Chapter 3

Effect of constitutive overexpression of *ppc* gene of *Synechococcus elongatus PCC* 6301 *on production of organic acid in B. japonicum* USDA110 and *M. loti* MAFF0300669

3.1: Introduction

PEP carboxylase (PPC) is the principal enzyme found in *Escherichia coli* for the key reaction in formation of succinate during mixed-acid fermentation of glucose. It catalyses the carboxylation of three-carbon intermediates such as phosphoenolpyruvate (PEP) to four-carbon oxaloacetate. In *E. coli,* PEP may also be converted to pyruvate which during anaerobic growth leads to the formation of lactate, formate, acetate, and ethanol. In other prokaryotes and many eukaryotes during glucose metabolism, oxaloacetate is synthesized by carboxylation of pyruvate by pyruvate carboxylase, an enzyme that is absent in *E. coli* (PYC) (Attwood et al., 1995; Payne et al., 1996; Peters et al., 1997). PEP is also required for glucose consumption *via* the PEP-phosphotransferase system (PEP-PTS) and for the synthesis of aromatic amino acids (Gottschalk et al., 1985; Clark et al., 1989). PEP partitioning is highly regulated by cellular mechanisms because of its central position in glucose metabolism.

Increased succinate production has been shown to result from overexpression of *ppc* gene in *E. coli* and *pyc* gene in *Rhizobium etli* (Millard et al., 1996; Gokarn et al., 1998). Similarly, the expression of gene for malic enzyme in *E. coli* strains lacking the enzymes pyruvate formate lyase (PFL) and lactate dehydrogenase (LDH) yielded succinate as the major fermentation product (Slots et al., 1997). Each of these genetic perturbations directly affects the central metabolic network and therefore impacts the carbon flow through the metabolic branches. Flux analysis methodologies are used to understand succinate production changes in metabolic fluxes. Genetic perturbations affecting the activities of PPC and PYC was done to improve the understanding of anaerobic succinate production in *E. coli*, fermentation patterns suggested that the cell adapted to these genetic alterations by adjusting the flux to lactate, ethanol and acetate (Gokarn et al., 2000).

Thus, one of the highly explored junctions in the *E. coli* carbon metabolism is the phosphoenolpyruvate (PEP)-Pyruvate-Oxaloacetate (OAA) node which is the critical branch point between catabolism and anabolism. The carbon flux distribution is regulated

by dynamic equilibrium between the metabolites at this junction by the activities of the enzymes which are controlled at both transcriptional and allosteric levels. As compared to *E. coli*, the set of enzymes at the node differ in other organisms like *Bacillus*, *Corynebacterium* and *Pseudomonas citronellolis*. Thus, the flux to and from anaplerotic node in these organisms is differentially regulated which is evident from the nature of metabolic responses to a specific genetic manipulation. In *E. coli* phosphoenolpyruvate kinase (PEPCk) mutant, the anaplerotic flux through phosphoenolpyruvate carboxylase (PPC) was reduced while the glyoxylate shunt was activated where as in *C. glutamicum* it drastically increased the production of lysine and glutamate (Yang et al., 2003; Sauer and Eikmanns, 2005).

Overexpression of *pyc* gene in wild type *E. coli* reduced acetate overflow and improved recombinant protein production while in *ppc* mutant *E. coli* and alcohol dehydrogenase (*adh*E)-lactate dehydrogenase (*ldh*A) double mutant *E. coli* resulted in increased succinic acid production (Gokarn et al., 2000; March et al., 2002; Sanchez et al., 2005a). On the contrary, *pyc* overexpression in *C. glutamicum* resulted in growth enhancement or lysine overproduction depending on aspartate kinase activity (Koffas et al., 2002). Similarly, genetic engineering in the form of overexpression of *ppc* gene involved in OAA biosynthesis has been a frequent target for altering the flux at PEP-Pyruvate-OAA node.

3.1.1: Effects of *ppc* gene overexpression in *E. coli* and other organisms.

The main goals in recombinant protein production processes with *E. coli* are high gene expression levels in high cell density cultures, those two goals can seldom be obtained simultaneously. This is caused by the excretion of considerable amounts of acetic acid under the high cell density culture conditions that are useful in industrial processes (De Anda et al., 2006). Koo and Park (1999) observed a 60% increase of recombinant protein production in *E. coli* after elimination of the acetate production. Generally, saturation of the tricarboxylic acid cycle (TCA cycle), and/or the electron

transport chain are considered to be the main causes of this phenomenon (Contiero et al., 2000).

Elevated acetate concentrations are highly detrimental for growth rate and recombinant protein yield (De Anda et al., 2006). Since citrate synthase (CS, *gltA*) and PPC provide a link between glycolysis and TCA cycle, they are considered to be important metabolic control points (Park et al., 1994; Sauer and Eikmanns 2005). Both CS and PPC direct metabolites into the TCA cycle and by diminishing the pool of PEP, pyruvate and acetyl CoA, they prevent these metabolites to participate in the acetate production (**Fig. 3.1**). Moreover, overexpression of *gltA* gene can increase the flux through the TCA cycle in glucose medium. Most of the molecular approaches used to date do not completely eliminate acetate production and have a deleterious effect on growth rate or lead to undesirable accumulation of byproducts (De Anda et al., 2006).

Expression of *gltA* gene is tightly regulated since CS combines the requirement of biomass synthesis and energy production under different culture conditions. CS is an important control point for the metabolic speed of the cell and the rate of this reaction limits the turnover of the TCA cycle under certain conditions (Walsh and Koshland, 1985). This leads to the hypothesis that controlled (over-) expression of the enzyme could decrease acetate accumulation.

PPC plays an anaplerotic role in replenishing oxaloacetate (OAA) and keeping the TCA cycle intermediates from depletion (Peng et al., 2004). By converting PEP to OAA, PPC prevents the accumulation of pyruvate and provides OAA, which is further converted by CS. The accumulation of pyruvate creates, according to Ponce (1999), the highest flux to acetate and thus is the central cause of acetate production. Thus, these findings lead to the assumption that over-expression of PPC can lead to less acetate formation. Lowering the pyruvate pool was also one of the means suggested by others to reduce acetate production (Chao and Liao 1993; Farmer and Liao 1997; Gokarn et al., 2001; Lin et al., 2005).

Maeseneire et al., (2006) demonstrated that over-expression of *ppc* and *gltA* genes in *E. coli* MC1061 can completely eliminate acetate production and simultaneously increase the final cell density of the cultures. Knock-out and over-expression mutants were constructed and characterised at the level of expression, enzyme activity, growth and metabolite production. Over-expression of *ppc* gene clearly had a positive effect on growth to higher cell densities, which is accompanied by the elimination of acetate formation and thus, with a better pH-profile of the cultures. Over-expression of *ppc* and *gltA* genes can lead to a higher flux through the TCA cycle and thus it can eliminate the acetate production by eliminating saturation of this cycle **(Fig. 3.1)**.

