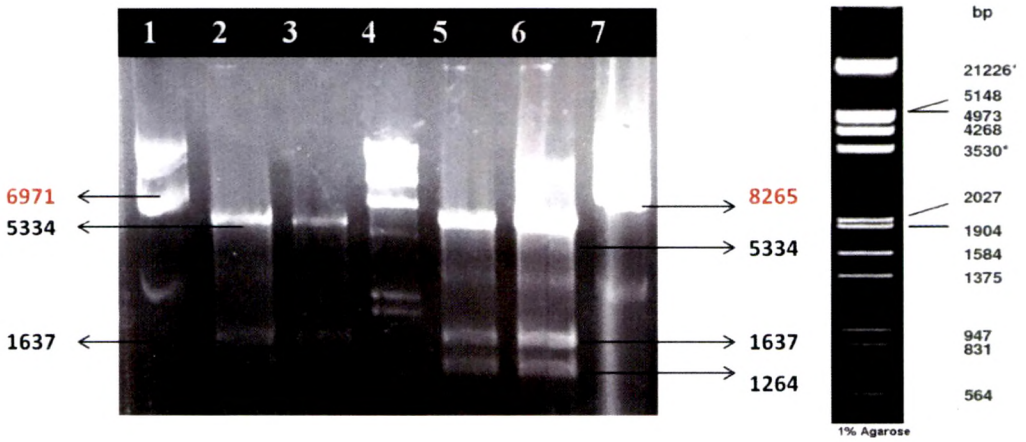


Fig. 4.5: Restriction Digestion pattern of plasmids containing *cs* gene isolated from transformants of *B. japonicum* USDA110 and *M. loti* MAFF030669: Lane 1: pAB8 plasmid Undigested (6971 bp); Lane 2 and 3:pAB8 plasmid digested with XbaI and HindIII (5334 bp and 1637 bp); Lane 4 : MWM-Lambda DNA cut with HindIII ; Lane 5 and 6: pAB7 plasmid digested with XbaI and HindIII (5334 bp, 1637 bp and 1264 bp); Lane 7 : pAB7 plasmid Undigested (8265 bp).

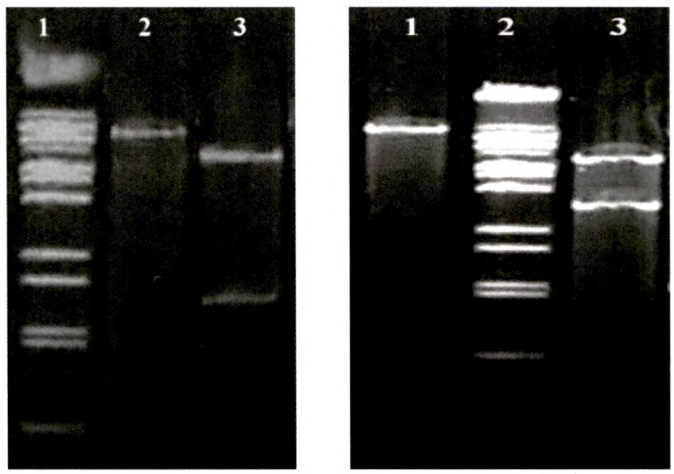


Fig. 4.6: Restriction digestion pattern of plasmids containing *cs* gene isolated from transformants of analysis of *M. loti* MAFF030669: Lane 1: Molecular Weight Marker (MWM)- Lambda DNA cut with BstEII; Lane 2: pAB8 linearised with EcoR I(8.26 kbp); Lane 3: pAB8 digested with EcoRI-HindIII (5,298bp, 1,673bp);Lane 1: pAB7 linearised with EcoR I(8.26 kbp); Lane 2: (MWM)-Lambda DNA cut with BstEII; Lane 3: pAB7 digested with EcoRI-HindIII (5,298bp, 2,967bp).

The citrate synthase activity in *Bj* (pAB7) and *Ml* (pAB7) transformants grown on TRP medium with 50 mM glucose, was ~3 fold higher (56.69 ±.1.55 U and 42.07 ±0.81 U) in both the strains compared to control levels of CS activity (18.64 ± 1.26 U) and (13.89 ± 0.83 U).

To check MPS ability a zone of clearance and acidification was observed on PVK and TRP plates respectively and the maximum zone of clearance and acidification was shown by *Bj* (pAB7) and *Ml* (pAB7) as compared to the control *Bj* (pAB8) and *Ml* (pAB8). P-solubilizing ability of wild type *B. japonicum* USDA110 and *M. loti* MAFF030669 and its transformants varied in the order of *Bj* (pAB7) = *Ml* (pAB7) > *Bj* (pAB8) = *Ml* (pAB8) >*Bj*=*Ml* on PVK medium after 3 days of incubation at 30°C (**Fig. 4.7**).

