Development of mineral phosphate solubilization ability in *Rhizobium* spp. by metabolic engineering of tricarboxylic acid cycle

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STATEMENT UNDER O. Ph.D. 8/(iii) OF

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This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Dr. G. Naresh Kumar.**

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This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph. D. degree of The M. S. University of Baroda by Ms. Praveena G.Bhandari is her original work. The entire research work and the thesis have been built up under my supervision.

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Dedicated
To
One and All
Near and Dear
To
P.P.Hariprasad
Swamiji

Expression of Gratitude:

If I could express my gratitude adequately enough, this would turn out to be the longest chapter of this thesis. My name on the cover of this dissertation seems inadequate by itself; for without the invaluable contribution of a great number of people, this research and thesis would not have been possible. I would therefore like to express my indebtedness to every individual who has been a crucial part of this endeavour.

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- "I do not believe in giving the answer; but I believe in drawing out the answer from the student."
- "I don't want you to simply get the degree, but I want you to imbibe a training that would enable you to guide others."
- "I always believe in solutions."
- "Always live in the present, and think of a bright future."
- "One has to change himself with the changing time."

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Praveena

List of Abbreviations

6-PG	6-phosphogluconate
Ap	Ampicillin
ATP	Adenosine triphosphate
bp	base pair (s)
cAMP	Cyclic adenosine monophosphate
CS	Citrate synthase
CTAB	Cetyl trimethyl ammonium bromide
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DTT	1, 4-Dithiothreitol
ED /EDD	Entner-Doudoroff Pathway
EDA	KDPG Aldolase
EDTA	Ethylene diamine tetra acetic acid
EMP	Embden–Meyerhof–Parnas pathway
ETC	Electron transport chain
G-6-P	Glucose-6-phosphate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GADH	Gluconate dehydrogenase
GDH	Glucose dehydrogenase
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
ICDH	Isocitrate dehydrogenase
ICL	Isocitrate lyase
kb	kilobase (s)
kDa	Kilodalton
KDPG	2-keto-3-deoxy-6-phosphogluconate
Km	Kanamycin
LDH	Lactate dehydrogenase
LB	Luria broth
MDH	Malate dehydrogenase
ME	Malic enzyme
mM/M	Millimolar/Molar
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
ng	Nanogram
nm	Nanometer
OAA	Oxaloacetate
OD	Optical Density
Ori	Origin
P/Pi	Phosphate / inorganic phosphate

PCR	Polymerase Chain Reaction
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PEPCk	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PGPR	Plant Growth Promoting Rhizobacteria
POX	Pyruvate oxidase
PPC	Phosphoenolpyruvate carboxylase
PPP	Pentose phosphate pathway
PQQ	Pyrolloquinoline quinine
PSM	Phosphate solubilizing microorganisms
PTS	Phosphotransferase system
PYC	Pyruvate carboxylase
PYK	Pyruvate kinase
rpm	Revolutions per minute
Str	Streptomycin
Tc	Tetracycline
TCA	Tricarboxylic acid
Tmp	Trimethoprim
Tris	Tris (hydroxymethyl) aminomethane buffer
TYE	Tryptone Yeast Extract
WT	Wild type
YEMB	Yeast Extract Mannitol Broth
µl/ml	Microlitres/milliliters

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Chapter 1

Review of Literature and Introduction

1.1 Rhizobia: The master microbe

Nitrogen fixing bacteria in legume nodules collectively designated as rhizobia have been known since 1888 (Quispel, A.1988). They were the first biofertilizers produced and allowed savings of millions of dollars in chemical fertilizers that may contaminate soil and water (Hungria et al 2006). In 1888, Beijerink obtained the first pure bacterial culture named by him *Bacillus radicicola* from nodule suspension. This isolate could nodulate *Pisum* and *Vicia* was later renamed as *Rhizobium leguminosarum* (Frank, 1889). The description of the first rhizobial species was mainly based on the legume, which acted as host. This fact led to the definition of the cross-nodulation groups, which was based on the nodulation specificity after infectivity experiments in several legumes (Baldwin and Fred, 1929).

1.1.1: General Taxonomy

The current taxonomy of rhizobia consists of several genera in the subclass Alpha- and Beta- Proteobacteria. *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Azorhizobium*, *Methylobacterium*, *Bradyrhizobium*, *Phyllobacterium*, *Devosia* and *Ochrobactrum* are genera that belong to rhizobial Alpha-Proteobacteria. Rhizobial Beta-Proteobacteria includes the following genera: *Burkholderia*, *Herbaspirillum* and *Cupriavidus* (Weir, 2012). This classification is based on taxonomically important strains that may not necessarily be important reference strains for legume growth improvement. Rhizobial strains commonly used in inoculants have good field performance and stability of symbiotic properties in culture, but are not necessarily well documented or used in taxonomy or molecular biology studies (Lindström et al., 2010).

1.1.2: Host Specificity and Nodulation

The legume-rhizobia association is specific (each rhizobial strain establishes a symbiosis with only a limited set of host plants and *vice versa*). Thus, a restricted number of inoculants fit with a leguminous plant and farmers need to be familiar with the

suitability of inoculants for application according to the characteristics of plant and soil (Mabrouk and Belhadj, 2010). Plants mutually compatible with the same species of rhizobia were listed in earlier years in so-called *cross inoculation groups* (**Table 1.1**).

Table 1.1: Cross-inoculation group and *Rhizobium*-legume association (Morel et al., 2012)

Rhizobia	Legume Cross-inoculation group
Ensifer meliloti	Alfalfa Group: alfalfa (*Medicago sativa*), sweet clover (*Melilotus* spp.) (yellow and white), fenugreek (*Trigonella* spp.)
R. leguminosarum bv *trifolii*	Clover Group (Clover I, II, III and IV): clovers (*Trifolium* spp.)
B. japonicum	Soybean Group: soybean (*Glycine max*)
Bradyrhizobium spp.	Cowpea Group: pigeon pea (*Cajanus cajan*); peanut (*Arachis hypogaea*); cowpea, mungbean, black gram, rice bean (*Vigna* spp.); lima bean (*Phaseolus lunatus*); *Acacia mearnsii; A. mangium; Albizia* spp.; *Enterlobium* spp., *Desmodium* spp., *Stylosanthes* spp., Kacang bogor (*Voandzeia subterranea*), *Centrosema* sp., winged bean (*Psophocarpus tetragonolobus*), hyacinth bean (*Lablab purpureus*), siratro (*Macroptilium atropurpureum*), guar bean (*Cyamopsis tetragonoloba*), calopo (*Calopogonium mucunoides*), puero (*Pueraria phaseoloides*)
R. leguminosarum bv *viciae*	Pea Group: peas (*Pisum* spp.), lentil (*Lens culinaris*), vetches (*Vicia* spp.), faba bean (*Vicia faba*)
R. leguminosarum bv *phaseoli*	Bean Group: beans (*Phaseolus vulgaris*), scarita runner bean (*Phaseolus coccineus*)
Mesorhizobium loti	Chickpea Group: chickpea (*Cicer* spp.), Birdsfoot trefoil (*Lotus corniculatus* L.)
Rhizobium lupini	Group Lupines
Rhizobium spp.	Crown vetch

The specificity of symbiotic interactions is achieved by exchange of molecular signals. In the early steps of symbiosis, a diverse array of compounds is exuded into the rhizosphere, including flavonoids, isoflavonoids, and non-flavonoid inducers (Peters et al. 1986; Caetano-Anolle´s et al., 1988; Dharmatilake and Bauer 1992; Spaink et al., 1991, 1995; Hungria and Stacey 1997).

Molecular determinants of host specificity during nitrogen-fixing symbiosis are depicted in **Fig. 1.1** (Wang et al., 2012) – (i) the plant produce flavonoid signals (such as the luteolin from *M. truncatula*) to free-living soil bacteria, activating the bacterial NodD proteins. NodD proteins, bind to the conserved *nod*-box in the promoters of bacterial nodulation genes to induce their expression.

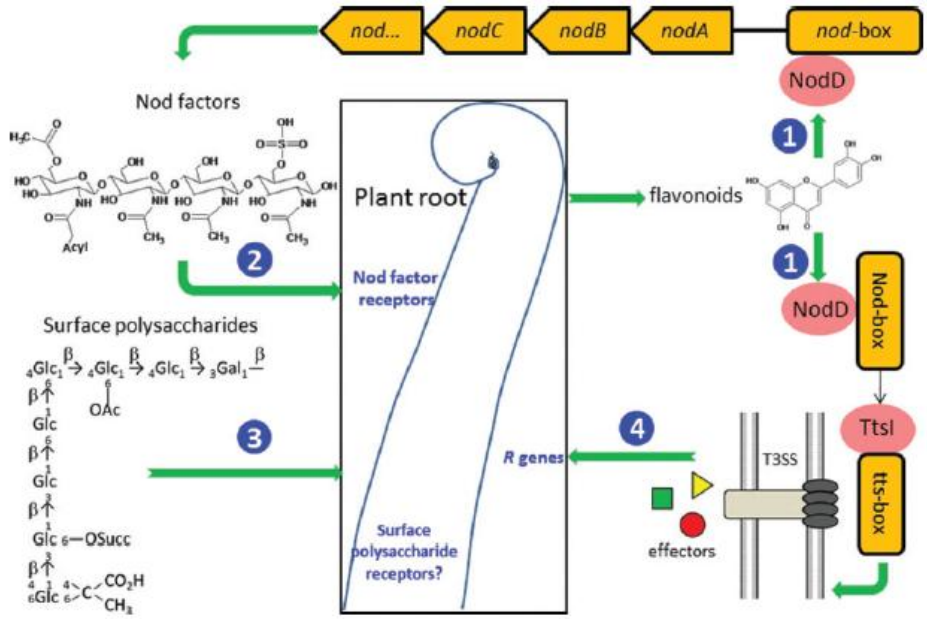


Fig. 1.1: Molecular determinants of host specificity during nitrogen-fixing symbiosis (Wang et al., 2012).

(ii) The *nod* genes code for enzymes for the synthesis of Nod factors, which are recognized on the plant surface by transmembrane Nod factor receptors in a strain- and ecotype-specific fashion. Modifications on the Nod factor such as the length and

saturation of the acyl group determine host specificity. Activation of Nod factor receptors triggers growth changes in the root hair to trap a small number of bacteria, which would give rise to the entire population colonizing the resulting nodule. (iii) Possibly downstream of Nod factors, rhizobia also use their surface polysaccharides (such as EPS from *S. meliloti*, depicted) to modulate host range. The plant receptor(s) are unknown, but may resemble animal receptors for surface polysaccharides from bacterial pathogens. (iv) In certain rhizobial strains, NodD also induces the expression of *TtsI*, which codes for a transcriptional regulator that binds to highly conserved promoter elements, called *tts* boxes, upstream of operons encoding the type III secretion machinery and effectors. Recognition of these effector proteins by *R* genes present in some varieties of plants limits host range.

The legume secretes flavonoids which induce the rhizobia to produce Nod factors and attract them to the plant root hair cells **(Fig. 1.2)** (Haag et al., 2012). Nod-factor signaling triggers a number of developmental changes, including root hair curling which traps the rhizobia in Shepherd's crooks. Inward growth of the root hair tip results in tubular structures called infection threads, which allow the rhizobia to enter the cortical cell layers of the plant root. The rhizobia escape the infection thread and are taken into the host cell *via* an endocytosis-like process **(Fig. 1.2, c-1),** forming vesicles composed of host-derived membrane and are known as symbiosomes. In legumes of the Inverted Repeat-Lacking Clade (IRLC) (consisting of legumes such as *Medicago, Pisum, Vicia, Trifolium, Galega* and *Astragalus*), the rhizobia are challenged with nodule-specific cysteine-rich peptides (NCR) peptides **(Fig. 1.2, c-2),** and differentiated into elongated bacteroids **(Fig. 1.2, c-3).** The bacterial BacA protein is essential for protecting the rhizobia against the antimicrobial activity of NCR peptides **(Fig. 1.2, c-2).** In contrast, BacA is dispensable for rhizobia infecting legumes of the phaseoloid clade that do not produce NCR peptides. In these host plants, rhizobia do not differentiate terminally and often multiple bacteroids can be found inside a single symbiosome membrane.

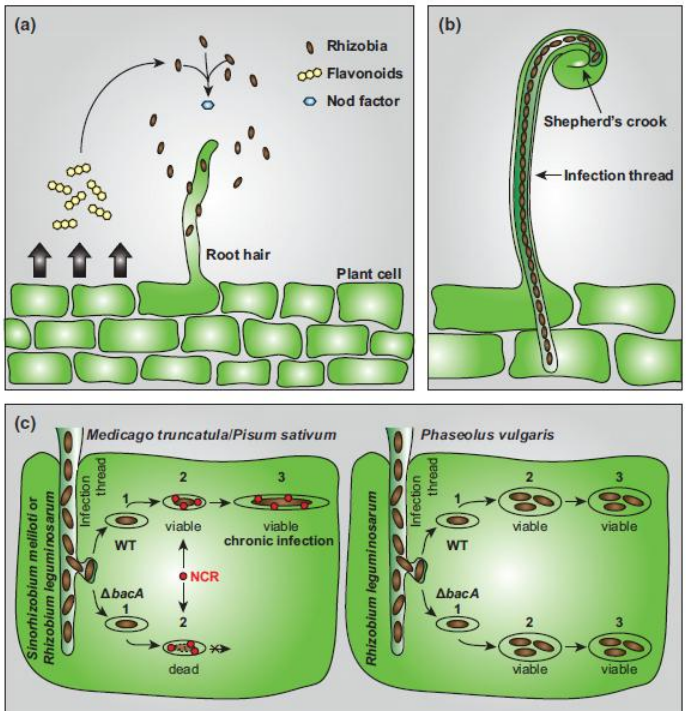


Fig. 1.2: Rhizobia interacting with legumes (Haag et al., 2012). (WT: wild-type).