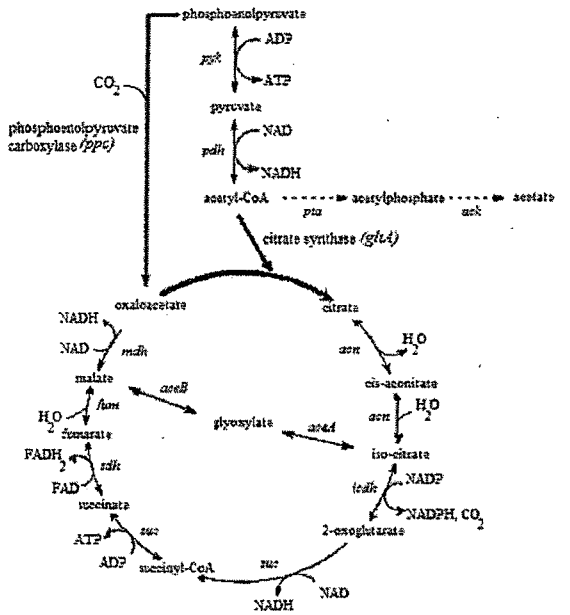


Fig. 3.1: Summary of the effects of overexpression of TCA cycle genes in *E. coli*. Over-expressed enzymes are indicated with bold arrows (De Maeseneire et al., 2006)

Several metabolic engineering approaches have been proposed to understand the metabolic rigidity of the biochemical network at the PEP branch point. Under anaerobic conditions, *ppc* overexpression in *E. coli* altered carbon flux towards fermentation products leading to a significant increase in the yield of succinic acid on glucose which otherwise is a minor product (Millard et al., 1996). Under aerobic condition

overexpression of *ppc* decreased the rates of glucose consumption and organic acid excretion, but the growth and respiration rates remained unchanged; thereby resulting into improved growth yield on glucose (Chao and Liao, 1993). This result indicated that the wild-type level of PPC was not optimal for the most efficient glucose utilization in batch cultures. Under aerobic conditions in presence of excess glucose, *ppc* overexpression in *E. coli* did not affect the growth and the glucose consumption rates but reduced the acetate excretion by 60% (Farmer and Liao, 1997; Abdel-Hamid et al., 2001). Simultaneous overexpression of *ppk* and *pck*, or *pps* alone in the presence of glucose lead to futile cycling, which did not affect the growth rate significantly (Liao et al., 1994).

E. coli ppc gene when expressed in *Synechococcus* PCC 7942 *ppc* mutant showed lower PPC activity with reduced growth, chlorophyll-a content and photosynthetic activity (Luinenburg and Coleman, 1993). Overexpression of *ppc* gene in combination with ornithine carbamoyltransferase and carbamoylphosphate synthetase genes triggered the biosynthesis of cyanophycin in *Acinetobacter sp.* strain ADP1 (Elbahloul and Steinbüchel, 2006). Overexpression of *ppc* gene resulted in lysine overproduction in *C. glutamicum* containing feedback-resistant aspartate kinase while it did not contribute much in glutamate overproduction (Cremer et al., 1991; Shirai et al., 2007).

Synechococcus elongatus PCC 6301 *ppc* gene was constitutively overexpressed in fluorescent pseudomonads, to increase the supply of oxaloacetate, a crucial anabolic precursor and an intermediate in biosynthesis of organic acids implicated in phosphate (P) solubilization (Buch et al., 2010) (**Fig 3.2**). *Pseudomonas fluorescens* ATCC 13525, transformed with pAB3 plasmid containing the *ppc* gene showed a 14-fold increase in PPC activity under P-sufficiency. It also resulted in increased carbon flow through the direct oxidative pathway and reduced metabolic overflow. Under P-limitation, the direct oxidative pathway significantly increased in *P. fluorescens* ATCC 13525; however, *ppc* gene overexpression enhanced glucose catabolism through intracellular phosphorylative pathway. These results showed correlation with gluconic, pyruvic and acetic acid levels as well as the activities of key glucose catabolic enzymes. Irrespective of the P-status,

ppc gene overexpression improved biomass yield without altering growth rate, resulting in improved P- solubilizing abilities of *P. fluorescens* ATCC 13525 and of the wheat rhizosphere fluorescent pseudomonads isolates Fp585, P109 and Fp315. This work presented a feasible genetic engineering approach for developing efficient P-solubilizing bacteria, illustrated in **Fig.3.2.**

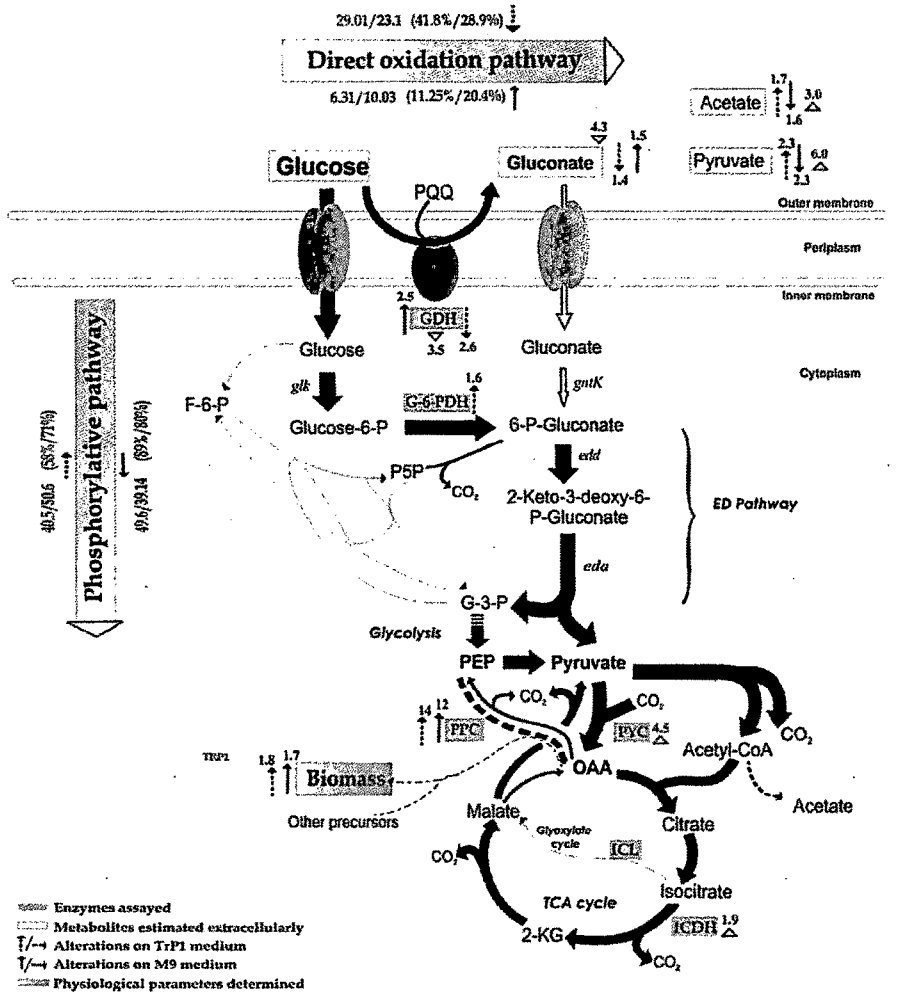


Fig. 3.2: Glucose metabolism of *P. fluorescens* and media dependant alterations due to *S. elongatus* PCC 6301 *ppc* overexpression. Numbers depicted on the arrows represent the fold change in response to ppc overexpression on M9 and TRP1 minimal media. Numbers on the arrow heads (D) depict the fold variations in *Pf* (pAB4) on M9 as

compared toTRP1 medium. The parameters depicted are organic acid yields, enzyme activities and physiological parameters while unchanged parameters are not indicated. The values represented against the arrows depicting two pathways, represent the Mean amount of glucose distributed in phosphorylative and direct oxidation pathways of the initial 100 mM supplemented, in *Pf* (pAB4)/*Pf* (pAB3). The glucose concentration remaining unutilized in the spent medium was obtained by subtracting from 100 mM, the sum of the values of direct oxidation pathway and phosphorylative pathway. The adjacent % values depict the percentage contribution of each pathway in *Pf* (pAB4)/*Pf* pAB3) on the two media (Buch et al., 2010).