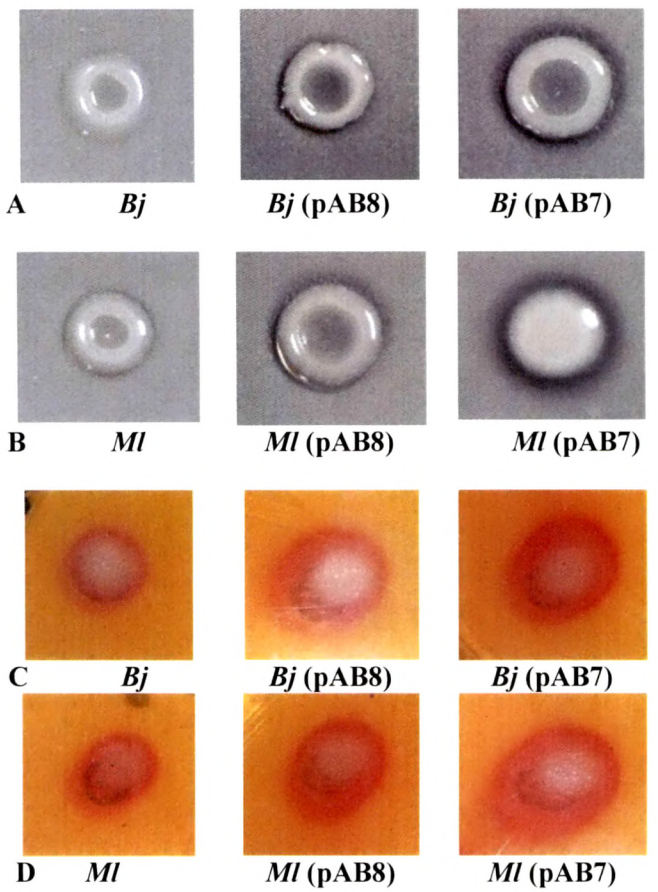


Fig. 4.7: MPS phenotype of (A),(C) *B. japonicum* USDA110 and (B),(D) *M. loti* MAFF030669 harboring pAB7 plasmid expressing *E. coli cs* gene. Zone of clearance formed by the transformants on Pikovskyas agar and TRP agar containing 50mM glucose was monitored noted after an incubation of 3 days at 30 °C.

Table 4.2: P solubilization index on Pikovskyas agar of *B. japonicum USDA110* and *M. loti MAFF030669* transformants during 3 days of growth, *Bj* and *Ml*: wild type strain; *Bj* (pAB8) and *Ml* (pAB8) : *B. japonicum* USDA110 and *M. loti* MAFF030669 with vector control and *Bj* (pAB7) and *Ml* (pAB7) : *B. japonicum* USDA110 and *M. loti* MAFF030669 with *cs* gene. The results were noted after an incubation of 3 days at 30 °C and are given as mean ± S.D. of three independent observations as compared to native *Bj and Ml.*

***Rhizobium* Strains**	**Diameter of zone of clearance (mm)**	**Diameter of colony (mm)**	**Phosphate Solubilizing Index**
Bj	12.17 ± 0.29	11.17 ± 0.29	1.09
***Bj* (pAB8)**	11.17 ± 0.29	9.50 ± 0.50	1.22
***Bj* (pAB7)**	13.05 ± 0.50	10.17 ± 0.29	1.30
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
***Ml* (pAB8)**	12.17 ± 0.29	10.17 ± 0.29	1.22
***Ml* (pAB7)**	12.50 ± 0.50	9.17 ± 0.29	1.33

The pAB7 transformants of *B. japonicum* USDA110 and *M. loti* MAFF030669 showed maximum enhanced zone of clearance as compared to the control pAB8. Phosphate Solubilizing Index was calculated as described in 2. And it was highest in *Bj* (pAB7) and *Ml* (pAB7) **(Table 4.2).**

4.3.2: Effect of *E. coli cs* gene overexpression on growth pattern and pH profile in presence of 50 mM glucose concentrations

The growth profiles and organic acid secretion of *Bj* (pAB7), *Bj* (pAB8), *Ml* (pAB7) and *Ml* (pAB8) along with native, on TRP medium with 50 mM glucose demonstrated that maximum O.D. was reached faster in transformants (12 h) compared to 20 h of {*Bj* (pAB8)} and{*Ml* (pAB8)}. pH decreased ~ 6.8 and 6.6 within 20 h in the

native and control vector, respectively, while pH decreased to 4.40 and 4.15 in {*Bj* (pAB7)} and {*Ml* (pAB7)} within 12 h (**Fig. 4.8).**