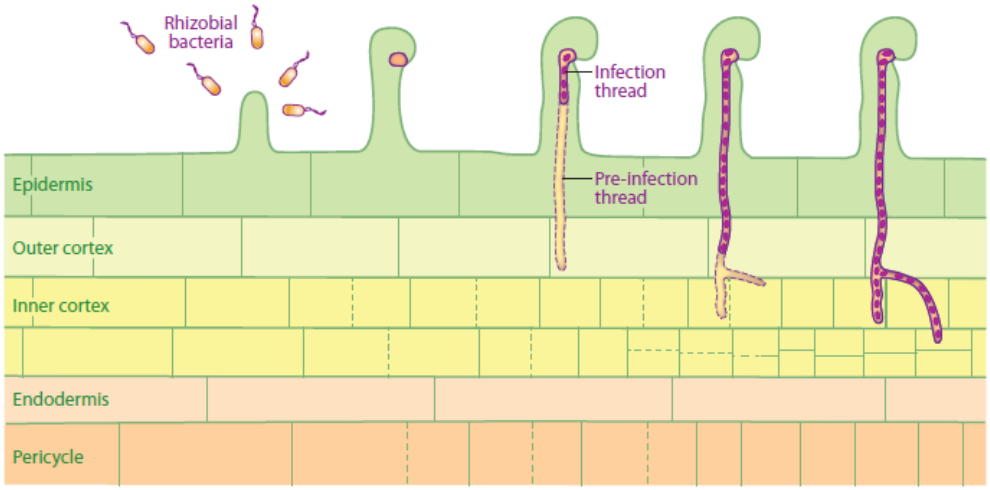


Fig. 1.3: Nodulation involves the coordinated development of bacterial infection and nodule organogenesis (Oldroyd et al., 2011). Cell division (*indicated with dotted lines*) in the inner or mid-cortex and pericycle is initiated early in the interaction between the root and rhizobial bacteria and precedes the initiation of infection events. Bacteria are entrapped in a curled root hair, and from this site infection threads (ITs) are initiated. The route of the IT is predicted by pre-infection threads that are densely cytoplasmic

subdomains with aligned cytoskeleton. Its progress into the inner cortex where the nodule primordium has formed is through a series of cell divisions. From these divided cells, the nodule meristem forms.

Depending on the host plant, changes in rhizobial metabolism are accompanied by changes in rhizobial cell size and shape and three distinct types of bacteroids (Vasse et al., 1990; Mergaert et al., 2006; Bonaldi et al., 2011; Haag et al., 2011b). The first type of bacteroids, develop in legumes of the IRLC. Infecting rhizobia undergo repeated rounds of genome amplification, increase their cell size by elongation and can even be branched **(Fig. 1.2 c)** (Bisseling et al., 1977; Paau et al., 1979; Kobayashi et al., 2001; Mergaert et al., 2006). Bacteroids that undergo such type of metamorphosis are terminally differentiated and are no longer viable once extracted from the nodule (Mergaert et al., 2006). The second type of bacteroids is found in legumes such as those of the Dalbergoid clade (*Aeschynomene* and *Arachis* species). Bacteroids are enlarged by either elongation as for example in *Aeschynomene afraspera* or by forming large spheres as in *Arachis*, *Aeschynomene indica* or *Aeschynomene evenia* (Sen and Weaver, 1984; Bonaldi et al., 2011). In contrast, the third type of bacteroids is found in rhizobia that infect phaseoloid legumes (i.e. *Phaseolus, Vignia*, Lotus and Glycine species). In this case, rhizobia do not undergo terminal differentiation and retain a cell shape and size and DNA content similar to free-living bacteria (**Fig. 1.2c**) (Bisseling et al., 1977; Paau et al., 1979; Mergaert et al., 2006). *Rhizobium* strains nodulate legumes of different clades and adopt a bacteroid morphotype according to the host, it was concluded that bacteroid metamorphosis was induced by host factors rather than being encoded in the bacterial genome (Sen and Weaver, 1984; Mergaert et al., 2006; Bonaldi et al., 2011). A class of nearly 600 genes encoding nodule-specific cysteine-rich peptides (NCRs) has been discovered and the expression of more than 300 NCRs confirmed (Mergaert et al., 2003; Graham et al., 2004; Young et al., 2011).

The list of metabolites produced by symbiotic enhancers is as follows: vitamins that may supplement the nutritional requirement of rhizobia (Marek-Kozaczuk and

Skorupska, 2001); hydrolytic enzymes that assist during rhizobial penetration in the root hair, or attack phytopathogenic fungi (Sindhu et al., 2002; Egamberdieva et al., 2010); or P-solubilizing acids that increase phosphorus availability (Elkoca et al., 2008). The use of Nitragina, as a donor of effective *Rhizobium,* for pea plants resulted in slightly higher Green Area Index values (Zając et al., 2013).

The growth of rhizobial infection threads (ITs) in root hairs may be further understood by the given model **(Fig. 1.3; Fig. 1.4)** (Oldroyd et al., 2011). Nod factors (NFs) produced by rhizobia within root hairs are perceived by an NF receptor complex that resides in signaling microdomains at the infection thread apex. One of the mechanisms may be that NFs induce the production of reactive oxygen species (ROS). Although their role is not clear, NADPH oxidases is required for both pollen tube and root-hair growth, so they may play an analogous role in IT growth. One of the main roles of ROS is the activation of calcium channels. The cytoskeleton is also essential for IT growth. Microtubules help to determine the direction of growth at the site of Ca^{2+} influx and actin provides the infrastructure for vesicle delivery at the site of growth root hairs For example, F-actin, which is found in the sub-apex of growing root hairs, along with microtubules participate in the recycling and endocytosis of PM sub domains, plays a key role for the cytoskeleton in signaling. Small G proteins also play crucial roles in support of tip growth. The proximity of the nucleus to the site of root-hair growth is significant, and its movement is mediated by actin. This invagination is associated with bacterial infection, and the promotion of cell division in the cortex leading to the nodule meristem. The nodules and associated symbiosomes are structured for efficient nitrogen fixation (Oldroyd et al., 2011). Infection thread is major key and check point for selection of competitive rhizobia, mutation in LPS and EPS which are cell surface components failed to be released IT, which fails to show symbiosis of rhizobia to plant (Gibson et al., 2008)

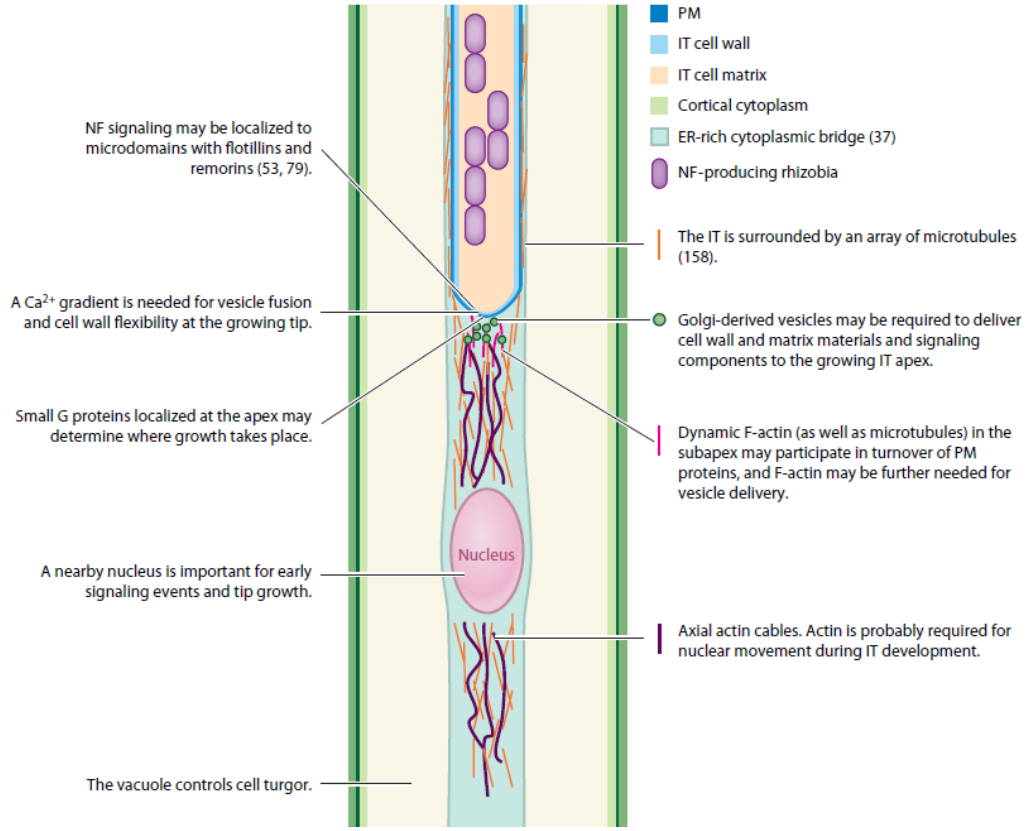


Fig. 1.4: A model for the growth of rhizobial infection threads (ITs) in root hairs (Oldroyd et al., 2011).

1.1.3: *Rhizobium* as nitrogen-fixer

Legume crops are not only used as human diet but also for improving soil fertility through biological nitrogen fixation (Anjum, 2006). Biological nitrogen (N) fixing microbes are free living in soil media that are also found in association with rhizosphere and the tissues (endophytes) of the healthy plant are beneficial for plants systems (**Fig. 1.5**) (Bashan and de-Bashan, 2005; Herridge et al., 2008). N fixing organisms having ability to enhance the N fixation performance, as well as they may also increase nutrient level in soil, which is due to the production of substances like hormones, siderophores, phosphate solubilization, improvement of nutrients, water uptake and also these microbes are also helps to enrich soil fertility and counteract agro environmental problems (Badawi

et al., 2011; Mader et al., 2011). The different N fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in **Fig. 1.5**.

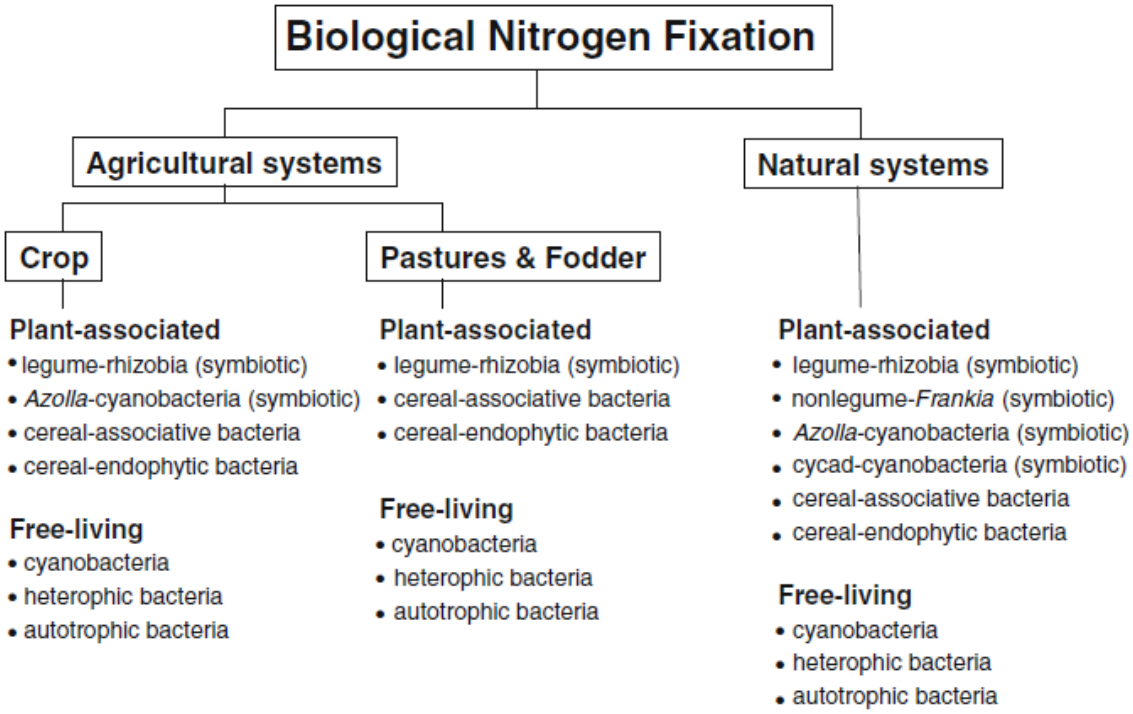


Fig. 1.5: The different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems (Herridge et al., 2008).