3.1.2.: Why heterologous *ppc* gene?

PPC, being one of the key enzymes at the critical anaplerotic as discussed earlier, is highly regulated under physiological conditions. Majority of the PPC enzymes of non-photosynthetic bacteria including *E. coli* and *P. citronellolis* belong to class I which get allosterically activated by acetyl-CoA and inhibited by L-aspartate (Newaz and Hersh, 1975; O'Brien *et al.*, 1977). In *E. coli*, additionally PPC is activated by fructose 1,6-bisphosphate, GTP and long chain fatty acids while is inhibited by L-malate (Morikawa et al., 1980). On the contrary as rare case, PPC in *Pseudomonas* AM-1 and *Pseudomonas* MA grown on methylamine as sole carbon source belonged to Class III as they were independent of acetyl-CoA and aspartate mediated allosteric regulations (Large et al., 1962; Newaz and Hersh, 1975). PPC in *Pseudomonas* MA was also activated by NADH and inhibited by ADP (Millay et al., 1978).

In order to avoid such allosteric regulations exerted at the anaplerotic node by the host metabolism, *ppc* gene from a heterologous host *Synechococcus elongatus* PCC 6301 (*Anacystis nidulans*, cyanobacteria) was selected for the present study. This PPC is known to be non-allosteric and has been demonstrated to be insensitive to the allosteric effectors including dioxane (non-physiological activator) and L-aspartate (Ishijima et al., 1985; Kodaki et al., 1985). Cyanobacterial PPC is not activated by acetyl-CoA

(Luinenburg and Coleman, 1993). *S. elongatus ppc* gene codes for a 1053 amino acid residue polypeptide with the codon usage not so markedly different from that of the *E. coli ppc* (Katagiri et al., 1985). Like most of the known PPCs, this cyanobacterial PPC functions as a homotetramer of ~95–110-kDa subunits, and is more closely related to bacterial PPCs due to presence of conserved bacterial type (including *E. coli*) catalytic domain and lack of N-terminal phosphorylation domain typical of plant PPC (Sanchez and Cejudo, 2003; Xu et al., 2006).

Fuhrer et al. (2005) investigated glucose metabolism in seven bacterial species and found that compared to those of the model bacteria *Escherichia coli* and *Bacillus subtilis*, metabolisms of the investigated species differed significantly in several respects. Among them, the ED pathway and the TCA cycle were the almost exclusive catabolic pathways in *P. fluorescens* and *S. meliloti.* With a view to increase the flux through the anaplerotic node for increasing oxaloacetate levels, this chapter dealt with developing *Rhizobium* strains expressing *S. elongatus* PCC 6301 *ppc* gene and monitoring its effects on the glucose metabolism.

3.2 EXPERIMENTAL DESIGN

The experimental plan of work includes the following-

3.2.1: Bacterial strains used in this study

All wild type and genetically modified *E. coli* and *Rhizobium* strains used in this study are listed in **Table 2.1 and 2.2**. The plasmids used in the present study and their restriction maps are given in **Table 2.3** and **Fig. 2.1**. *E. coli* JM101 was used for all the standard molecular biology experiments wherever required. The *ppc* mutant strain, *E. coli* JWK3928, was a generous gift from NARA Institute of Science and Technology (Japan) due to kind recommendation of Prof. H. Mori.(**Table 3.1**)

Table 3.1: List of bacterial strains used. Detailed characteristics of these strains are given in Section 2.1 Parent strains and the transformants of *E. coli* and *Rhizobium* were respectively grown at 37°C and 30°C with variations in tetracycline and erythromycin concentrations for rich and minimal media as described in Section 2.2 and 2.3.

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 JWK3928 *ppc*-strain	*lacI^q rrnBT14 DlacZWJ16 sdR514 DaraBADAH33,DrhaBADLD78*	Peng et al., 2004
JM101 (pAB3) *E. coli*	JM101 with pAB3 plasmid; Amp^r, Tc^r	Buch et al., 2008
JM101 (pAB4) *E. coli*	JM101 with pAB4 plasmid; Amp^r, Tc^r	Buch et al., 2008
	***Rhizobium* strains**	
Bradyrhizobium japonicum USDA110	NC_004463.1	NCBI
Mesorhizobium loti MAFF030669	NC_002678.2	NCBI
Bj (pAB4)	*Bradyrhizobium japonicum* USDA110 with pAB4 plasmid; Amp^r, Tc^r (control vector)	This study
Bj (pAB3)	*Bradyrhizobium japonicum* USDA110 with pAB3 plasmid; Amp^r, Tc^r (*ppc*)	This study
Ml (pAB4)	*M. loti* MAFF030669 with pAB4 plasmid; Amp^r, Tc^r (control vector)	This study
Ml (pAB3)	*M. loti* MAFF030669 with pAB3 plasmid; Amp^r, Tc^r (*ppc*)	This study

3.2.2: Development of *B. japonicum* USDA110 and *M. loti* MAFF030669 strains harboring *ppc* gene of *S. elongatus* PCC6301.

The recombinant plasmids pAB3 and pAB4 (control) were transformed in *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 by electroporation (Section 2.4.2.2). The transformants were selected on tetracycline selection plates and were confirmed by restriction endonuclease digestion pattern.

3.2.3: Growth and MPS phenotype of transformant strains of *Rhizobium*

The MPS ability of transformants of *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 and its transformants were monitored on Pikovaskya's (PVK) agar and 100 mM Tris buffered RP (TRP) agar as described in Chapter 2.

3.2.4: Effect of heterologous *ppc* gene expression on the physiology and glucose metabolism.

Bradyrhizobium japonicum USDA110 and *M. loti* MAFF030669 transformants were subjected to physiological experiments involving growth and organic acid production profiles on TRP medium with 50 mM glucose as carbon source (Section 2.2.). The samples withdrawn at regular interval were analyzed for O.D.600nm, pH, extracellular glucose, and organic acid (Section 2.9.3). The physiological parameters were calculated as in Section 2.8. The enzyme assays were performed as described in Section 2.9; 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.

3.3: RESULTS

3.3.1: Heterologous overexpression of *S. elongatus* PCC 6301 *ppc* gene in *Rhizobium* strains.

The plasmids incorporated *in Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 transformants were isolated from the transformants and were confirmed

based on restriction digestion pattern (**Fig. 3.3**) before studying the effect of overexpression of *S. elongatus* PCC 6301 *ppc* gene.