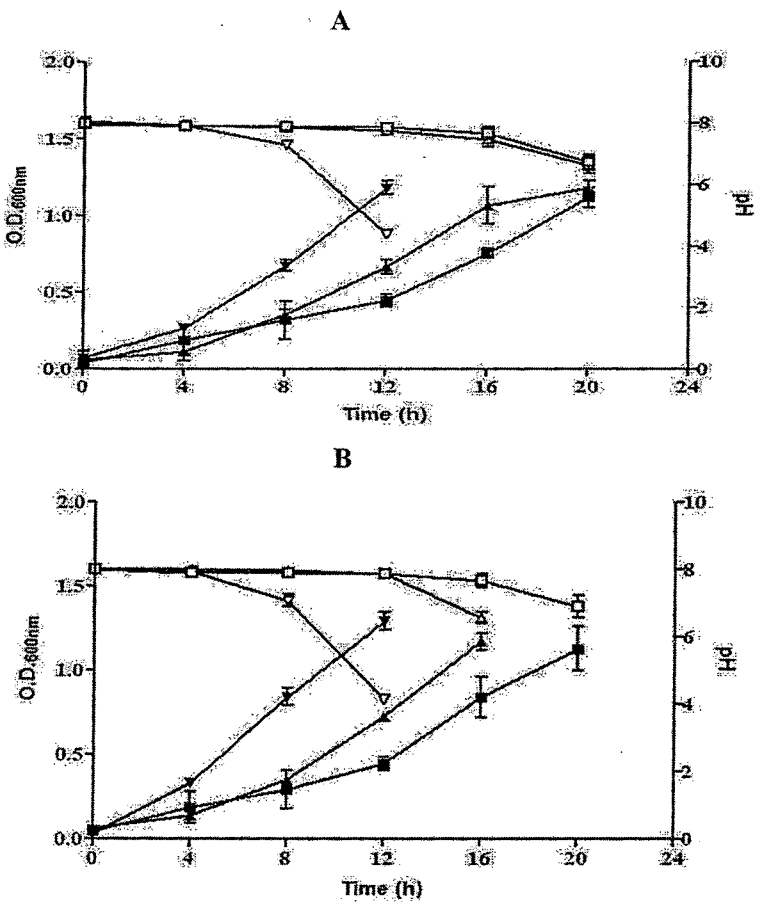


Fig. 4.8: Effect of *cs* gene overexpression on extracellular pH (□, Δ, ▽,) and growth profile (■, ▲, ▼) of (A) *B. japonicum* USDA110 and (B) *M. loti* MAFF030669, with 50 mM glucose and 100 mM Tris-Cl pH 8, 1mg/ml of rock phosphate medium. (□, ■, *Bj*, *Ml* wild type); {Δ, ▲, *Bj* (pAB8), *Ml* (pAB8)}; {▽, ▼, *Bj* (pAB7), *Ml* (pAB7)}. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

4.3.3: Physiological effects of *E. coli cs* overexpression on TRP medium

Specific growth rate was increased by ~1.5 and ~1.3 fold in *Bj* (pAB7) and *Ml* (pAB7) The total glucose utilization rate showed no change and the total amount of glucose consumed showed ~1.4 fold decrease at the time of pH drop. CS activity increased biomass yield by ~1.4 fold and ~1.4 fold decrease was seen in specific glucose utilization rate in the transformant of *B. japonicum* USDA110 (**Table 4.3**). However biomass yield and specific glucose utilization rate remain unaffected in the transformant of *M. loti* MAFF030669.

Table 4.3: Physiological variables and metabolic data from of *B. japonicum* USDA110 and *M. loti* MAFF030669 *cs* transformants grown on TRP medium. The results are expressed as Mean ± S.E.M of 6 independent observations. *a* Biomass yield $Y_{dcw/Glc}$, specific growth rate (k) and specific glucose utilization rate (Q_{Glc}) were determined from mid log phase of each experiment. *b* Total glucose utilized and glucose consumed were determined at the time of pH drop. The difference between total glucose utilized and glucose consumed is as explained in Section 2.9.3. * P<0.05,** P<0.01 and *** P<0.001.

Rhizobium Strains	Specific Growth Rate $k(h^{-1})$ [a]	Total Glucose Utilized (mM) [b]	Glucose Consumed (mM) [b]	Biomass Yield $Y_{dcw/Glc}$ (g/g) [a]	Specific Glucose Utilization Rate Q_{Glc} $(g.g\ dcw^{-1}.h^{-1})$ [a]
Bj	0.186 ±0.03	46.20 ±0.20	38.23 ±1.33	1.78 ±0.14	0.14 ±0.01
Bj (pAB8)	0.229 ±0.02	46.01 ±0.31	37.11 ±0.33	1.57 ±0.29	0.17 ±0.04
Bj (pAB7)	0.332 ±0.04***	48.29 ±0.16	36.36 ±0.51	2.19 ±0.16***	0.12 ±0.01
Ml	0.221 ±0.03	45.91 ±0.64	37.07 ±0.55	1.36 ±0.26	0.19 ±0.04
Ml (pAB8)	0.258 ±0.02	46.01 ±0.51	37.07 ±0.71	1.06 ±0.07	0.24 ±0.02
Ml (pAB7)	0.333 ±0.02***	48.31 ±0.09	36.24 ±1.02	1.08 ±0.11	0.23 ±0.02

4.3.4: Biofilm, exopolysaccharide and indole acetic acid production by in *Bj* (pAB7) and *Ml* (pAB7) transformants in TRP medium.