In many tropical forests, N inputs via free-living N-fixers, which are ubiquitous and responsive across space and time, are critical for meeting high N demands and loss rates (Houlton et al., 2008; Hedin et al., 2009; Brookshire et al. 2012). Free-living N fixations are an important source of new N to ecosystems (Reed et al., 2011). Based on this nitrogen-fixing symbiosis, legume crops require 35–60% less fossil-based energy than conventional, N-fertilized crops (Jensen et al., 2012). On a global scale, biological nitrogen fixation in the legume–rhizobia symbiosis accounts for roughly 200 million tons of fixed nitrogen per year. Thus, legumes are agriculturally and ecologically very important and account for 25% of the world's primary crop production (Ferguson et al., 2010).

The soil borne, gram-negative *Rhizobia* bacteria have nitrogen reduction capacity catalyzed by nitrogenase, an enzyme not found in plants (Kessel and Hartley, 2000; Raven and Johnson, 2008; Taiz and Zeiger, 2010; Valentine *et al*., 2011).

$$N_2 + 16ATP + 16H_2O + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi + 8H^+$$

This nitrogen fixation process is essential for life, because fixed nitrogen is required to biosynthesize the basic building blocks of life, e.g. nucleotides for DNA and RNA and amino acids for proteins (Raven and Johnson, 2008; Taiz and Zeiger, 2010). The family Fabaceae establishes symbiosis with diazatrophic bacteria (for example, the genus *Rhizobia*, which has the unique ability to establish N_2-fixing symbiosis on legume roots) in exchange for metabolites and reduced carbon (Kessel and Hartley, 2000; Valentine et al., 2011).

Nitrogen fixation is the most energetically expensive reaction known to occur in any plant cell (Raven and Johnson, 2008). Each symbiotically fixed ammonium molecule utilizes 8 ATPs for the reaction and the cost of symbiotic N_2 reduction in legumes is between 2 and 3 mg carbon (C) per mg fixed N, varying according to the species and specific genotypes (Valentine et al., 2011). Symbiotic nitrogen fixation required significantly more energy per N fixed than NO_3^- uptake and reduction. Abiotic stresses account for major reductions in nitrogen fixation, where more than 50% of legume crops are lost worldwide due to drought, salinity, aluminium toxicity and nutrient deficiencies.

1.1.4: Plant Growth Promoting Rhizobzcteria (PGPR)

Interactions between plants and micro-organisms in the rhizosphere can clearly affect crop yields. The most studied PGPR belong to gram-negative genera, and large number of strains belongs to fluorescent pseudomonads (Kloepper, 1993). Plant Growth Promoting Rhizobzcteria (PGPR) are divided into two groups according to the mode of action: PGPR that directly affect plant growth and biocontrol that indirectly benefit the plant growth (**Fig. 1.6; Fig. 1.7; Table 1.3**) (Glick, 1995; 1999). Direct mechanisms of plant growth by PGPR include (i) the provision of bioavailable phosphorus for plant

uptake, (ii) nitrogen fixation for plant use, (iii) sequestration of iron for plants by siderophores, (iv) production of plant hormones like auxins, cytokinins and gibberellins, and (v) lowering of plant ethylene levels (Podile and Kishore, 2006; Bhattacharyya and Jha, 2012). Indirect mechanisms used by PGPR include (i) antibiotic protection against pathogenic bacteria, (ii) reduction of iron available to phytopathogens in the rhizosphere, (iii) synthesis of fungal cell wall-lysing enzymes, and (iv) competition with detrimental microorganisms for sites on plant roots.

Phytohormones like indole-3-acetic acid (IAA), gibberellic acid and cytokinins PGPR increase root surface and length and promote plant development (Kloepper et al., 2007). Several PGPR as well as some pathogenic, symbiotic and free living rhizobacterial species produce IAA and gibberllic acid in the rhizospheric soil and plays a significant role in increasing the root surface area and number of root tips in many plants (Vessey, 2003; Bhattacharya and Jha, 2012). Ethylene, a gaseous phytohormone commonly induced by wounding in plants, causes root growth inhibition. Many PGPR have the capability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which contributes to growth promotion even in stressful conditions such as flooded or soils contaminated with cadmium (Podile and Kishore, 2006).

Microorganisms and plants have evolved specific mechanisms to chelate insoluble iron through the release of siderophores and uptake of iron-siderophore complexes through specific outer membrane receptor proteins to meet their iron requirement (Sharma and Johri, 2003). These siderophores can be of different types: hydroxamates, phenol-catecholates, and carboxylates (Podile and Kishore, 2006). Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability all over world (Compant et al., 2005). Diverse PGPR antagonize the root pathogens through one or more different mechanisms, for example by production of bacterial allelochemicals such as volatile or non-volatile antibiotics, siderophores, detoxification enzymes, lytic enzymes and other secondary metabolites like HCN (Podile and Kishore, 2006).

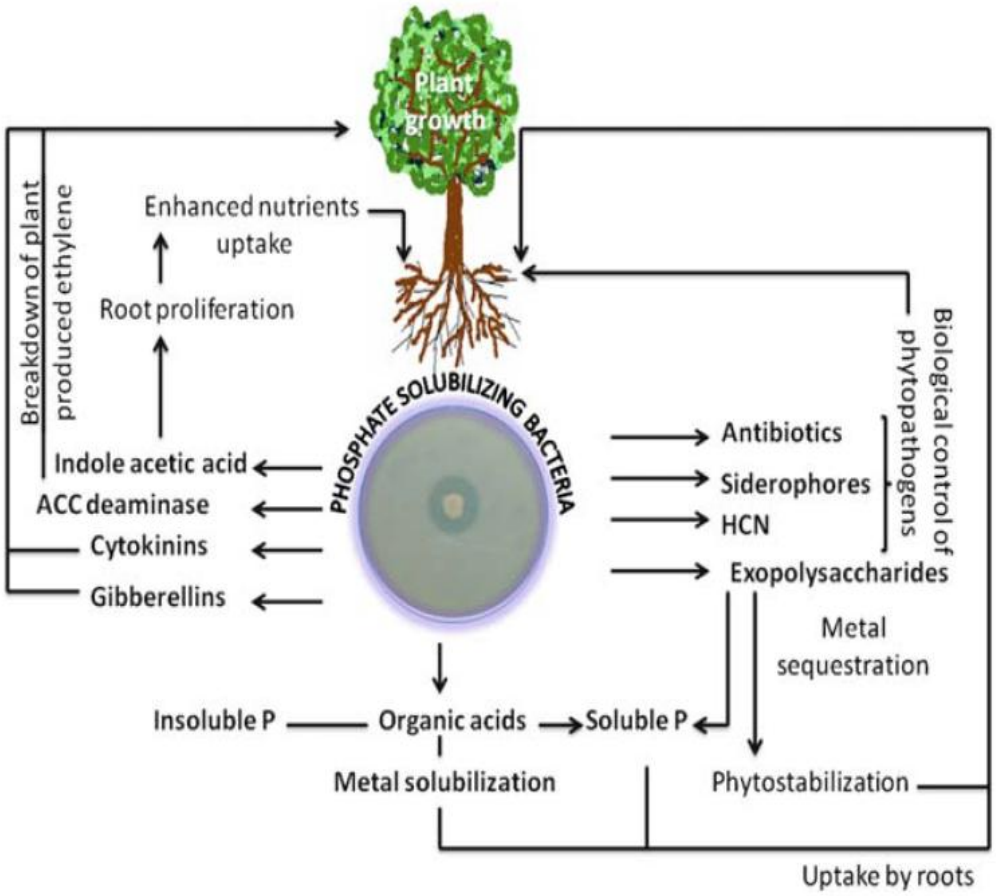


Fig. 1.6: Mechanism of growth promotion by phosphate solubilizing bacteria (Zaidi et al., 2009).

Production of these compounds is highly influenced by the qualitative and quantitative nutrient availability and is also subjected to quorum sensing (Haas and Keel, 2003). Many PGPR present the ability to produce peptide antibiotics, which are oligopeptides that inhibit synthesis of pathogens cell walls, influence membrane structures of cells, and inhibit the formation of initiation complex on small subunit of ribosomes system of the plant (Maksimov, et al., 2011). It was revealed that *B. subtilis* surfacine is able to stimulate induced systemic resistance by activation of components like lipoxygenases, lipid peroxidases and the formation of reactive oxygen species (Maksimov et al., 2011).

Table 1.2: Growth-promoting substances released by phosphate solubilizing bacteria (Zaidi et al., 2009).

Phosphate solubilizing bacteria	Plant growth-promoting traits	References
Pseudomonas sp., Bacillus sp.	IAA, siderophore	Rajkumar et al. (2006)
Bacillus spp.	IAA, siderophores, ammonia production, HCN, chromium reduction, metal solubilization	Wani et al. (2007a, 2007b, 2007c)
Mesorhizobium loti MP6	HCN, IAA	Chandra et al. (2007)
Dyella ginsengisoli, Burkholderia kururiensis, Pandoraea sp. ATSB30	Siderophore, IAA, salicylic acid, ACC Deaminase	Anandham et al. (2008)
Pseudomonas sp.	siderophore ACC deaminase, IAA,	Poonguzhali et al. (2008)
Bacillus subtilis	IAA, siderophore, antifungal activity	Singh et al. (2008)
Serratia marcescens	IAA, siderophore, HCN	Selvakumar et al. (2008)
P. fluorescens	ACC deaminase	Shaharoona et al. (2008)
Acinetobacter sp., Pseudomonas sp.	ACC deaminase, IAA, antifungal activity, N_2-fixation	Indiragandhi et al. (2008)
Enterobacter sp.	ACC deaminase, IAA, siderophore	Kumar et al. (2008)
Burkholderia	ACC deaminase, IAA, heavy metal solubilization siderophore,	Jiang et al. (2008)
P. jessenii	ACC deaminase, IAA, heavy metal solubilization siderophore,	Rajkumar and Freitas (2008)
P. aeruginosa	ACC deaminase, siderophore, IAA	Ganesan (2008)
Mesorhizobium sp., Bacillus sp., Azotobacter sp., Pseudomonas sp.	IAA, siderophore, antifungal activity, ammonia production, HCN	Ahmad et al. (2008)
P. aeruginosa, P. mosselii, P. plecoglossicida	Siderophore, IAA, protease, cellulose and HCN	Jha et al. (2008)

Some PGPR can induce plant growth promotion through a combination of modes of action and act synergistically to stimulate the growth of the host plant (Antoun et al., 1998; Belimov et al., 2001; Vessey, 2003; Dey et al., 2004; Khan et al., 2009). ACC deaminase activity along with expression of one or more of the traits such as suppression

of phytopathogens, solubilization of tricalcium phosphate, production of siderophore and/or nodulation promotion by the PGPR contributed to the enhancement of growth, yield, and nutrient uptake of peanut (Dey et al., 2004). Seed inoculation of rice (cv. Naveen) by the six individual PGPR isolates had a considerable impact on different growth parameters including root elongation that was positively correlated with ACC deaminase activity and IAA production and also showed other plant growth attributes including ammonia production and at least two isolates produced siderophores (Bal et al., 2013).

1.1.4.1: *Rhizobium* as PGPR for non-legumes.

Rhizobia also have an excellent potential to be used as PGPR and PSM with non-legume plants (Chabot, 1996). Beyond nitrogen fixation, rhizobia also colonize roots of non-legume species and promote their growth without forming any nodule-like structure (Mehboob et al., 2009; Mia and Shamsuddin, 2010). Inoculation with *Rhizobium* had significant effect on the plant height, number of branches, root and shoot dry weight, number of nodule, seed and biomass yields, number of pod, crude protein rate and phosphorus content of seed (Erman et al., 2009). Increased rice production was seen by inoculation with a *Rhizobium leguminosarum* bv. trifolii strain (Yanni and Dazzo, 2010). *R. leguminosarum* PETP01 and TPV08 are excellent biofertilizers for tomato and pepper in different production steps leading to increased yield and quality (Garcı´a-Fraile1 et al., 2012). Rhizobia strains establish endophytic relationships with rice plants to promote shoot growth and enhance grain production (Biswas et al., 2000a, b; Mia and Shamsuddin, 2010; Costa, et al., 2013). One native rhizobia, POA3 isolated from the Porto Alegre locality promoted growth of white clover (*Trifolium repens*) and rice plants (*Oryza sativa*) (Granada, et al., 2013).

The symbiotic rhizobia isolated from leguminous plants also promote plant growth *via* their inherent PGP capacities: siderophores and indolic compound production and nutrient solubilization (Ashraf et al., 2013; de Souza et al., 2013; Jida and Assefa, 2013). Indolic compounds production was the most common characteristic of the rhizobia

species isolated from *Cajanus cajan* (Dubey et al., 2010). Crop enhancement, plant nutrients like P, K, Ca, Mg and even Fe accumulation and biofertilizer attributes were observed in cereal crops due to rhizobial inoculation (Mia and Shamsuddin, 2010) Rhizobia also promotes plant growth by synthesis of vitamins, phytohormones and enzymes, producing siderophores, dissolving phosphates and other nutrients and prevention deleterious effects of phytopathogenic microorganisms besides biological nitrogen fixation (Boiero et al., 2007; Hayat et al., 2010; Ahemad and Khan, 2011).