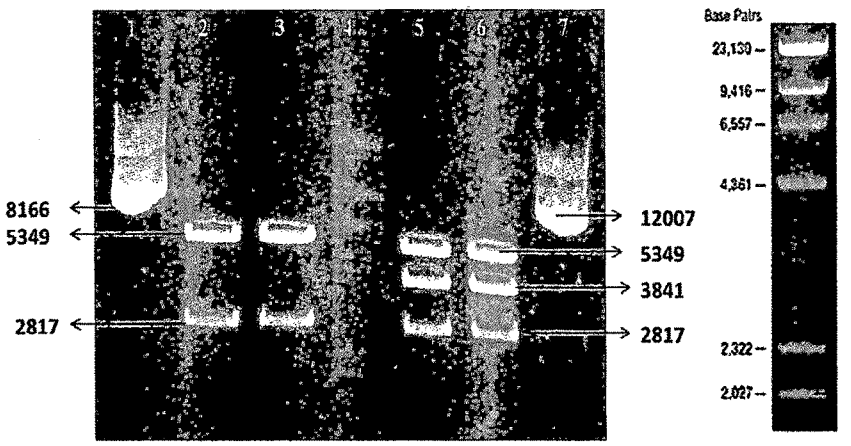


Fig. 3.3: Restriction digestion pattern of plasmids containing *ppc* gene isolated from transformants of *Bradyrhizobium japonicum USDA110* and *M. loti MAFF030669.* Lane 1: pAB4 plasmid Undigested (8166 bp); Lane 2 and 3: pAB4 plasmid digested with BamHI and HindIII (5349 bp and 2817 bp); Lane 4 : MWM-Lambda DNA cut with HindIII; Lane 5 and 6: pAB3 plasmid digested with BamHI and HindIII (5349 bp, 3841 bp and 2817 bp); Lane 7: pAB3 plasmid Undigested (12007 bp).

The PPC activity in *Bradyrhizobium japonicum USDA110* and *M. loti MAFF030669* containing pAB3 {*Bj* (pAB3)} and {*Ml* (pAB3)} grown on TRP medium with 50 mM glucose, was 45.55 ± 1.68 U and 35.61 ± 0.59 U, respectively, and the increase was ~9.2 and ~6.2 fold higher than that in control {*Bj* (pAB4)} and {*Ml* (pAB4)} which possessed very negligible levels of PPC activity (4.98 ± 0.38 U) and (5.82 ± 0.24 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* (pAB3) and *Ml* (pAB3) as compared to the control *Bj* (pAB4) and *Ml*

(pAB4).P-solubilizing ability of wild type *Bradyrhizobium japonicum USDA110* and *M. loti MAFF030669* and its transformants varied in the order of *Bj* (pAB3) = *Ml* (pAB3) > *Bj* (pAB4) = *Ml* (pAB4) >*Bj*=*Ml* on PVK medium after 3 days of incubation at 30°C (**Fig. 3.4; Table 3.2**).

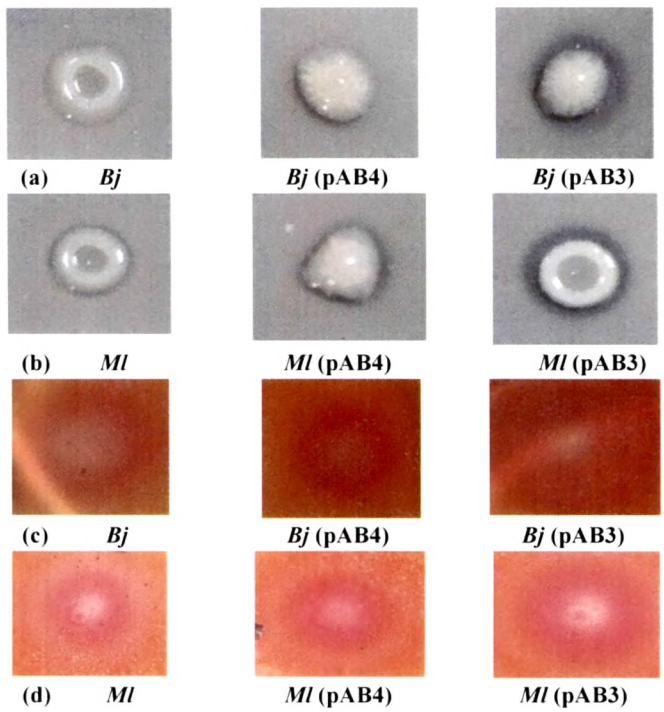


Fig. 3.4: MPS phenotype of *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 strains harboring pAB3 plasmid. (A) and (B) on Pikovskaya's agar and **(C) and (D)** Tris rock phosphate agar containing 50 mM glucose and 100 mM Tris HCl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 °C. Media composition and other experimental details are as described in Sections 2.2.4 and 2.7.

The pAB3 transformants of *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 showed maximum enhanced zone of clearance as compared to the control pAB4 (**Table 3.2).** Phosphate Solubilizing Index was calculated as described in 2.5. And it was highest in *Bj* (pAB3) and *Ml* (pAB3). There was ~1.2-fold increase in PSI of *Bj* (pAB3).

Table 3.2 P solubilization index on Pikovskyas agar of *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 transformants during 3 days of growth *Bj* and *Ml*: wild type strain; *Bj* (pAB4) : *Bradyrhizobium japonicum* USDA110 with vector control and *Bj* (pAB3) : *Bradyrhizobium japonicum* USDA110 with *ppc* 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* (pAB4)**	13.50 ± 0.50	11.17 ± 0.29	1.18
***Bj* (pAB3)**	15.17 ± 0. 29	11.50 ± 0.50	1.36
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
***Ml* (pAB4)**	13.17± 0.29	11.17 ± 0.29	1.18
***Ml* (pAB3)**	14.17 ± 0.29	11.17 ± 0.29	1.28

3.3.2: Effect of *S. elongatus* PCC 6301 *ppc* overexpression on growth pattern and pH profile in TRP medium.

The growth profiles and organic acid secretion on TRP medium with 50mM glucose demonstrated that maximum O.D. was reached in 16 h {*Bj* (pAB3)} and {*Ml* (pAB3)} transformants compared to 20 h of the controls {*Bj* (pAB4)} and {*Ml* (pAB4)}. Slight pH drop was found within 20 h in native and control vector transformant while pH drop to 4.7 and 4.2 was seen within 16 h in {*Bj* (pAB3)} and {*Ml* (pAB3)}, respectively **(Fig. 3.5)**.