Biofilm formation showed significant increase by ~1.67 and ~2.04 fold in *Bj* (pAB7) and *Ml* (pAB7) transformants, respectively, compared to control (Table 4.4). Exopolysaccharide production was increased by ~1.74 fold in *Bj* (pAB7) compared to *Bj* (pAB8) while there was no significant increase in *M. loti* MAFF030669 transformants. Indole acetic acid production was increased by ~1.23 fold in *Bj* (pAB7) **(Table 4.4).**

Table 4.4: Biofilm, exopolysaccharide and indole acetic acid production by *Bj* (pAB7) and *Ml* (pAB7) transformants in TRP medium. The results are expressed as Mean ± S.E.M of 6-10 independent observations. * P<0.05, ** P<0.01and *** P<0.001,

***Rhizobium* Strains**	**Biofilm O.D.at 550nm**	**EPS (g/100ml)**	**IAA (µg/ml)**
Bj	1.96 ±0.03	12.48 ±0.24	20.14 ±1.33
***Bj* (pAB8)**	2.08 ±0.03	13.41 ±0.63	25.54 ±0.81
***Bj* (pAB7)**	3.48 ±0.11***	23.33 ±1.58***	31.42 ±0.37**
Ml	1.51 ±0.06	13.55 ±2.78	30.16 ±2.34
***Ml* (pAB8)**	1.54 ±0.06	15.65 ±0.51	26.65 ±2.18
***Ml* (pAB7)**	3.14 ±0.02***	18.14 ±0.47**	31.23 ±1.08**

4.3.5: P Solubilization and Organic acid secretion by *Bj* (pAB7) and *Ml* (pAB7) transformants in TRP medium

Release of P by *Bj* (pAB7) and *Ml* (pAB7) transformants increased by ~7.4 (0.37 mM) and ~8.3 fold (0.36 mM) as compared to 0.05 mM and 0.04 mM by *Bj* (pAB8) and *Ml* (pAB8) transformants in TRP medium containing 50 mM glucose **(Fig. 4.9)**.

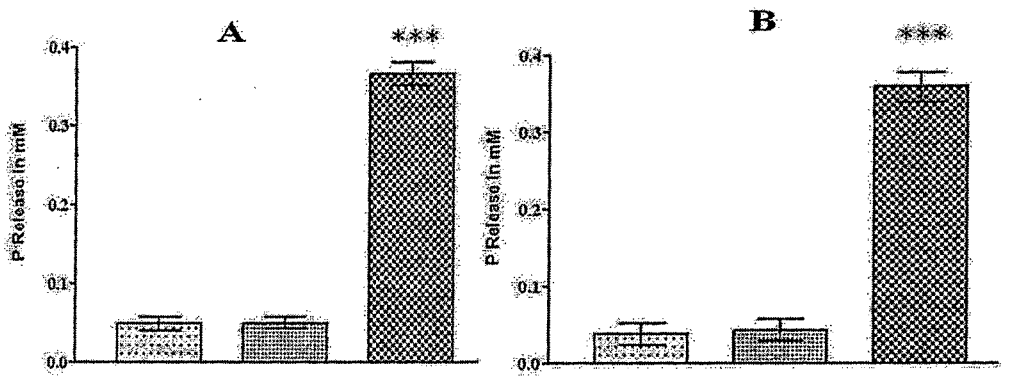


Fig. 4.9: P solubilization by (A) *B. japonicum* USDA110 , (B) *M. loti* MAFF030669 transformants on TRP medium. (*Bj*, *Ml* wild type); { *Bj* (pAB8), *Ml* (pAB8)}; { *Bj* (pAB7), *Ml* (pAB7)}; the values are depicted as Mean ± S.E.M of 7-10 independent observations. *** P<0.001.

Extracellular medium of *Bj* (pAB7) and *Ml* (pAB7) contained ~1.44 and ~1.47 fold higher amount of gluconic acid, respectively, as compared to *Bj* (pAB8) and *Ml* (pAB8). Additionally, ~9.94 and ~7.94 fold increase was found in citric acid secretion with corresponding increase in yield ($Y_{C/G}$) by ~7.36 and ~5.31 fold in *Bj* (pAB7) and *Ml* (pAB7) transformants, respectively (**Fig. 4.10**). Intracellular citric acid levels remained unchanged(**Table 4.5**).