The effects of *Rhizobium* inoculation, lime and molybdenum supply on yield and yield components of *Phaseolus vulgaris* L. significantly improved the number of pods per plant, number of seeds per plant, 100-seed weight and seed yield (Bambara and Ndakidemi, 2010). On average, an increase of 4-5% in crop yield has an important impact in agricultural production.

1.1.5: Effect of P on nodulation

Next to nitrogen, phosphorus is the most important element for adequate grain production. The evolution of science, particularly in the past century, has clearly demonstrated the significance of phosphorus for all animal and plant life on the earth (Ryan et al., 2012). Calcium increased root growth, number of nodule primordia, nodules, and growth of the soybean plant (Waluyo et al., 2013). Ca and P had a synergistic effect on BNF of soybean in acid soils. Ca is important for the establishment of nodules, whilst P is essential for the development and function of the formed nodules. P increased number of nodule primordia, thus it also has an important role in the initiation of nodule formation. This effect of P supply on nodule formation is because P supply affects the production of root-exudates including flavonoids that trigger nod-gene expression to form nodules, and also plays a role in nodule cell metabolism that affects nodule development (Raghothama et al., 1999; Abel et al., 2002). Improved soil P status enhanced the positive effect of elevated [CO_2] on grain yield, biomass and shoot total N and P contents of the legumes tested (Lam et al., 2012). Besides contributing to plant growth by making

soluble phosphorus more available, the legume-nodulating strains increased levels of soluble phosphate, thus improving the efficiency of biological nitrogen fixation (Silva et al., 2006). A positive correlation is observed between BNF and P availability in natural soils (Pearson and Vitousek, 2002; Labidi et al., 2003). Wherever soil P availability is low, elevated CO_2 does not increase BNF, and pasture quality decreased because of a reduction in above ground; at low P availability, there is a limited response of biomass production by grass community (Edwards et al., 2005; 2006).

Crop growth and yield reduce greatly due to low P availability especially for legumes, since legume nodules responsible for N_2 fixation have high P requirements (Sulieman and Tran, 2012). P is often the limiting element for biological productivity (Sato and Miura, 2011; Lopez-Arredondo and Herrera-Estrella, 2012). P deficiency is one of the critical limiting factors, adversely affecting nodulation and N_2 fixation, and thus legume growth and productivity, worldwide (Tesfaye et al., 2007). *M. truncatula* plants inoculated with either the *S. meliloti* 102F51 or 2011 strain but due to P deficiency severely inhibited plant growth and development of nodules as well as N and P assimilation (Sulieman and Schulze, 2010*a*). Plants engaged in symbiotic N_2 fixation have high P demand (Vance et al., 2003; Sulieman et al., 2013a). Low levels of phosphorus affected symbiosis by decreasing the supply of photosynthates to the nodule, which reduced the rate of bacterial growth and the total population of legume-nodulating microorganisms (Moreira et al., 2010). P availability dominantly controlled free-living N fixation in tropical rain forest (Reed et al., 2013). Thus, the efficiency of nitrogen fixation by the strains approved as inoculants may be related to a greater ability to solubilize low soluble phosphates.

1.1.6: Phosphorus deficiency and Nitrogen fixation

Phosphorus deficiency is commonly reported along with Al^{3+} toxicity as 40% of the world's arable soil is considered acidic. Phosphorus deficiency and Aluminium (Al^{3+}) toxicity are associated with each other in acid soils, and they both have major effects on legume plant growth and function and are collectively considered as inseparable factors

that limit crop productivity on such soils (Ward et al., 2008). Nodule biomass is strongly correlated to P availability to plants as about 3 times more P is required by nodules than the surrounding root tissues. An increase in P supplied to host legume plants led to a 4-fold increase in nodule mass (Olivera et al., 2004). P deficiency in soil severely limits plant growth productivity, in legumes, and this has a deleterious effect on nodule formation, development and function (Haque et al., 2005). Nodule construction cost and growth respiration of soybeans increased with P deficiency (Andrews et al., 2009). In the case of legumes, more P is required by symbiotic than non-symbiotic plants. Symbiotic nitrogen fixation (SNF) has a high demand for P, with up to 20% of total plant P being allocated to nodules during N_2 fixation. The process consumes large amounts of energy, such that the energy generating metabolism is depended upon the availability of P (Schulze et al., 1999; Schenk, 2012). The effects of P deficiency may be direct, as P is needed by nodules for their growth and metabolism, or indirect. The high requirement of P are linked to its role in nodule carbon and energy metabolism, therefore as the deficiency may affect the supply of carbon to the nodules, the bacteria will have greater respiratory demand on the host plant during nitrogen fixation (Sar and Israel, 1991; Valentine et al., 2011).

1.1.7: *Rhizobium*- PSM co-inoculation

Considering the main limitations to the biological N_2 fixation with soybeans and common beans inoculated with rhizobia and the benefits to crop growth attributed to *Azospirillum*, co-inoculation with both microorganisms might improve plant's performance. This approach is current with modern demands of agricultural, economic, social and environmental sustainability (Chaparro et al., 2012). Many evidences are there to show that co-inoculation with *Rhizobium* and PSM have additive or synergistic effect on plant growth and crop yield (**Table 1.4**) (Morel and Brana, 2012). The results confirm the feasibility of using rhizobia and PSM such as azospirilla as inoculants in a broad range of agricultural systems, replacing expensive and environmentally unfriendly N-fertilizers (Hungria et al., 2013).

Table 1.3: Ten years of studies on legume co-inoculation (2002-2012). Increase in legume symbiotic parameters and yield by co-inoculation compared to single-inoculation with rhizobia. Abbreviations are as follows: RDW: root dry weight; SDW: shoot dry weight; RL: root length; NN: nodule number; NFW: Nodule fresh weight; PDW: plant dry weight; PFW: plant fresh weight (Morel et al., 2012).

Rhizobium and PSM	Host plant	Observation (% increase)	Reference
Rhizobium and PSB	chickpea	enhanced nodulation, plant growth, yield and nutrient uptake	Rudresh et al., 2005.
R. leguminosarum D293 and AM fungi	Pea	increased plant biomass, nodulation parameters, N_2 fixation activity, increased significantly total P content in plant tissues and percentage of root colonization	Stancheva et al., 2006.
Bradyrhizobium sp. (*Vigna*) and *B. subtilis*	green gram plants	increased dry matter yield, chlorophyll content in foliage and N and P uptake	Zaidi and Khan, 2006
Bradyrhizobium spp./ *Rhizobium* and PGPR	legumes	increased root and shoot biomass, nodule dry matter, nitrogenase activity, N_2-fixation, and grain yield	Elkoca et al., 2008.
B. japonicum USDA110- P. putida	Mung bean (*Vigna radiata*	Increase in total Biomass and in Nodule Number	Shaharoona et al., 2006
Rhizobium sp. *-P.putida/ P. fluorescens/B. cereus*	Pigeon pea (*Cajanus cajan*)	Increase in Nodule Number	Tilak et al., 2006
Rhizobium sp.*-Bacillus* spp.	Pigeon pea (*Cajanus cajan*)	Increase in plant fresh weight and in Nodule Number	Rajendran et al., 2008
R. leguminosarum- B. thuringeinsis	Lentin (*Lens Culinaris L.)*	Increase in plant fresh weight and in Nodule Number	Mishra et al., 2009
R. leguminosarum- B. thuringeinsis	Pea (*Pisum sativum* L. cv. Capella	Increase in plant fresh weight and in Nodule Number	Mishra et al., 2009
R. leguminosarum bv viceae -P. fluorescens	Pea (*Pisum sativum* L. cv. Capella	Increase in plant dry weight	Kumar et al., 2001
R. leguminosarum bv. *viciae - A. brasilense* 30	Vetch (*Vicia sativa*)	Increase in SDW, nod gene induction and decrease in indoles content	Star et al., 2011

R. galegae bv. *orientalis* - *Pseudomonas* spp.	Galega (*Galega orientalis*)	Increase in SDW RDW and in Nodule Number	Egamberdieva et al., 2010
S. meliloti - *Delftia* sp.	Alfalfa (*Medicago sativa*)	increase in SDW and in nodulation rate	Morel et al., 2011
Bradyrhizobium sp. -*Pseudomonas* sp./ *Ochrobactrum cytisi*	Altramuz (*Lupinus luteus*)	66 in SDW and 20-40, 25, and 30-50 decrease in Cd, Cu and Zn – accumulation in roots, respectively	Dary et al., 2010
R. leguminosarum bv.trifolii - *P. fluorescens*	Clover (*Trifolium repens*)	20 in SDW; 100 in Nodule Number	Marek-Kozaczuk and Skorupska, 2001
R. leguminosarum bv. trifolii - *Delftia* sp	Clover (*Trifolium repens*)	50 in SDW and 80 in nodulation rate	Morel et al., 2011
Rhizobium sp. - *Thiobacillus* sp.	Peanut (*Arachis hypogaea*)	50 in PDW; 80 in Nodule Number	Anandham et al., 2007
M. ciceri - *Azotobacter chroococcum*	Chickpea (*Cicer arietinum*)	15 in Nodule Number; 25 in P-soil availability	Qureshi et al., 2009
M. ciceri - *Pseudomonas* sp/*Bacillus* sp	Chickpea (*Cicer arietinum*)	20 in PDW; 30 in Nodule Number; 100 in P-uptake	Wani et al., 2007
Mesorhizobium sp. *Cicer* - *Pseudomonas* spp.	Chickpea (*Cicer arietinum*)	70 in Nodule Number; 30 in SDW, 30 in Nuptake	Goel et al., 2002
Mesorhizobium sp. *Cicer* -*Pseudomonas* spp.	Chickpea (*Cicer arietinum*)	1,2-1,86 in Nodule Number; 1,3-2,11 NFW; 1-2,93 in PDW	Malik and Sindhu, 2011
Rhizobium -*B. subtilis/ megaterium*	Chickpea (*Cicer arietinum*)	18 in SDW; 16-30 in RDW; 14 in total biomass yield	Elkoca et al., 2008
Rhizobium spp. -*A. brasilense*	Common bean (*Phaseolus vulgaris*)	30 total yield	Remans et al., 2008b
R. tropici - *Paenibacillus Polymyxa*	Common bean (*Phaseolus vulgaris*)	50 in Nodule Number; 40 in N uptake in non-drought stress	Figuereido et al., 2008
R. tropici/etli - *A. brasilense*	Common bean (*Phaseolus vulgaris*)	18-35 and 20-70 in RDW; 29 and 28 in SDW under non saline and saline conditions, respectively.	Dardanelli et al., 2008
R. etli - *C. balustinum*	Common bean	35 in SDW; 35 in Nodule Number under non-saline	Estevez et al., 2009

	(*Phaseolus vulgaris*)	conditions; and 39 in SDW; 63 in RDW under saline conditions	
Rhizobium spp. - *P. putida /B. subtilis/A. brasilense*	Common bean (*Phaseolus vulgaris*)	30 in Nodule Number; 20 in SDW; 30-45 in RDW	Remans et al., 2007
Rhizobium spp. - *A. brasilense*	Common bean (*Phaseolus vulgaris*)	70 in Nodule Number	Remans et al., 2008a
R. tropici - Paenibacillus polymyxa	Common bean (*Phaseolus vulgaris*)	50 in Nodule Number; 40 in N uptake in non-drought stress	Figuereido et al., 2008
Rhizobium spp. - *P. fluorescens /A. lipoferum*	Common bean (*Phaseolus vulgaris*)	25 in Nodule Number; 13 in SDW; 74 in seed yield	Yadegari et al., 2010
S. meliloti B399 and the *Bacillus* sp. M7c	alfalfa plants	increase in root and shoot dry weight, length, and surface area of roots, number, and symbiotic properties	Guiñazú et al., 2010; Lorena et al 2010.
E. fredii - Chryseobacterium balustinum	Soybean (*Glycine max*)	56 and 44 in SDW; 100 and 200 in RDW; 155 and 286 in Nodule Number	Estevez et al., 2009
B. japonicum USDA110- P. putida	Soybean (*Glycine max*)	40 in SDW; 80 in Nodule Number; 45 in RDW	Rosas et al., 2006
B. japonicum USDA110- B. subtilis/ S. proteamaculans	Soybean (*Glycine max*)	12 in SDW; 10 in P-uptake	Han and Lee, 2005
B. japonicum USDA110- A. brasilense	Soybean (*Glycine max*)	47 in Nodule Number	Cassán et al., 2009
Rhizobium and PSB	grass	enhanced nodulation and increased the number and weight of nodules	Abusuwar and Omer, 2011.
Pseudomonas fluorescens P-93 and *Azospirillum lipoferum S-21* *Rhizobium* strains Rb-133 and Rb-136	Common bean seeds	increased nodule number and dry weight, shoot dry weight, amount of nitrogen fixed as well as seed yield and protein content.	Yadegari et al., 2010

*B. japonicum USDA110*RCR 3407 strain *B. subtilis*	Common bean seeds	influence plant growth, vitality, and the ability of the plant to cope with pathogens	Elkoca et al., 2010; Tsigie et al., 2012
Rhizobium and phosphate solubilizing bacteria	faba bean plants	increased yield and seed quality decreased seeds carbohydrate content	Rugheim and Abdelgani, 2012
Pseudomonas and *Rhizobium* isolates	common bean	improved growth and yield production	Samavat et al., 2012
Rhizobium and AM fungi	Chick pea plant	significantly increased fresh and dry weights of shoot and root	Moradi et al., 2013
P. chlororaphis and A. pascens amendment with RP	Walnut	highest plant height, shoot and root dry weight, P and nitrogen (N) uptake of walnut seedlings, and the maximum amounts of available P and N in soils	Xuan Yu et al., 2012
*B. japonicum USDA110*with *A. brasilense*	Soybean and common bean	increased seed yield, improved nodulation	Hungria et al., 2013
tetra inoculants *R. leguminosarum* + *A. chroococcum* + *P. aeruginosa* + *T. Harzianum*, tri inoculants of *R. leguminosarum* + *A. chroococcum* + *P. aeruginosa* and *R. leguminosarum* + *A. chroococcum* + *T. harzianum*	Common bean seeds	significant nodulation, grain yield and nutrient uptake	Varma and Yadav, 2012

1.1.8: Phosphate solubilization by *Rhizobium* spp.