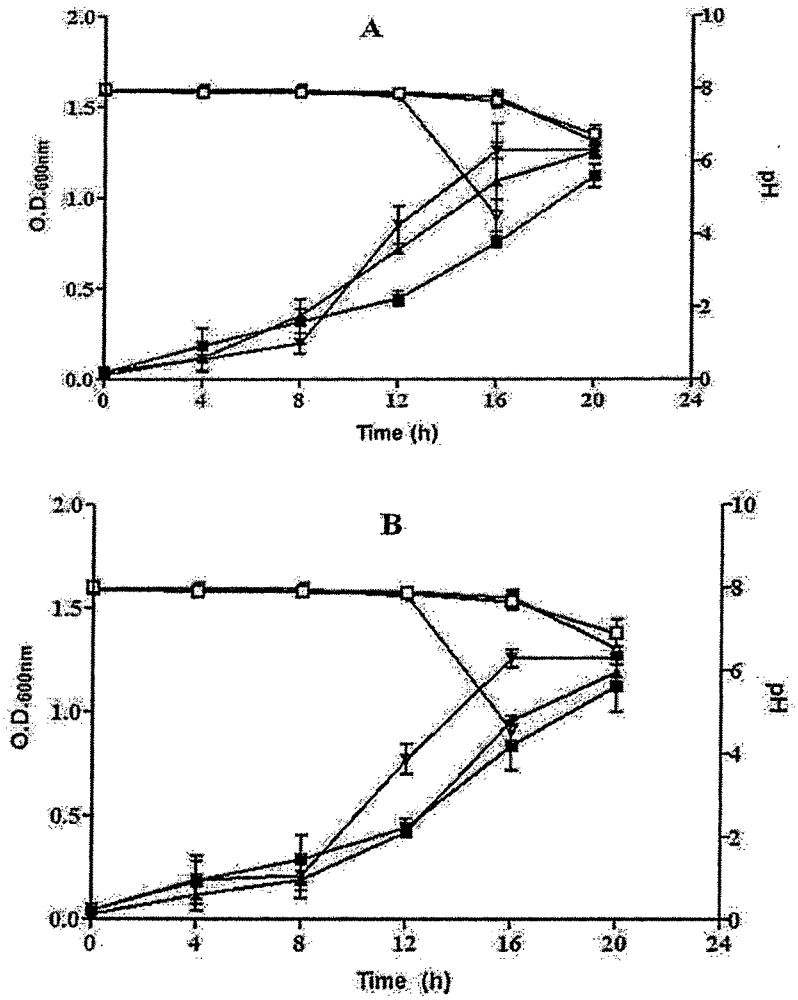


Fig. 3.5 Effect of *ppc* overexpression on extracellular pH (□, Δ, ▽,) and growth profile (■, ▲, ▼) of (A) *Bradyrhizobium japonicum* USDA110 and (B) *M. loti* MAFF030669, with 50 mM glucose and 100 mM Tris-Cl pH 8, 1mg/ml of rock phosphate medium. (□, ■, *Bj, Ml* wild type); {Δ, ▲, *Bj* (pAB4), *Ml* (pAB4)}; {▽, ▼, *Bj* (pAB3), *Ml* (pAB3)}. **A** – *Bradyrhizobium japonicum USDA110* and **B** - *M. loti* MAFF030669. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations

3.3.5: Physiological effects of *ppc* overexpression on TRP medium with 50mM glucose.

In presence of 50 mM glucose, increase in PPC activity significantly affected growth profile (**Table 3.5**). The total glucose utilization rate and the total amount of glucose used at the time of pH drop remained unaffected (**Table 3.5**). However, the Specific Glucose Utilization Rate Q_{Glc} (g.g $dcw^{-1}.h^{-1}$) decreased ~1.4 and ~1.2 fold in {*Bj* (pAB3)} and {*Ml* (pAB3)}, respectively. Additionally, the increase in PPC activity increased the specific growth rate by ~2 fold and ~1.7 fold, and improved the biomass yield by 1.4 and 1.2 fold in the transformants of {*Bj* (pAB3)} and {*Ml* (pAB3)}, respectively, compared to control {*Bj* (pAB4)} and {*Ml* (pAB4)}.

Table 3.3: Physiological variables and metabolic data from of *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 *ppc* transformants grown on TRP medium. The results are expressed as Mean ± S.E.M of six 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. * $P<0.05$ 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	0.19 ± 0.03	46.20 ± 0.20	38.23 ± 0.33	1.78 ± 0.14	0.14 ± 0.01
Bj (pAB4)	0.26 ± 0.04	45.99 ± 0.22	36.56 ± 1.29	1.67 ± 0.26	0.15 ±0.02
Bj (pAB3)	0.53 ± 0.05***	48.18 ± 0.16	29.59 ± 2.39	2.23±0.29*	0.11 ±0.01
Ml	0.22 ± 0.03	45.91 ± 0.64	37.07 ± 0.55	1.36 ± 0.26	0.19 ± 0.04
Ml (pAB4)	0.29 ± 0.01	46.30 ± 0.12	36.09 ± 0.18	1.63 ± 0.06	0.15 ± 0.01
Ml (pAB3)	0.48±0.09***	48.41 ± 0.17	31.56 ± 1.16	1.9 ± 0.17*	0.13 ± 0.01

3.3.6: Biofilm, exopolysaccharide and indole acetic acid production by *Bj* (pAB3) and *Ml* (pAB3) transformants in TRP medium.

Biofilm, exopolysaccharide and indole acetic acid production on an average showed significant increase by ~1.7 fold in *Bj* (pAB3) as well as *Ml* (pAB3) transformants in TRP medium in comparison to control *Bj* (pAB4) and *Ml* (pAB4) (**Table 3.4**).

Table 3.4: Biofilm, exopolysaccharide and indole acetic acid production by *Bj* (pAB3) and *Ml* (pAB3) transformants in TRP medium. The results are expressed as Mean ± S.E.M of six independent observations. *** P<0.001.

Rhizobium Strains	Biofilm O.D.at 550nm	EPS (g/100ml)	IAA (µg/ml)
Bj	1.39 ± 0.02	11.48 ± 0.1	20.14 ± 1.33
Bj (pAB4)	1.54 ± 0.02	12.36 ± 0.4	24.87 ± 1.86
Bj (pAB3)	2.65 ± 0.01***	21.30 ± 3.3***	41.78 ± 0.53***
Ml	1.51 ± 0.06	11.34 ± 0.05	28.17 ± 1.35
Ml (pAB4)	1.61 ± 0.10	13.54 ± 1.60	29.79 ± 1.66
Ml (pAB3)	2.74 ± 0.12***	23.97 ± 0.39***	45.74 ± 1.59***

3.3.7 : P solubilization and organic acid secretion in 100mM Tris-Cl Buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml

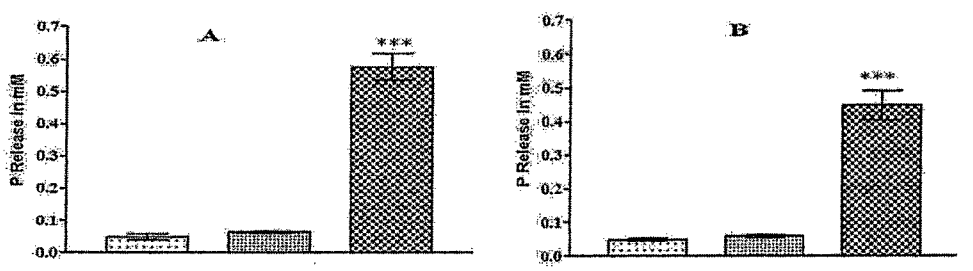


Fig. 3.6: P solubilization by (A) *Bradyrhizobium japonicum* USDA110 and (B) *M. loti* MAFF030669 transformants on TRP medium.

The values are depicted as Mean ± S.E.M of 7-10 independent observations. *** P<0.001.

There was significant increase in P release by ~7.4 and ~7.9 fold in {*Bj* (pAB3)} and {*Ml* (pAB3)}, respectively, compared to control {B*j* (pAB4)} and {M*l* (pAB4)} respectively.