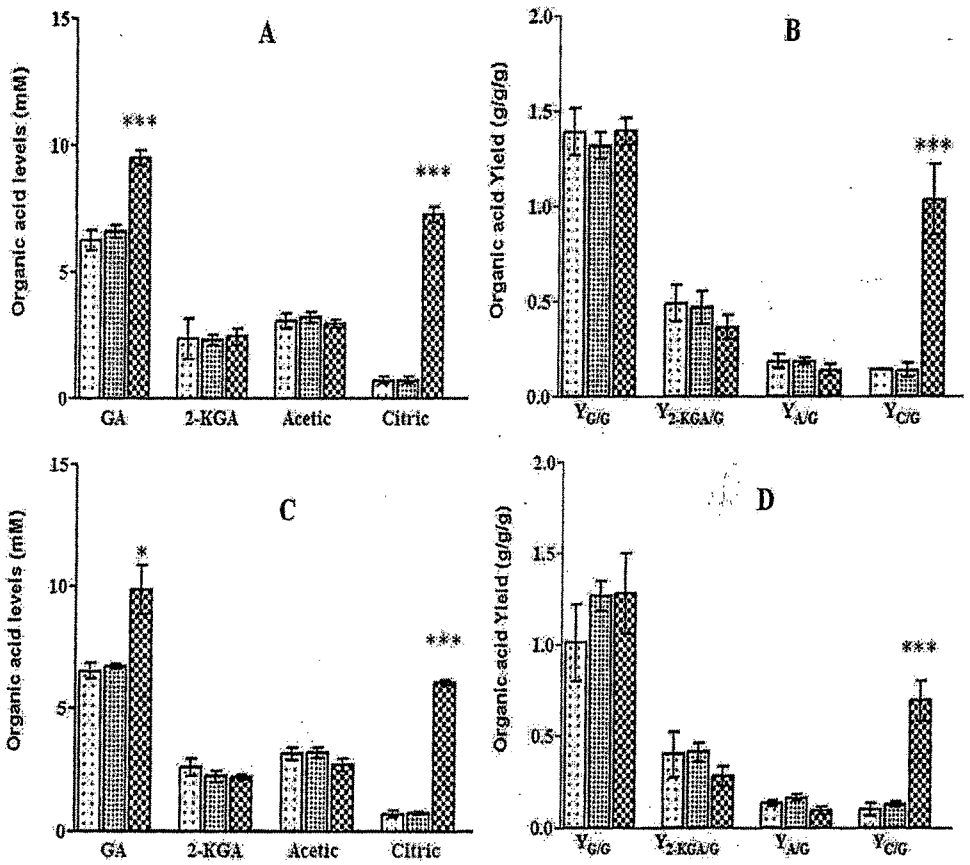


Fig. 4.10 : Organic acid production from *B. japonicum* USDA110 *and M. loti* MAFF030669 *cs* gene (A), (C) organic acids in mM (Gluconic, 2-keto gluconic, acetic and citric acids); (B), (D) Organic acid Yields ($Y_{G/G}$ $Y_{2\text{-}KGA/G}$, $Y_{A/G}$ and $Y_{C/G}$ in *Bj, Bj* (pAB8), *Bj* (pAB7), *Ml* , *Ml* (pAB8) and *Ml* (pAB7). All organic acids are estimated from stationary phase cultures (at the time of pH drop) grown on TRP medium with 50 mM glucose. Results are expressed as Mean ±S.E.M of 4-6 independent observations. * $P<0.05$, and *** $P<0.001$.

Table 4.5: Intracellular citric acid production by *Bj* (pAB7) and *Ml* (pAB7) transformants in TRP medium.

Rhizobium Strains	Intracellular Citric acid in mM	*Rhizobium* Strains	Intracellular Citric acid in mM
***B.japonicum* USDA110**	0.83 ± 0.06	***M. loti* MAFF030669**	0.85 ± 0.04
***Bj* (pAB8)**	0.75 ± 0.05	***Ml* (pAB8)**	1.15 ± 0.06
***Bj* (pAB7)**	1.15 ± 0.06	***Ml* (pAB7)**	0.90 ± 0.04

4.3.6: Alterations in enzyme activities in *Bj* (pAB7) and *Ml* (pAB7) transformants.

In order to correlate the alterations in physiological variables and organic acid profile, enzymes involved periplasmic direct oxidation and intracellular phosphorylative were estimated. GDH activity increased by about ~1.5 and ~1.2 fold as compared to the control PYC showed ~2.0 and ~2.4 fold increase in the transformants. In response to *cs* gene overexpression, about ~ 3 fold increase is seen in CS activity in both *Bj* (pAB7) and *Ml* (pAB7). The activity of G-6-PDH, PPC and ICDH, in *Bj* (pAB7) and *Ml* (pAB7) did not alter significantly as compared to the control. Glyoxylate pathway enzyme ICL, showed slight increase in activity in *Bj* (pAB7) and remain unaltered in *Ml* (pAB7) (**Fig. 4.11**).