The phosphate-solubilizing activity of *Rhizobium* is associated with the production of 2-ketogluconic acid, indicating that phosphate-solubilizing activity of the organism is entirely due to its ability to reduce pH of the medium (Halder and Chakrabarty, 1993). Since 1950s it is reported that P-solubilizing bacteria release phosphorus from organic and inorganic soil phosphorus pools through mineralization and solubilization **(Fig. 1.7)** (Khan et al., 2009).

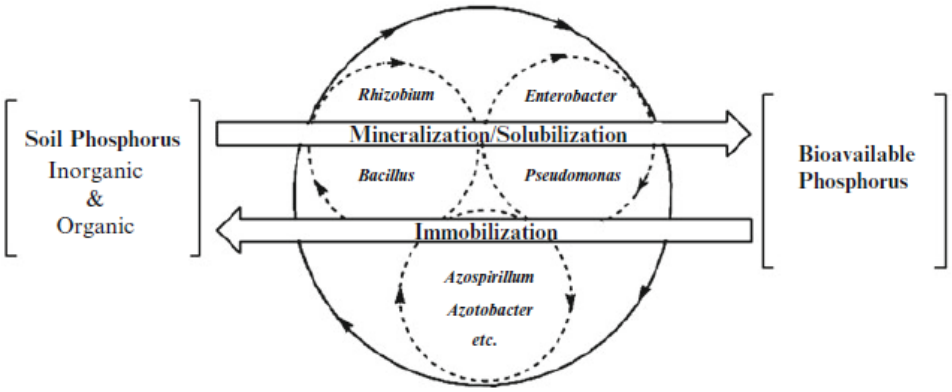


Fig.1.7: Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al. 2009)

General sketch of P solubilization in soil is shown in **(Fig. 1.8).** A wide range of microbial P solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey, 1982). Phosphatic rocks are solubilized by acid producing microorganisms to release more P for plant uptake (Gyaneshwar et al., 2002). A few strains or species of *Rhizobium* are involved in phosphate solubilization also along with symbiotic nitrogen fixation (Deshwal et al., 2003). Lowering of soil pH by microbial production of organic acids such as acid phosphatases, lactate, citrate, and succinate, gluconic and 2-ketogluconic acids etc. and proton extrusion is the main principal mechanism of mineralization of organic form of phosphorus (Goldstein, 1995; Deubel et al., 2000). Phosphate availability in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes (Alikhani et al., 2006). Many phosphate-solubilizing bacteria are

found in soil and in plant rhizospheres and potentially represent 40% of the culturable population (De Freitas, et al., 1997; Richardson, 2000; Chen et al., 2006). PBS produces a range of organic acids such as citrate, lactate, and succinate that solubilize mineral phosphates. To make P available for plant nutrition *Bacillus*, *Pseudomonas*, *Klebsiella* and *Enterobacter* spp. are involved in the stepwise degradation of phytate to lower phosphate esters of myo-inositol and phosphorous by means of acid and alkaline phosphatase enzymes (Podile and Kishore, 2006). Bacteria also enhance phosphorus availability to crops by solubilizing precipitated forms of phosphorus (Chen et al., 2006). Single and dual inoculation with *Rhizobium* along with P fertilizer is 30-40% better than only P fertilizer for improving grain yield of wheat, where *Rhizobium* with non-legumes could act as phosphate solubilizer, hormone producer and to some extent as N-fixer (Afzal and Bano, 2008). Bacteria assimilate soluble phosphorus, and make it available by preventing it from adsorption (Khan and Joergensen, 2009). Phosphate solubilization activity of rhizobia is related with the production of 2-ketogluconic acid, due to its ability to reduce pH of the medium (Hayat et al., 2010). During phosphate solubilization, the nature of organic acid produced by rhizobia is more important than the quantity (Hayat et al., 2010).

Rhizobium ciceri inoculation and phosphorus application in combination increased growth rate and P utilization of chickpea cultivars as compared to the control, greatly affected the P Efficiency Index (EI) and P utilization performance of chickpea cultivars.(Karaman et al., 2013). Certain strains of *R. leguminosarum* (bv. viciae, bv. phaseoli, bv. trifolii), *R. leguminosarum* sp, *B. japonicum, Mesorhizobium ciceri, Mesorhizobium mediterraneum* and *S. meliloti* are good P-solubilizers (Antoun et al., 1998; Peix et al., 2001; Alikhani et al., 2006; Daimon et al., 2006; Rivas, 2006; Boiero et al., 2007). *B. japonicum USDA110*518 strain showed the ability to solubilize insoluble tricalcium phosphate (Marinkovic et al., 2013).

1.1.9: Phosphorus in agriculture

Phosphorous is going to be plant nutrient that will limit the agricultural production in the next millennium**.** It is a major growth-limiting nutrient, and unlike the

case of nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa et al., 2002). As regards the role of P, it stimulates root development and growth, gives plant rapid and vigorous start leading to better tillering, essential for many metabolic processes in plant life and for seed formation and organization of cells, encourages earlier maturity. In most soils, its content is about 0.05% of which only 0.1% is plant available (Achal et al., 2007). About 20-25% of total phosphorous in arid soils of India is organic in nature and 68% organic phosphorous in the soil is present as phytin (Yadav and Tarafdar, 2007), which are not directly available to plants. Phosphorous is taken up from soil in the form of soluble orthophosphate ions; $H_2PO_4^{-1}$, HPO_4^{-2} and PO_4^{-3} and generally the availability of these ions to the plants is in the order of $H_2PO_4^{-1} > HPO_4^{-2} > PO_4^{-3}$. Only about 20% of the phosphorus used in agriculture reaches the food we consumed, most of the rest is lost in inefficient steps along the phosphorus cycle (Cordell et al, 2011) (**Fig. 1.8**).

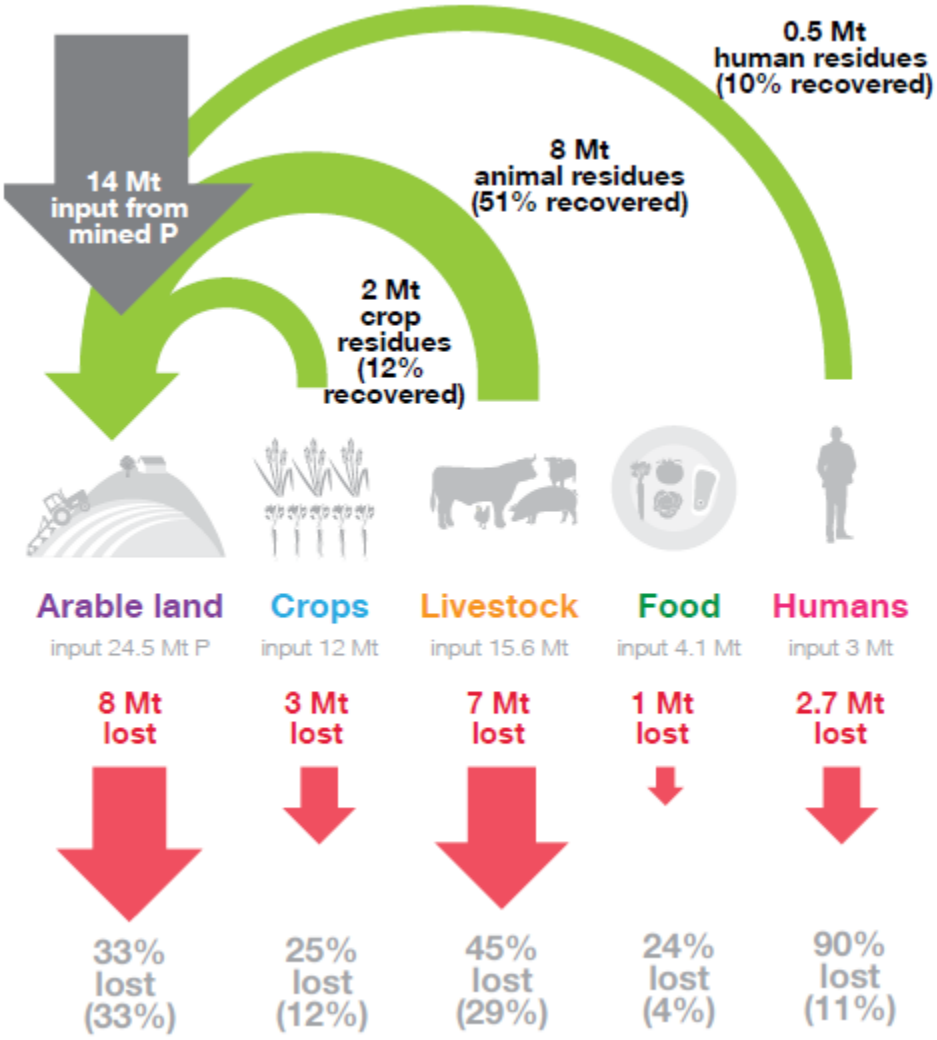


Fig. 1.8: Simplified cycle of phosphorus in agriculture (based on data from Cordell et al., 2009 and 2011). Red arrows represent losses into water systems ultimately, and green arrows represent current recoveries into arable land from the different subsystems. The percentages under the red arrows represent the percentage losses from each subsystem, and shown in brackets are the percentage losses relative to the total input into agriculture land. For example, the livestock system loses about 45% of the phosphorus entering the livestock system itself, and this represents about a 29% loss of the phosphorus entering the agriculture system overall.

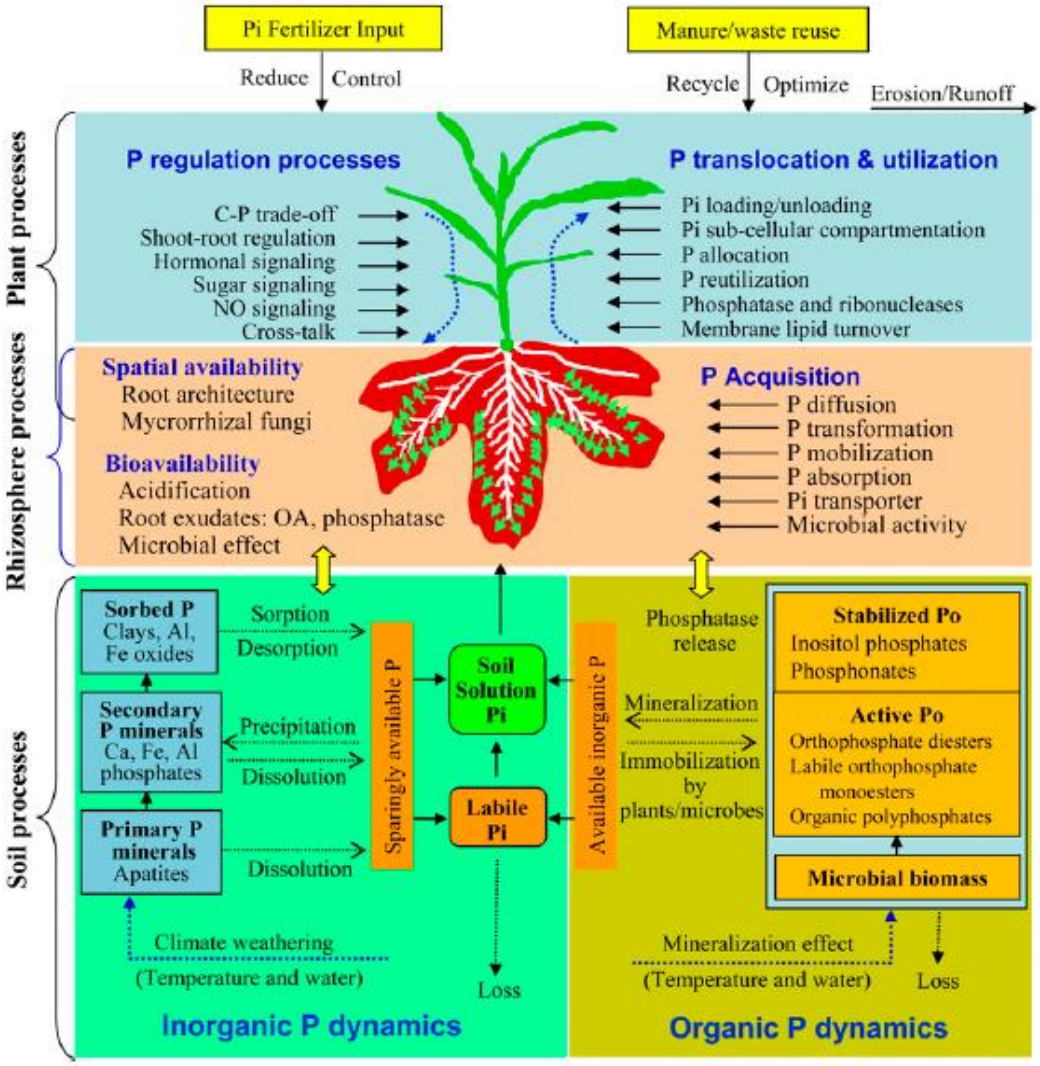


Fig. 1.9: P dynamics in the soil/rhizosphere-plant continuum. C-P, Carbon- P; NO, nitric oxide; OA, organic acids (Shen,et al 2011).