On TRP medium in presence of 50 mM glucose and 100 mM Tris Cl Buffer pH 8.0, the organic acids identified were mainly gluconic, 2-ketogluconic, acetic and citric acids. Extracellular medium of *Bj* (pAB3) and *Ml* (pAB3) contained ~9.3 and ~8.9 folds higher amounts of citric acid, respectively, with its specific yield ($Y_{C/G}$) increasing by ~5.6 and ~7.6-fold and it also contained ~2.2 and ~2.1-fold higher amounts of gluconic acid as compared to *Bj* (pAB4) and *Ml* (pAB4) with its specific yield ($Y_{G/G}$) increasing by ~1.32 and ~1.75-fold. Levels of 2-ketogluconic and acetic acids were unaltered as compared to *Bj* (pAB4) and *Ml* (pAB4) respectively (**Fig. 3.7**).The intracellular citric acid level remained unaltered. **(Table.3.5)**

Table 3.5: Intracellular Citric acid levels of *Bj* (pAB3) and *Ml* (pAB3) 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* (pAB4)**	0.80 ± 0.10	***Ml* (pAB4)**	0.83 ± 0.06
***Bj* (pAB3)**	0.93 ± 0.06	***Ml* (pAB3)**	1.15 ± 0.13

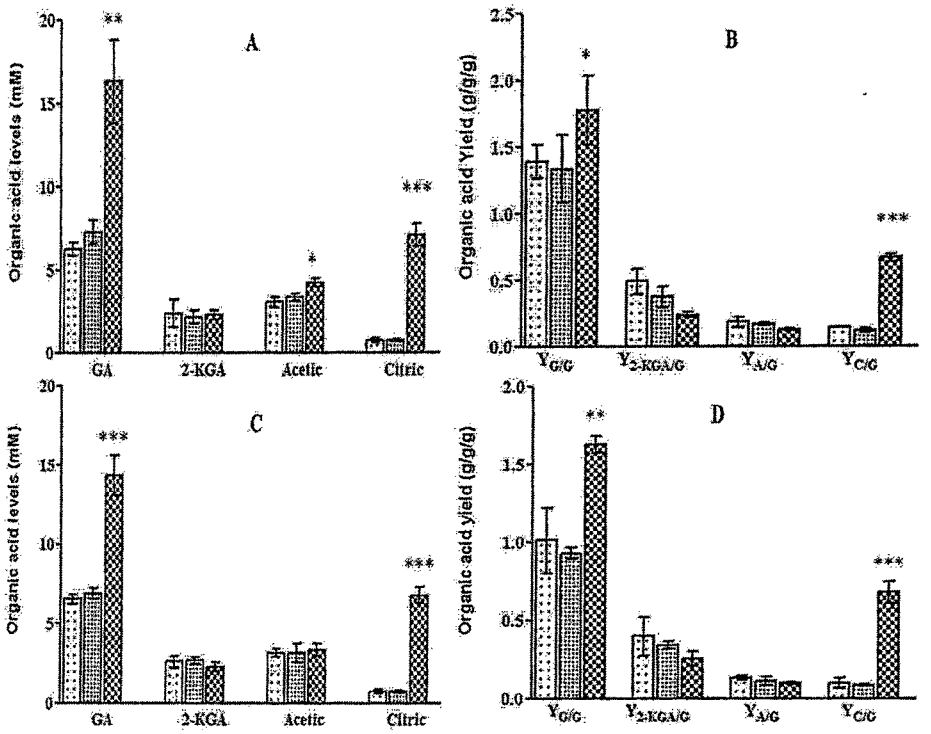


Fig. 3.7: Organic acid production from *Bradyrhizobium japonicum* USDA110 *and M. loti* MAFF030669 *ppc* gene: A and C : Gluconic, 2-keto gluconic, acetic and citric acids levels and B and D: Organic acid Yields ($Y_{G/G}$ $Y_{2\text{-}KGA/G}$, $Y_{A/G}$ and $Y_{C/G}$ in *Bj*, *Bj* (pAB4), *Bj* (pAB3), *Ml* , *Ml* (pAB4) and *Ml* (pAB3), 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. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

3.3.8: Alterations in enzyme activities in *Bj* (pAB3) and *Ml* (pAB3) transformants.

Alterations in physiological variables and organic acid profile investigated with enzyme activities involved in periplasmic direct oxidation and intracellular phosphorylative pathways. In response to ~9.2 and ~6.2 fold increase in PPC activity in *Bj* (pAB3) and *Ml* (pAB3), GDH activity increased by about ~1.4 and ~1.7 fold,

respectively, as compared to the control (**Fig. 3.8**). Additionally, G-6-PDH, representing phosphorylative pathway increased by ~1.8 and ~2 fold in *Bj* (pAB3) and *Ml* (pAB3), respectively, Also there was ~1.8 and ~2 fold increase in ICDH activity while PYC, and CS and activities remained unaltered . in *Bj* (pAB3) and *Ml* (pAB3), as compared to *Bj* (pAB4) and *Ml* (pAB4) .Glyoxylate pathway enzyme ICL showed very low activity in all transformants.

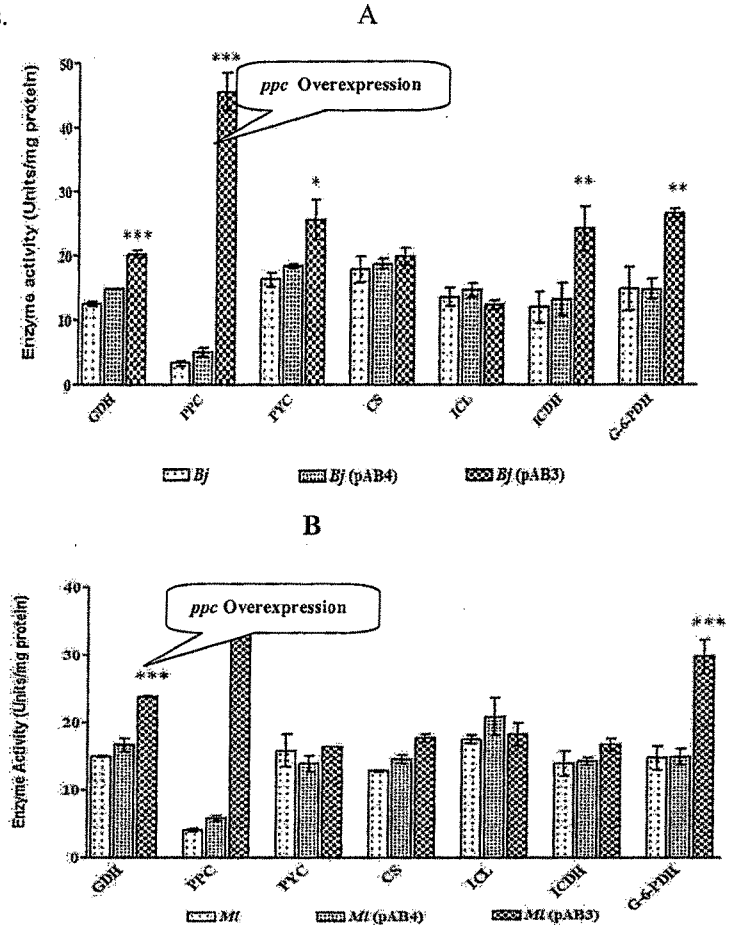


Fig. 3.8: Activities of enzymes PPC, PYC, GDH, G-6-PDH, ICDH and ICL in (A) *Bradyrhizobium japonicum* USDA110 and (B) *M. loti* MAFF030669 *ppc* transformant. The activities have been estimated using cultures grown on TRP medium with 50mM glucose. All the enzyme activities were estimated from mid log 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<0.01and *** P<0.001.