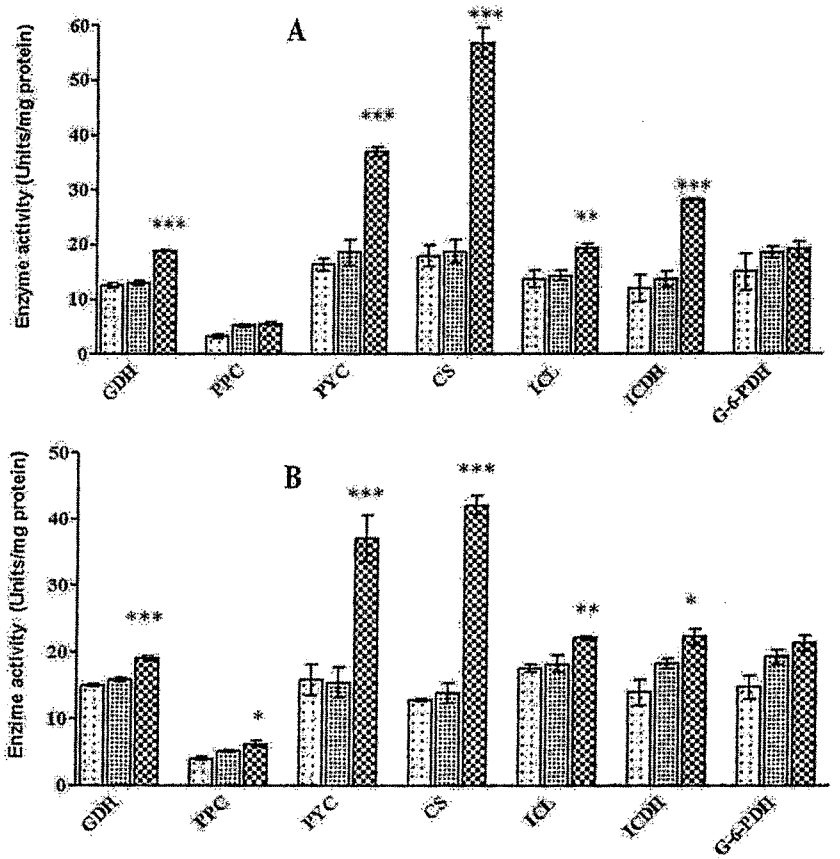


Fig. 4.11: Activities of enzymes. A- *B. japonicum* USDA110; B- *M. loti* MAFF030669. PPC, PYC, GDH, G-6-PDH, ICDH and ICL in and *cs* transformant. (A): *B. japonicum* USDA110 and (B): *M. loti* MAFF030669: The activities have been estimated using cultures grown on TRP medium with 50mM glucose. All the enzyme activities were estimated from mid log phase to late log phase cultures except CS, ICDH and ICL which were estimated in stationary phase (Section 2.10). All the enzyme activities are represented in the units of nmoles/min/mg total protein. The values are depicted as Mean ± S.E.M of 7-10 independent observations. * $P<0.05$, ** $P<0.01$and *** $P<0.001$,

4.4: DISCUSSION

The TCA cycle is used to generate energy and also produce precursors for the biosynthesis of amino acids, purines, pyrimidines and vitamins. The cycle has been intensively studied in *Escherichia coli* and *Bacillus subtilis* and a complex network of genetic and metabolic controls have been elucidated in these organisms. TCA cycle enzyme activities have been measured in bacteroids of many rhizobia and have been tentatively correlated with symbiotic efficiency (Dunn 1998).

Genetic and molecular mechanisms of organic acid secretion by MPS bacteria are confined to involvement of genes responsible for gluconic acid and 2-ketogluconic acid biosynthesis in bacteria like *Enterobacter intermedium*, *Pseudomonas cepacia* and several *Bacillus* spp. The PQQ-GDH and gluconate dehydrogenase (GAD) catalyzes the formation of gluconic and 2-ketogluconic acids and are localized in the periplasm Multiple studies suggest that the periplasmic conversion of glucose to gluconate may be a significant route for carbon flux in glucose-grown *S. meliloti* (Portais et al., 1997; Bernardelli et al., 2001) On the other hand, other commonly secreted organic acids including citric, succinic, oxalic, tartaric, lactic, fumaric, glyoxylic, acetic and malic acids, are metabolites of intracellular catabolic pathways. Yet very few efforts have been made to genetically manipulate the central metabolic pathways for MPS ability in *Pseudomonas* (Buch et al., 2010). The present work describes the effect of heterologous overexpression of *E. coli cs* gene on MPS ability of *B. japonicum* USDA110 and *M. loti* MAFF030669 strains.