Rhizobacteria secrete organic acids as end products or by-products of primary metabolism. In most cases, sugars are catabolized by glycolytic or Entner-Doudroff pathway. The amount of the organic acid secretion differs between members of the same genus and sometimes between strains of the same species due to presence or absence of enzymes (Vyas and Gulati, 2009; Buch et al., 2010). Organic acids of aerobic or anaerobic respiration such as gluconic acid, 2-ketogluconic acid are directly formed extracellularly or in the in the periplasm by the membrane bound enzymes (**Fig. 1.10)** (Archana et al., 2012). However, organic acids formed by intracellular enzymes require

specific transport proteins that aid in their extracellular secretion. Mono-, di- and tri-carboxylate transporters are located in the plasma membrane mediate their secretion.

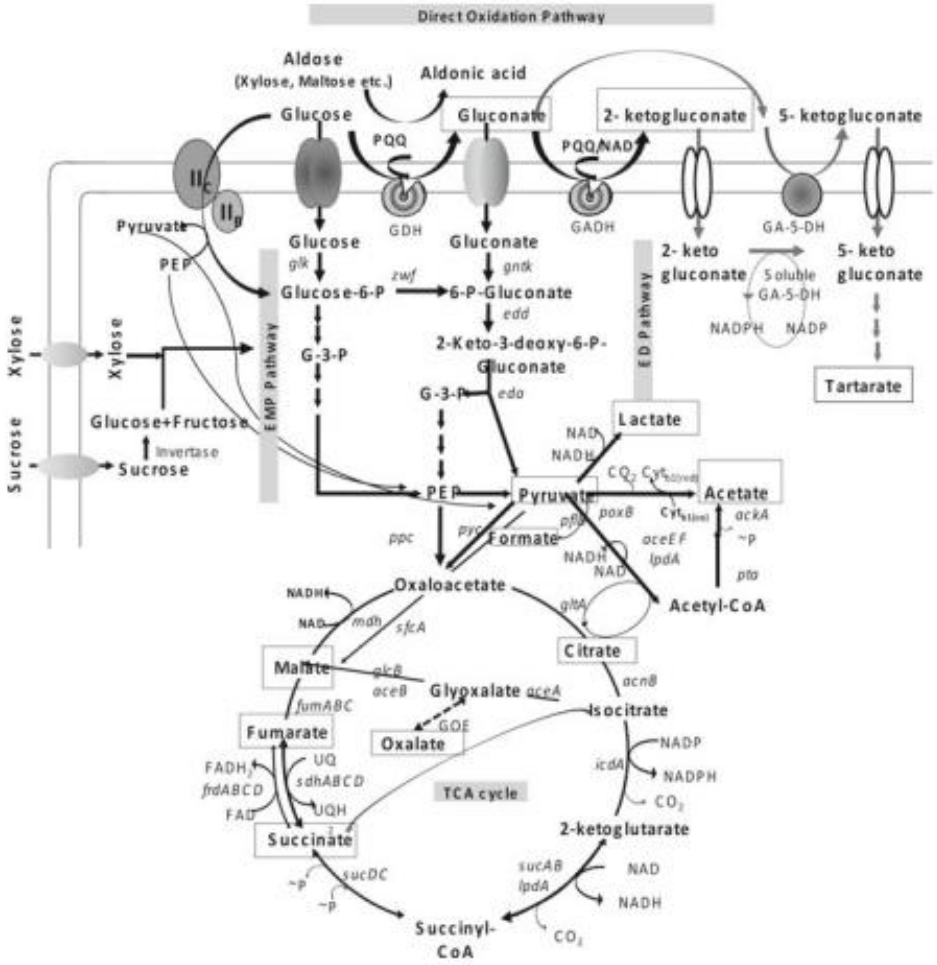


Fig.1.10: Pathways and enzymes involved in organic acid biosynthesis by rhizobacteria (Archana et al., 2012). The organic acids secreted are depicted in boxes. The diagram depicts a comprehensive set of pathways – all may not be present in any given organism. Abbreviations: GDH glucose dehydrogeanse, GADH gluconate dehydrogenase, GA-5-DH gluconate-5-dehydrogenase, glk Glucokinase, zwf Glucose-6-phosphate dehydrogenase, gntk Gluconate kinase, edd 6-phosphogluconate dehydratase, eda 2-keto-3-deoxy-6-phosphogluconate aldolase, ppc phosphoenolpyruvate carboxylase, pyc pyruvate carboxylase, gltA citrate synthase, acnB Aconitase, icdA Isocitrate dehydrogenase, icl Isocitrate lyase, sucABa ketoglutarate dehydrogenase, sucDC succinyl-CoA synthetase, sdhABCD succinate dehydrogenase, fumABC Fumarase, frdABCD fumarate reductase, mdh Malate dehydrogenase, sfcA malic enzyme, aceA Isocitrate lyase, aceB/glcB Malate synthase, GOE Glyoxalate oxidizing enzyme, ldh Lactate dehydrogenase, aceEF-lpdA pyruvate dehydrogenase, pta phosphotransacetylase, ackA acetate kinase A, poxB pyruvate oxidase, pfl pyruvate formate lyase.

Table 1.4: Organic acids involved in P-solubilization and produced by PS bacteria (Zaidi et al., 2009).

Bacterial communities	Organic acids produced	References
Burkholderia cepacia DA23	Gluconic	Song et al. (2008)
Pseudomonas corrugata (NRRL B-30409)	Gluconic, 2-ketogluconic	Trivedi and Sa (2008)
Citrobacter sp. DHRSS	Acetic, gluconic	Patel et al. (2008)
Burkholderia, Serratia, Ralstonia and Pantoea	Gluconic	Elizabeth et al. (2007)
Bacillus, Rhodococcus, Arthrobacter, Serratia and one Chryseobacterium, Delftia, Gordonia, Phyllobacterium, Arthrobacter ureafaciens, Phyllobacterium myrsinacearum, Rhodococcus erythropolis and Delftia sp.	Citric, gluconic, lactic, succinic, propionic	Chen et al. (2006)
Enterobacter intermedium	2-ketogluconic	Hwangbo et al. (2003)
B. amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio proteolyticus, xanthobacter agilis, E. aerogenes, E. taylorae, E. asburiae, Kluyvera cryocrescens, P. aerogenes, Chryseomonas luteola	Lactic, itaconic, isovaleric, acetic isobutyric	Vazquez et al. (2000)
Pseudomonas cepacia	Gluconic, 2-ketgluconic	Bar-Yosef et al. (1999)
Bacillus polymyxa, B. licheniformis, Bacillus spp.	Oxalic, citric	Gupta et al. (1994)

Table 1.5 Organic acids produced by phosphate solubilizing fungi (Zaidi et al 2009)

Organism	Organic acids produced	References
Aspergillus niger	Gluconic, oxalic	Chuang et al. (2007)
Penicillium oxalicum	Malic, gluconic, oxalic	Shin et al. (2006)
Aspergillus flavus, A. niger, Penicillium canescens	Oxalic, citric, gluconic succinic	Maliha et al. (2004)
Penicillium rugulosum	Citric, gluconic	Reyes et al. (2001)
A. niger	Succinic	Vazquez et al. (2000)
Penicillium variabile	Gluconic	Fenice et al. (2000)
Penicillium rugulosum	Gluconic	Reyes et al. (1999)
Penicillium radicum	Gluconic	Whitelaw et al.(1999)
P. variabile	Gluconic	Vassilev et al. (1996)
A. niger	Citric, oxalic, gluconic	Illmer et al. (1995)

A. awamori, A. foetidus, A. tamari, A. terricola,A. amstelodemi,	Oxalic, citric	Gupta et al. (1994)
A. japonicus, A. foetidus	Oxalic, citric gluconic succinic, tartaric	Singal et al. (1994)

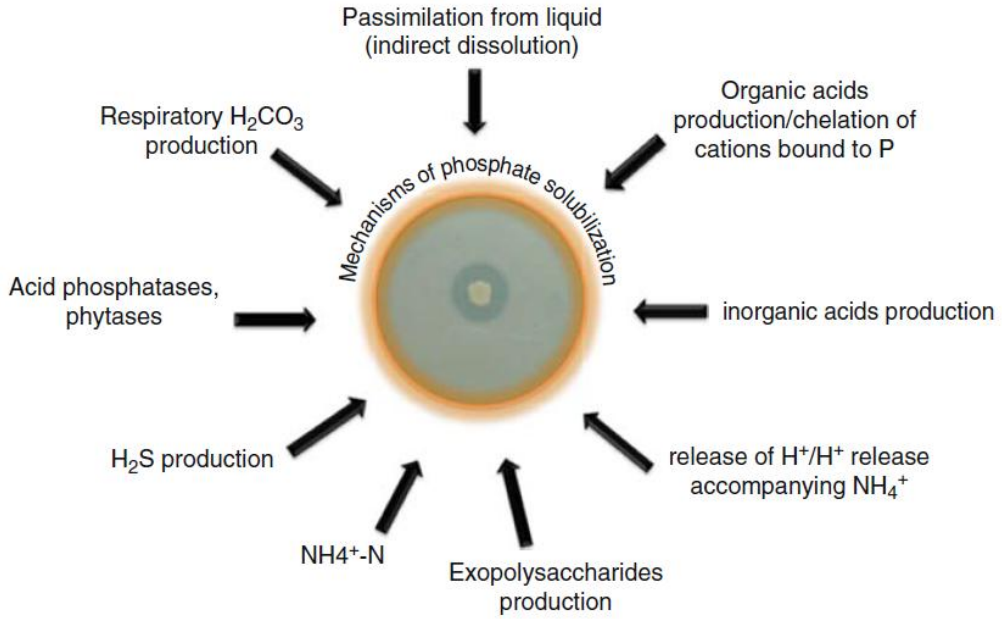


Fig. 1.11: Mechanisms of P-solubilization by phosphate solubilizing bacteria (Zaidi et al., 2009).

PS microbes are well known for making soluble P accessible for uptake by plants. They can also facilitate growth and development of plants by producing essential nutrients (Thomas et al., 2005) or by changing the concentration of plant growth promoting substances including phytohormones such as indoleacetic acid (Wani et al., 2007a, b), through asymbiotic or symbiotic N_2 fixation (Zaidi, 2003; Zaidi and Khan, 2007), soil conditioning, exhibiting bio-control activity (Pandey et al., 2006), by synthesizing siderophores (Vassilev et al. 2006), antibiotics, and cyanide (Lipping et al., 2008), by synthesizing an ACC deaminase that can modulate plant ethylene levels (Anandham et al., 2008; Poonguzhali et al., 2008), and by solubilizing or reducing the toxicity of metals (bioremediation) (Khan et al., 2009).

1.2: Central Carbon Metabolism

The term "central carbon metabolism" (CCM) describes the integration of pathways of transport and oxidation of main carbon sources inside the cell. In most bacteria, the main pathways of the CCM are those of the phosphotransferase system (PTS), glycolysis, ED pathway gluconeogenesis, pentose phosphate (PP) pathway, and the tricarboxylic acid cycle (TCA) with the glyoxylate bypass. As a whole, the system has a complex structure and it is regulated by complex networks of reactions. The knowledge about regulation in CCM has great industrial relevance as it allows the engineering of selected metabolic steps to reroute carbon fluxes toward precursors for industrially important metabolites (Nielsen, 2011).