3.4: DISCUSSION

PPC at the anaplerotic node has been a common genetic engineering target in strategies aiming at diverse bioprocesses like reducing acetate secretion in *E. coli* and improving glutamate production in *C. glutamicum* under biotin limitation and by control of 2-oxoglutarate dehydrogenase complex or by addition of Tween 40. (Farmer and Liao, 1997; Delaunay et al., 1999; Bott 2007; Shirai 2007). Genetic manipulations altering the carbon flow at the PEP branch-point has been well-studied in *E. coli,* with PPC being a frequent target (Sauer and Eikmanns, 2005). Over-expression of acetyl–CoA synthetase had enhanced recycling of the acetyl–CoA and acetyl–phosphate pools (Renilla et al., 2012). This chapter presents the consequences of overexpression of *S. elongatus* PCC 6301 *ppc* gene on glucose catabolism of *Bradyrhizobium japonicum USDA110* and *M. loti MAFF030669*. About ~9.1 and ~6.1 fold increase in PPC activity in {*Bj* (pAB3)}and {*Ml* (pAB3)}, respectively, was in accordance with about 14 fold increase in PPC activity in *P. fluorescens* harboring pAB3 plasmid {*Pf* (pAB3)} (Buch et al., 2009).

Growth parameters for *Bj* (pAB3) and *Ml* (pAB3) transformants were carried in the medium with 50mM glucose while {*Pf* (pAB3)} transformant had 100 mM glucose (Buch et al 2009). Additionally, *Bj* (pAB3) and *Ml* (pAB3) transformants showed enhanced biomass yield similar to that in {*Pf* (pAB3)} while increase in specific growth rate was better in *Rhizobium* transformants. The growth promotion of *Rhizobium* transformants could be attributed to the unique features of central metabolism. Amongst different bacteria, *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 strains appear belong to the group in which intracellular OAA is mainly supplied by PYC and contribution of PPC is minimal (**Table 1.1;** Fuhrer et al., 2005). Hence, heterologous *ppc* overexpression in *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 strains enhanced the carbon flow towards oxaloacetate. Additionally, *S. elongatus* PCC 6301 *ppc* gene with reduced influence of the allosteric regulation by malate and aspartate

operates efficiently towards anabolic and catabolic pathways at the PEP/OAA level. Also there was a significant increase in PYC activity supplying OAA in *Bj* (pAB3) and *Ml* (pAB3) which was not seen in *Pf* (pAB3).Increase in PPC activity is associated with increase in OAA which resulted in increased biomass due to the increased anaplerotic pathway. While in *P. fluorescens* increase in PYC is not seen suggesting no increase in anaplerotic pathway.

Increase in gluconic acid in *Bj* (pAB3) and *Ml* (pAB3) but not of 2-ketogluconic acid could be due to the low GAD activity in *Rhizobium* .Multiple studies suggest that the periplasmic conversion of glucose to gluconate may be a significant route for carbon flux in glucose-grown *S. meliloti* cells (Portais et al., 1997; Bernardelli et al., 2001). *Rhizobium ppc* transformants secreted low amounts of acetic acid. Similar levels of acetic acid secretion was found in *Pf* (pAB3) transformant (Buch et al., 2009). Similarly, expression of *pyc* gene in *E. coli* resulted in a 56% increase in biomass yield and a 43% decrease in acetate yield (Gokarn et a., 2001). Aerobic metabolism coupled with absence of pyruvate oxidase B could account for the low amounts of acetic acid secretion in both *Rhizobium* and *Pseudomonas* transformants. Increase in the gluconic acid yield in TRP medium could be due the presence of 0.1% yeast extract in the medium which contains 1-10 nmoles PQQ supporting GDH enzyme activity (Ohsuki et al., 1993).

Reduced glucose consumption of *Rhizobium* transformants could be a consequence of decreased intracellular phosphorylative pathway and enhanced direct oxidation pathway as demonstrated by alterations in activities of enzymes involved in glucose catabolism and organic acid profile and G-6-PDH, estimated to represent the contribution of phosphorylative pathway which was increased by ~1.8 and ~2.0-fold, respectively . Additionally, PYC activity showed slight increase in *Bj* (pAB3) while remained unaltered in *Ml* (pAB3). Comparison of G-6-PDH and GDH activities in *Rhizobium* and *Pseudomonas* transformants suggest that direct oxidative pathway is enhanced in *Pseudomonas* while phosphorylative is enhanced in *Rhizobium.*

Glucose flux through TCA cycle in *S. meliloti* is relatively higher than that of *E. coli* and *P. fluorescens* (Fuhrer et al., 2005). Such a metabolic and genetic background would be expected to allow the enforced increase in PPC activity to affect the flux distribution at the PEP-Pyruvate-OAA junction and the TCA cycle. In this study, there was a significant increase in citric acid levels ~9.3 and ~8.9-fold by *Bj* (pAB3) and *Ml* (pAB3), respectively, as compared to *Bj* (pAB4) and *Ml* (pAB4) with its specific yield ($Y_{C/G}$) increasing by ~5.6 and ~7.6-fold, respectively. The high level of citric acid secretion is a consequence of increased PPC enzyme activity in *Bj* (pAB3) and *Ml* (pAB3) which results in increased OAA facilitating a higher flow through the TCA cycle. OAA formation is the net result of PPC and PYC enzyme activities. In both *Pseudomonas* and *Rhizobium,* OAA seems to be higher due to increased PYC activity. In both these bacteria, normally PPC activity is low and PYC activity is high.

Intracellular levels of citric acid in *P. fluorescens* 13525 wild type as well as *ppc* transformant is high (~16 mM) appear to lesser efficiency of anaplerotic reactions to divert OAA towards anabolic pathways. On the other hand, *Rhizobium* strains wild type and *ppc* transformant had accumulated very low (less than 1 mM) level of citric acid which could be due to higher TCA cycle flux in *Rhizobium* as increase in ICDH activity in *Rhizobium* transformant was higher than *Pseudomonas* transformant. This is also substantiated by the fact that *S. meliloti* exhibited higher TCA cycle flux than *E. coli*, *B. subtilis*, *C. glutamicum*, *S. cerevisiae*, *Paracoccus versutus*, *R. sphaeroides Z. mobilis P. fluorescens* and *A. tumefaciens* (**Fig.1.21**) (Fuhrer et al., 2005). Additionally, *Rhizobium* transformant secreted citrate up to 7 mM while secretion in *Pseudomonas* was very low (70 μM) which indicates that *Rhizobium* possesses efficient efflux for citrate as compared to *Pseudomonas*.