In the last chapter, increase in citric acid was seen in response to overexpression of *ppc* gene. On the other hand, the overexpression of *E. coli cs* gene resulted in ~3 fold increase in the CS activity of both the transformants which is in accordance to ~3-fold and ~2-fold increase in *Pf* (pAB7) and *Pf* O1 (pAB7), respectively. This increase in CS activity resulted in secretion of higher citric acid i.e. 7.1 mM and 6.8 mM by *Bj* (pAB7) and *Ml* (pAB7), respectively, which is ~9.9 and ~7.9-f old higher as compared to *Bj* (pAB8) and *Ml* (pAB8), respectively. *Pf* (pAB7) and *Pf* O1 (pAB7) transformants could

only secrete 1.3 mM and 2 mM citric acid (Buch et al., 2009). In addition, *glt* gene overexpression did not increase intracellular citric acid levels in *Bj* (pAB7) and *Ml* (pAB7) but showed ~2-fold increase in *Pf* (pAB7) compared to *Pf* (pAB8). Accumulation of intracellular citric acid in *Pf* (pAB7) was similar to that of *E. coli* K and B *icd* mutants in which citrate accumulation was accompanied by ~3.8 and 2.5 fold increase in CS activity (Aoshima et al., 2003; Kabir and Shimizu, 2004). This suggested higher flux through TCA in *Rhizobium* transformants supported by increased activity of ICDH. Interestingly PYC activity increased with PPC and CS. Thus, the enzymes at the anplerotic node appear to be regulated in a coordinated manner. Similar observation was seen in *Pseudomonas* transformants (Buch et al., 2008; 2009).

Growth of *Bj* (pAB7) and *Ml* (pAB7) transformants showed much better growth performance as they grew in TRP medium containing 100 mM Tris, pH 8.0 and 50 mM glucose while {*Pf* (pAB7)} and *Pf* O1 (pAB7) transformants required 100 mM glucose to grow in this buffered medium (Buch et al., 2009; Adhikary's thesis, 2012). *Bj* (pAB7) transformants showed enhanced biomass yield similar to that in *Pf* O1 (pAB7) while specific growth rate was better in *Rhizobium* transformants compared to *Pseudomonas* transformants. The growth promotion of *Rhizobium* transformants could be attributed to the unique features of central metabolism directed towards anabolism. There was ~2 and ~2.4-fold increase in PYC activity supplying OAA in *Bj* (pAB7) and *Ml* (pAB7), respectively, which was similar to ~2 fold increase seen in *Pf* O-1 (pAB7) and ~1.4-fold increase seen in *Pf* (pAB7). Increase in PYC activity is associated with increase in OAA which resulted in increased biomass in *Bj* (pAB7) and *Pf* O1 (pAB7) due to the increased anaplerotic pathway. While in *Pf* (pAB7) and *Ml* (pAB7) increase in PYC is not seen suggesting no change in anaplerotic pathway.

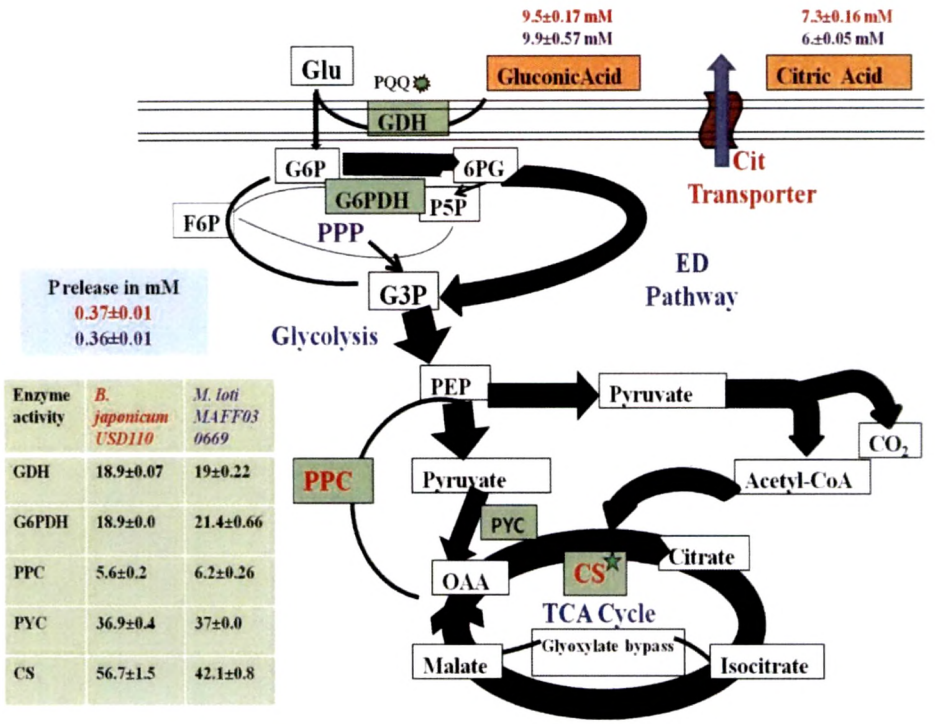
Increase in gluconic acid by *Bj* (pAB7) and *Ml* (pAB7) was ~1.4 and ~1.5 fold, respectively which was comparatively lower than the ~2.7 fold increase in gluconic acid by *Pseudomonas* transformants. This indicates that direct oxidative pathway showed an enhancement in *Pseudomonas* while remains unaltered in *Rhizobium* transformants. However, *Rhizobium* transformants secreted higher amount of citric acid could be