Carbon Metabolic networks of active reactions were identified using ^{13}C constrained metabolic-flux analysis that relies on the detection of mass isotopomer pattern in proteinogenic amino acids (Fischer and Sauer, 2003; Fischer et al., 2004). Based on the established amino acid biosynthesis schemes, intracellular-flux ratios was calculated from the labeling patterns of the amino acids by using the algebraic equations of METAFoR analysis (Fischer et al., 2003). These flux ratios represent direct, local evidence for the in vivo activity of particular pathways and reactions. Thus from the elucidated network topology of active reactions and from literature data, organism-specific metabolic-reaction models were deduced using the master network model **(Fig. 1.12).**

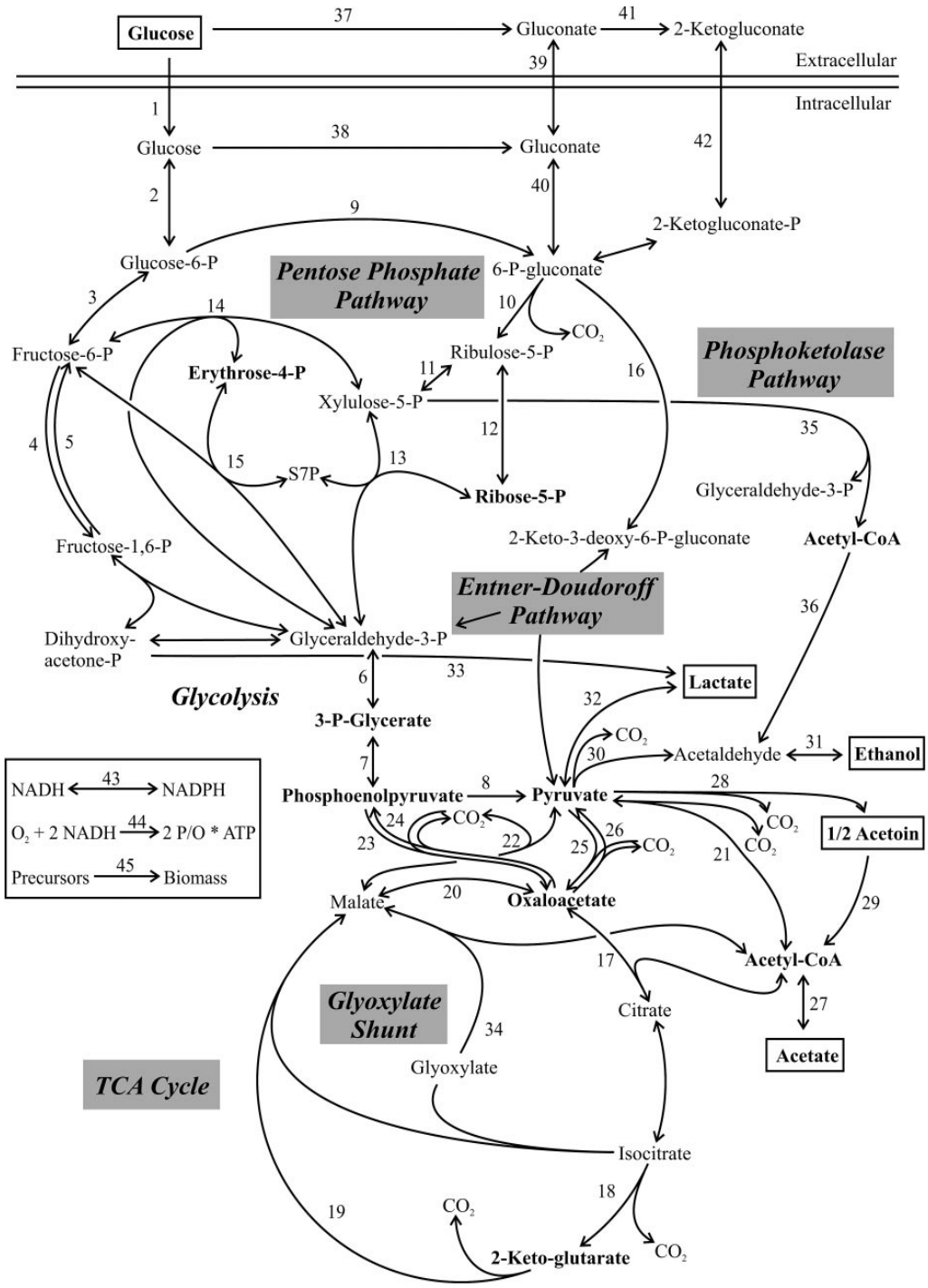


Fig. 1.12: Master network reaction that was used as the basis for net-flux analysis (Fuhrer et al., 2005). Metabolites in bold were precursors for amino acid biosynthesis, and metabolites in boxes were extracellular substrates or products. Doubled-headed arrows indicate reactions assumed to be reversible. Abbreviations: S7P, sedoheptulose-7-P; Acetyl-CoA, acetyl coenzyme A.

1.2.1: *Escherichia coli* –as the Model Organism

The CCM of *E. coli* and specifically the metabolism of glucose are intensively studied and well known topics (Raab et al., 2010; Blankschien, et al., 2010). Glucose metabolism starts with its uptake *via* the PTS and proceeds with several interconnected pathways with the major being: glycolysis, gluconeogenesis, the pentose- monophosphate bypass with the Entner-Dudoroff pathway, the TCA cycle with the glyoxylate bypass, anaplerotic reactions and acetate production and assimilation **(Fig. 1.13).**

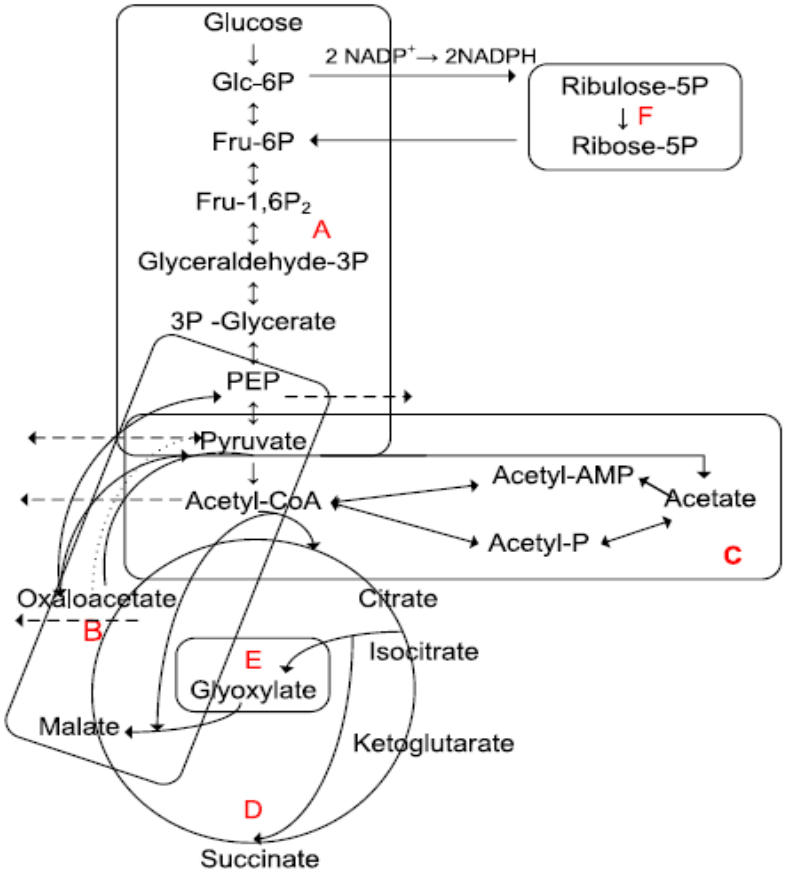


Fig. 1.13: Simplified representation of the central carbon metabolism of *E. coli.* (A) glycolysis and gluconeogenesis, (B) anaplerotic reactions, (C) acetate formation and assimilation, (D) TCA cycle, (E) Glyoxylate shunt, (F) PP pathway. The dotted line arrow from oxaloacetate to pyruvate indicates the anaplerotic reaction catalysed by pyruvate carboxylase. The broken line arrows indicate the removal of metabolites.

The extensive knowledge gained on *E. coli* CCM (Sauer and Eikmanns, 2005) offers key advantages in metabolic engineering efforts to achieve increased metabolite production. Such efforts have been focused intensively rather on the upper part of the carbon assimilation network, consisting of glycolysis and glyconeogenesis and their genetic and metabolic regulation.

The terminal stages of glycolysis in *E. coli* involve complex interplays. PEP conversion is coupled to two metabolic processes: PEP forms pyruvate by pyruvate kinase (PK) in the PTS or it forms oxaloacetate by the PEP carboxylase (ppc)-catalyzed anaplerotic reaction. The final products of glycolysis PEP and pyruvate enter the TCA cycle via acetyl-CoA and via the formation of oxaloacetate by carboxylation. This route, referred to as anaplerosis, replenish the intermediates of the TCA cycle that were used for anabolic purposes. Under gluconeogenic conditions, the TCA cycle intermediates oxaloacetate or malate are converted to pyruvate and PEP by decarboxylation and this way the PEP-pyruvate-axaloacetate node provides the precursors for gluconeogenesis. Therefore, the metabolic link between glycolysis, gluconeogenesis and the TCA cycle is represented by the PEP-pyruvate-oxaloacetate node (Sauer and Eikmanns, 2005)

1.2.2: Glucose catabolic pathways in pseudomonads

In pseudomonads although organic acids are the preferred carbon sources presence of glucose as the sole carbon source does induce the glucose metabolizing pathways. Pseudomonads do not catabolize glucose to triose phosphate via the traditional EMP pathway as they lack the key glycolytic enzyme PFK (Lessie and Phibbs, 1984). Unlike *E. coli*, pseudomonads generally lack PEP-PTS system for glucose uptake (Romano et al., 1970). Instead, pseudomonads catabolize glucose by two different routes: the direct oxidative pathway which acts on glucose extracellularly and the simultaneously operating intracellular phosphorylative pathway. Pseudomonads glucose oxidation occurs in two successive reactions forming D-gluconate and 2-keto-D-gluconate (2-KG) catalyzed by a membrane-bound PQQ-GDH and gluconate dehydrogenase (GADH) respectively (Lessie and Phibbs, 1984; Fuhrer et al., 2005) **(Fig.1.14).**

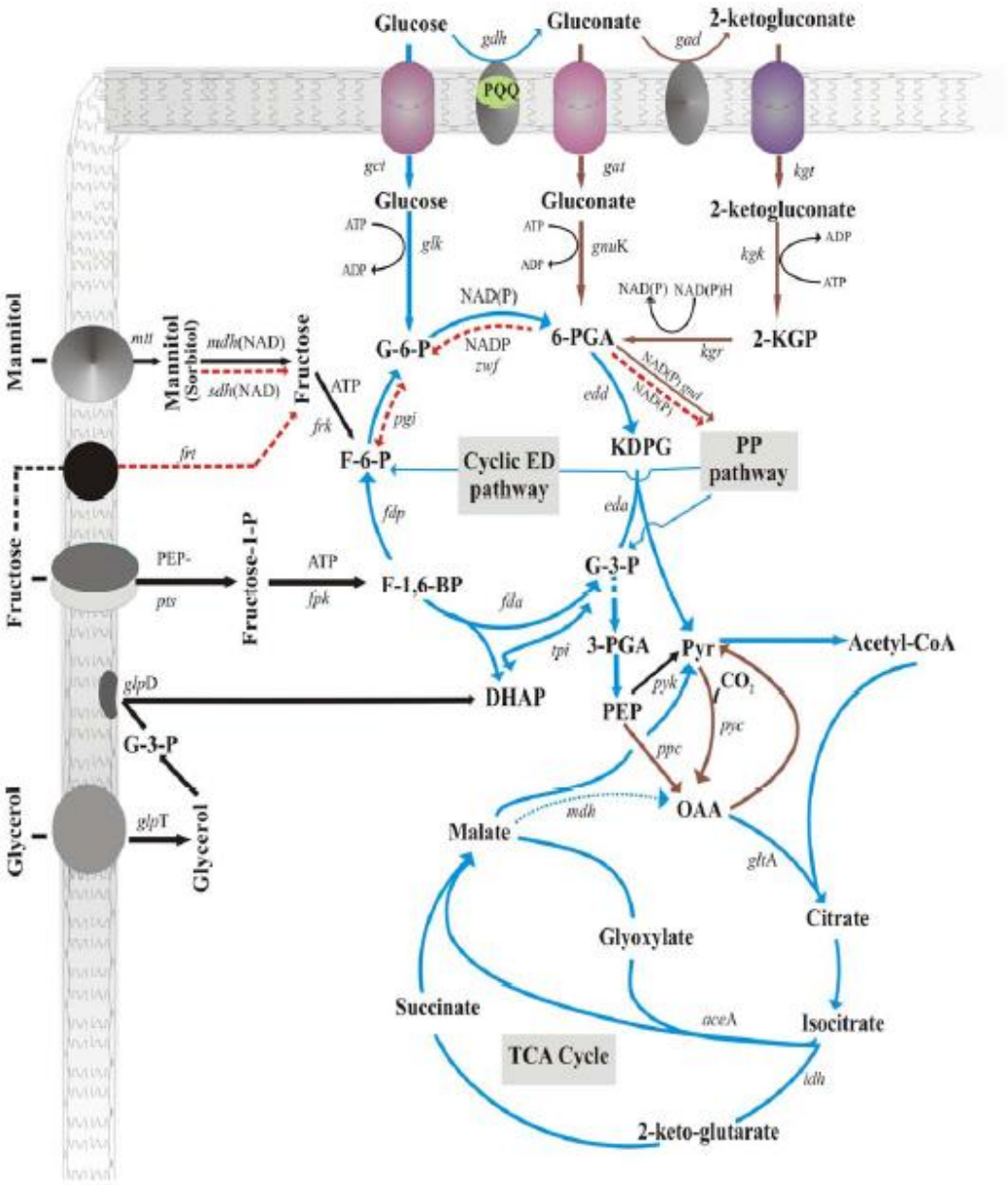


Fig.1.14: Carbohydrate metabolism in pseudomonads. Key to the pathway: Blue lines/arrows=Pathway operating in presence of glucose; **Bold Black arrows**=Reactions occurring when carbon source is other than glucose; Blue dashed arrow=Flux through that reaction is very low; Brown arrows=Occurrence of those genes is highly variable from strain to strain; Red dashed arrows=Reactions specific to *Pseudomonas cepacia*