P solubilization net result of higher levels of gluconic and citric acid secretion significantly increased by ~7.4 and ~7.9 fold by overexpression of *ppc* gene in {B*j* (pAB3)} and {M*l* (pAB3)} compared to control {B*j* (pAB4)} and {M*l* (pAB4)} respectively. Moreover this phenotype is under stringent condition, 50 mM glucose compared to 100 mM glucose in *Pseudomonas*. Though gluconic acid is more in

Pseudomonas compared to *Rhizobium* but increased citric acid secretion gives better phenotype. The enhanced di-calcium phosphate solubilizing ability in *ppc* transformants of inherently poor/moderate P-solubilizing isolates Fp585 and P109 as well as improved rock-phosphate solubilization by *ppc* transformant of inherently efficient P-solubilizer Fp315 suggested that *ppc* overexpression unambiguously improved the phosphate solubilizing ability on glucose and xylose (Buch et al., 2009). This suggests that *ppc* overexpression is giving better results in *Rhizobium* compared to *Pseudomonas.*

Meseniere's et al., (2006) found that overexpression of *ppc* and *gltA* genes in *E. coli* leads to a higher flux through the TCA cycle and thus it eliminated the acetate production by eliminating saturation of this cycle. The dry cell weight (dcw) was almost twice as high in cultures of the over-expression mutant as in cultures of the wild type or the knock-out strain. This confirmed that a higher PPC activity is associated with a higher flow through the TCA cycle a higher NADH availability and more ATP, resulting in a higher biomass production. Similar results were obtained in this study also where overexpression of *ppc* doubled the dcw in *Bj* (pAB3) compared to *Bj* (pAB4).

In addition to P solubilization, plant growth promoting activities are also enhanced by *ppc* transformants of both *Rhizobium* species. Biofilm, exopolysaccharide and indole acetic acid production on an average all showed a significant increase by ~1.7 fold in *Bj* (pAB3) as well as *Ml* (pAB3) transformants. As a consequence of more biomass going for increased biomass, more amount of EPS was produced, which increased colonization and gave better P solubilization.

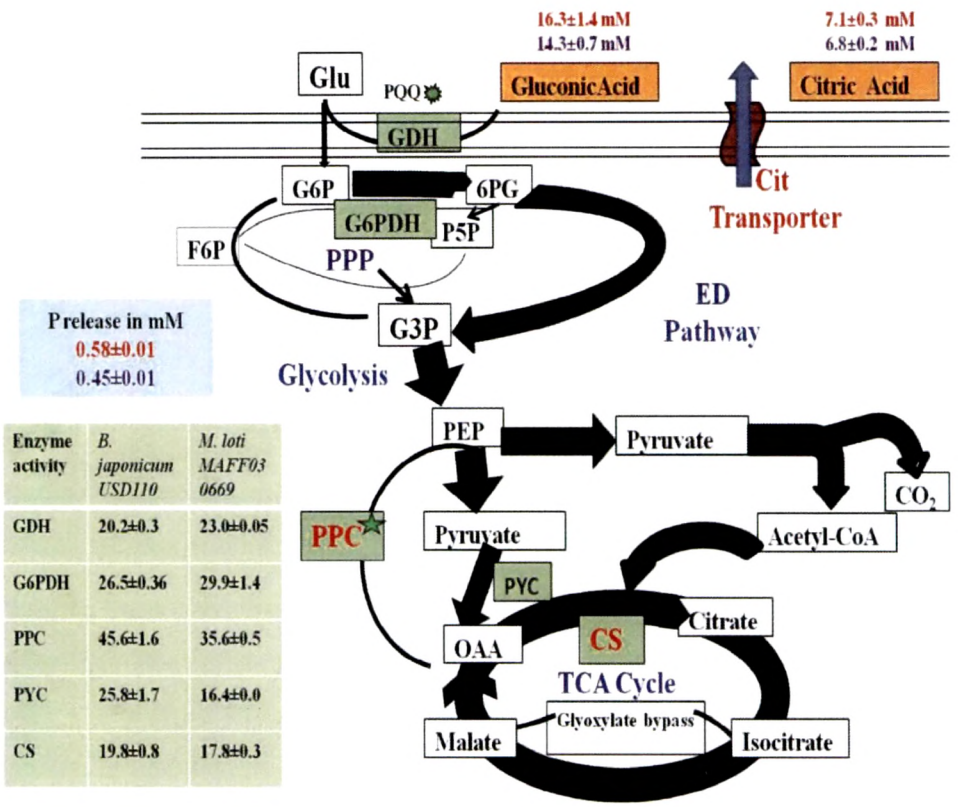
Biofilm formation in *Rhizobium*–legume N_2-fixing symbiosis contributes to effective root colonization by rhizobia and provides an effective mode for defense and helping rhizobia to survive under harsh and nutrient-limiting environments (Jebara et al., 2006). Diverse forms of the fungal bacterial biofilms (FBBs)/fungal rhizobial biofilms (FRBs) have been shown to improve nodulation and N_2 fixation in Rhizobium–legume symbiosis, colonize nonlegume plant roots, improve growth, increase soil nitrogen and phosphorus availabilities, solubilize rock phosphate, produce higher acidity and plant

growth-promoting hormones (Bandara et al.,2006; Seneviratne et al., 2009). *Rhizobium leguminosarum* bv. viciae and *Sinorhizobium meliloti* establish biofilms on both roots of its legume hosts, *Medicago sativa* L. and *Melilotus alba* Desr. and abiotic surfaces in the soil (Fujishige et al. 2006). In addition, bacterial surface polysaccharides e.g., exopolysaccharides are involved in the attachment process and production of EPS is characteristic to biofilmed form of bacteria including rhizobia (Pueppke et al., 1980; Kijne et al., 1988; Williams et al., 2008). Thus increase in biofilm and exopolysaccharide in this study suggests it to be a better P solubilizer.

IAA production was also increased by both the transformants. In spite of the direct effects of biofilm lifestyle of rhizobia in *Rhizobium*–legume symbiosis, some indirect effects could also affect the symbiosis. Increased production of IAA by an inoculated biofilm of *Penicillium* spp.–*Bradyrhizobium* spp. increased root growth of soybean (*Glycine max*) (Jayasinghearachchi and Seneviratne 2004a).

The Rhizobium– Arbuscular mycorrhizal (RAM) symbiosis, possibly forming FRB improved the nutrient availability where AM fungi supplied P while rhizobia provides N which together lead to increase in photosynthetic rates and concurrently the plant growth and thus plays an important role in Rhizobium–legume symbiosis and improved the performance and yields of legumes compared to nonsymbiotic plants (Lum and Hirsch 2003; Chalk et al. 2006; Seneviratne et al. 2008a; Kaschuk et al. 2009). So increase in IAA by the *ppc* gene transformants of *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 may help to improve its performance.

The growth and physiological effects of overexpression of *ppc* are similar to as observed in *P. fluorescens* ATCC 13525 but *Rhizobium* strains seem to be better P solubilizer due to secretion of gluconic and citric acid by its *ppc* gene transformants (Buch et al., 2009 and Adhikary, 2012).



Enzyme activity	*B. japonicum USDI10*	*M. loti MAFF03 0669*
GDH	20.2±0.3	23.0±0.05
G6PDH	26.5±0.36	29.9±1.4
PPC	45.6±1.6	35.6±0.5
PYC	25.8±1.7	16.4±0.0
CS	19.8±0.8	17.8±0.3

Fig. 3.9: Key metabolic fluctuations in *B. japonicum* USDA110 and *M. loti* MAFF030669 overexpressing *ppc* gene.