attributed to the increase in CS and PYC activities. As already mentioned, efficient TCA cycle flux in *Rhizobium* strains coupled with good efflux system account for extracellular levels of citric acid higher than in *Pseudomonas*. Increase in GDH activity and no change in G-6-PDH activity suggests increase in oxidative pathway but not in phosphorylative pathway occurs in *Rhizobium* transformants. Similar observations were seen in *Pf* (pAB7) while there was an increase in both the enzyme activities in *Pf* O1 (pAB7), suggesting increase in oxidative pathway as well as in phosphorylative pathway (Buch et al., 2010; Adhikary, 2012).

Reduction in CS activity by mutation in *S. meliloti* decreased growth rate in the free-living conditions (Grzemski et al., 2005). Mutants with approximately 3% of normal CS activity formed nodules with lower nitrogenase activity and a mutant with less than 0.5% of normal CS activity formed Fix^- nodules. Thus, CS activity was essential for nodule maintenance. In *R. tropici* there are two *gltA* genes, one on the chromosome and the other on a symbiotic plasmid. Loss of either gene lowered nodulation ability and loss of both resulted in ineffective, empty nodules (Hernandez-Lucas et al. 1995). Inactivation of an aconitase in *Bradyrhizobium japonicum* decreased enzyme activity by 70% and inhibited free-living growth but leads to nodules with normal fixation (Thony-Meyer and Kunzler 1996). A *B. japonicum* USDA110 mutant lacking 2-oxoglutarate dehydrogenase had some free-living and nodule development problems but the specific activity of bacteroids was near normal, a capability that was associated with a 2-oxoglutarate decarboxylase activity that bypassed 2-oxoglutarate dehydrogenase (Green et al., 2000). In *S. meliloti*, mutants in two TCA cycle genes, isocitrate dehydrogenase (*icd*) and *gltA*, differed in their nodulation phenotypes (McDermott and Kahn 1992; Mortimer et al., 1999). Mutations affecting bacterial surface components (particularly EPS and LPS), and mutations affecting TCA cycle enzymes and amino acid metabolism have been reported (Walshaw et al., 1997; Fraysse et al. 2003; Dymov et al. 2004)The *icd* mutants formed ineffective but normal-looking nodules that had abundant bacteroids within the infected cells, whereas *gltA* mutants formed empty nodules, completely lacking intracellular bacteria. *S. fredii gltA* mutants had a similar ineffective phenotype, although some bacteroids were present (Krishnan et al. 2003).

P solubilization was increased by *B. japonicum* USDA110 and *M. loti* MAFF030669 strains containing pAB7 plasmids due to increase in the production of gluconic acid and citric acid. Similar observation is also reported in *Pf* (pAB7) and *Pf* O-1 (pYF) as compared to their respective controls (Buch et al., 2010; Adhikary, 2012). P solubilization by *Rhizobium* transformants was less compared to overexpression by *ppc* gene due to lesser amount of gluconic acid while citric acid levels were similar.

Both *Rhizobium* transformants had enhanced growth promoting activities such as biofilm formation, exopolysaccharide and indole acetic acid production. Exopolysaccharide I (EPSI) biosynthesis is enhanced by succinate levels which in turn related to the CS activity in *Corynebacterium glutamicum* (**Zhu et al., 2013**). It is not clear whether EPS synthesis in *Rhizobium* transformants is related to CS activity. Increase in biofilm formation and IAA production could be a consequence of improved metabolism in TRP medium.



Enzyme activity	*B. japonicum USD110*	*M. loti MAFF030669*
GDH	18.9±0.07	19±0.22
G6PDH	18.9±0.0	21.4±0.66
PPC	5.6±0.2	6.2±0.26
PYC	36.9±0.4	37±0.0
CS	56.7±1.5	42.1±0.8

Fig. 4.12: Key metabolic fluctuations in *B. japonicum* USDA110 and *M. loti* MAFF030669 overexpressing *E. coli cs* gene.