1.2.3: Glucose Metabolism in *Bacillus subtilis*

B. subtilis is a gram positive spore forming bacterian and is the second most intensively studied bacteria after *E. coli.* Glucose is internalized via PTS *and* metabolizes

a large proportion of it to pyruvate and acetyl CoA, and subsequently converts these compounds to lactate, acetate and acetoin as by-products of metabolism which are excreted into the extracellular environment. The overall flux distribution done by ^{13}C metabolic flux analysis suggested glycolysis as the main catabolic pathway for glucose, acetate secretion, significant anaplerosis, and absent gluconeogenesis (**Fig. 1.15**) (Martin et al., 2011).

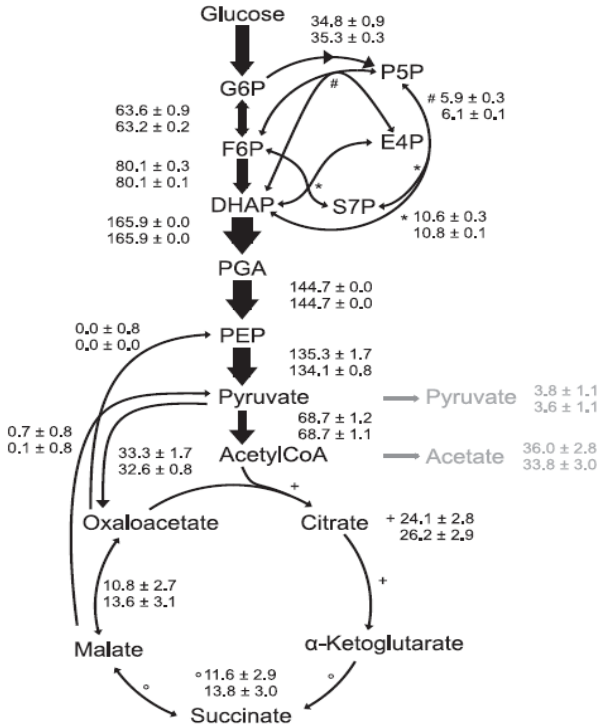


Fig. 1.15: Glucose Metabolism in *Bacillus subtilis* (Martin et al., 2011). Shown are relative flux values normalized to the glucose uptake rate of 8.2mmol g-1 h-1. Black arrows depict maximum and inner white arrows the minimum estimated flux value based on the Monte Carlo bootstrap error estimates with a confidence interval of 95%.

1.2.4: Glucose Metabolism in *Rhizobium*

The mechanism of glucose transport is established in both fast and slow-growing rhizobia, neither fast nor slow-growing rhizobia possessed a phosphoenolpyruvate phosphotransferase system and the uptake of glucose proceeded via an active process requiring an energized membrane state (Stowers et al., 1977; Mulongoy et al., 1978; DeVries et al., 1982). Carbohydrate supply is a major factor limiting nitrogen fixation by

the *Rhizobium*-legume symbiosis (Bethlenfalvay and Phillips, 1977; Hardy, 1977; Pate, 1977). Both fast- and slow-growing species possess the Entner-Doudoroff pathway (Katznelson and Zagallo, 1957; Keele et al., 1969; Martinez-de Drets and Arias, 1972; Mulongoy and Elkan, 1977 a). Fast-growing rhizobia also possess $NADP^+$-dependent 6-phosphogluconate dehydrogenase the key enzyme of the pentose phosphate pathway, but it was not found in slow-growing rhizobia (Katznelson and Zagallo, 1957; Keele et al., 1969; Martinez-de Drets and Arias, 1972; Mulongoy and Elkan, 1977a, b). The tricarboxylic acid cycle also operated in hexose catabolism in *B. japonicum USDA110*(Keele et al., 1969; Mulongoy and Elkan, 1977a). ED pathway was established as the presence of 6PG dehytratase (EC 4. 2. 1. 12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) activities were observed in glucose-grown cells (Keele et al.,1970; Stowers et al., 1985)(**Fig. 1.16.**)

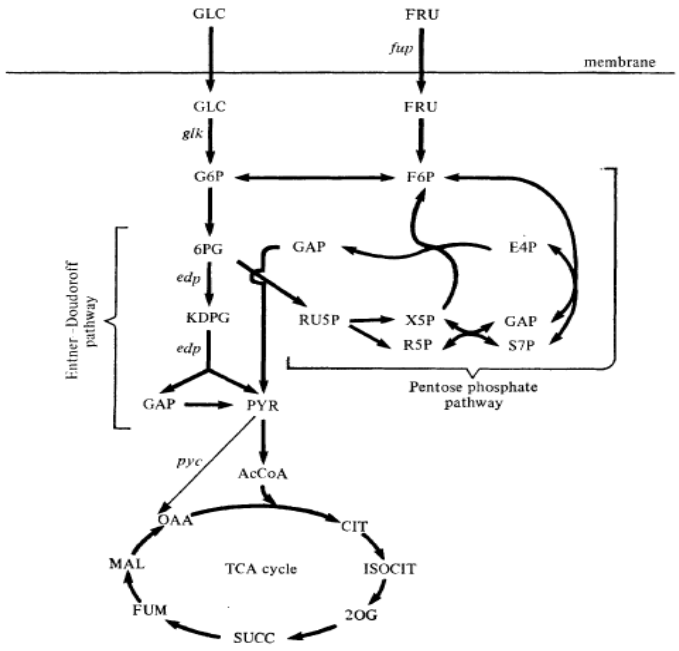


Fig. 1.16. Pathways of glucose and fructose catabolism available to *R. trifolii* strain 7000 (Ronson and Primrose, 1979). The mutants are blocked at the steps indicated: *glk*, strains 7009, 7013 and 7039; *fup*, strain 7039; pyc, strain 7049. Strain 7028 is blocked at one of the two steps labelled *edp*. Abbreviations: GLC, glucose; FRU, fructose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 6PG, 6-phosphogIuconate; KDPG, 2-keto-3-deoxy-6-phosphogluconatGe;A P, glyceraldehydes 3-phosphate; RUSP, ribulose 5-phosphate; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose

4-phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; OAA, oxaloacetate; CIT, citrate; ISOCIT, isocitrate; 20G, 2-oxoglutarate; SUCC, succinate; FUM, fumarate; MAL, malate; TCA, tricarboxylic acid.

Rhizobium trifolii strain 7000 contained key enzyme activities of the ED and PP pathways (**Fig. 1.16**). The lack of phosphofructokinase indicated that the EMP pathway was absent (Ronson and Primrose, 1979).

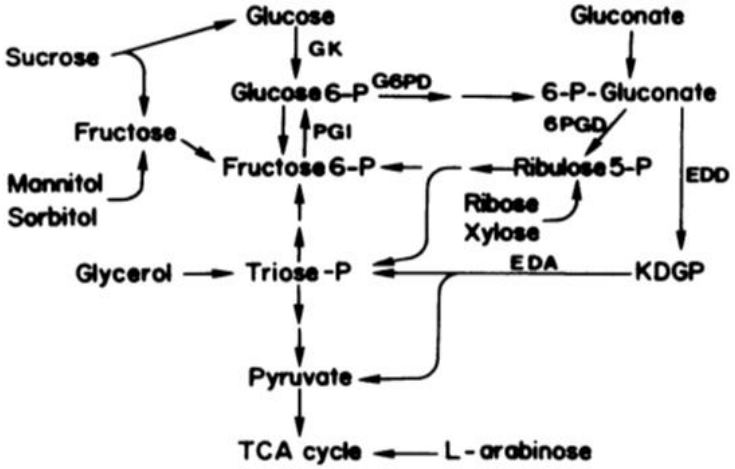


Fig.1.17 Outline of Possible pathways of Carbohydrate metabolism in *R. meliloti* (Arias et al., 1979)

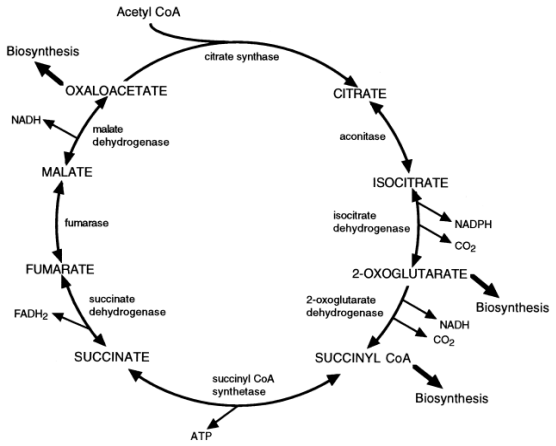


Fig.1.18: Reactions of the TCA cycle. (Dunn, 1998) Cycle intermediates are in capital letters. Additional products shown are for the forward (clockwise) reactions. Metabolites which are commonly used in biosynthetic reactions are also indicated.

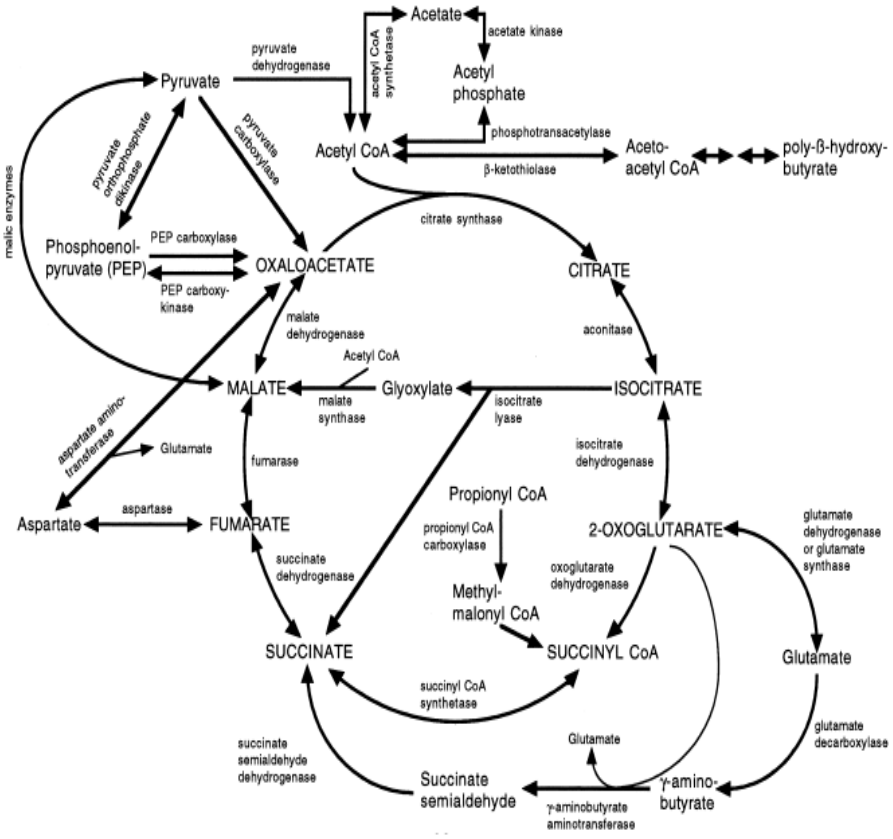


Fig. 1.19: Possible integration of anaplerotic and bypass pathways with the TCA cycle. (Dunn, 1998) Intermediates of the TCA cycle are in capital letters. Not all reaction products are shown.

The sources of acetyl-CoA are pyruvate and carbohydrate metabolism product in *S.meliloti* **(Fig. 1.20)** (Imperlini et al., 2009).. The main fates of acetyl-CoA are (i) its oxidation by the TCA cycle,(ii) its incorporation into PHB, and(iii) its utilization via the glyoxylate bypass. TCA cycle intermediates are commonly used in biosynthetic reactions and some reactions are inhibited by NADH.

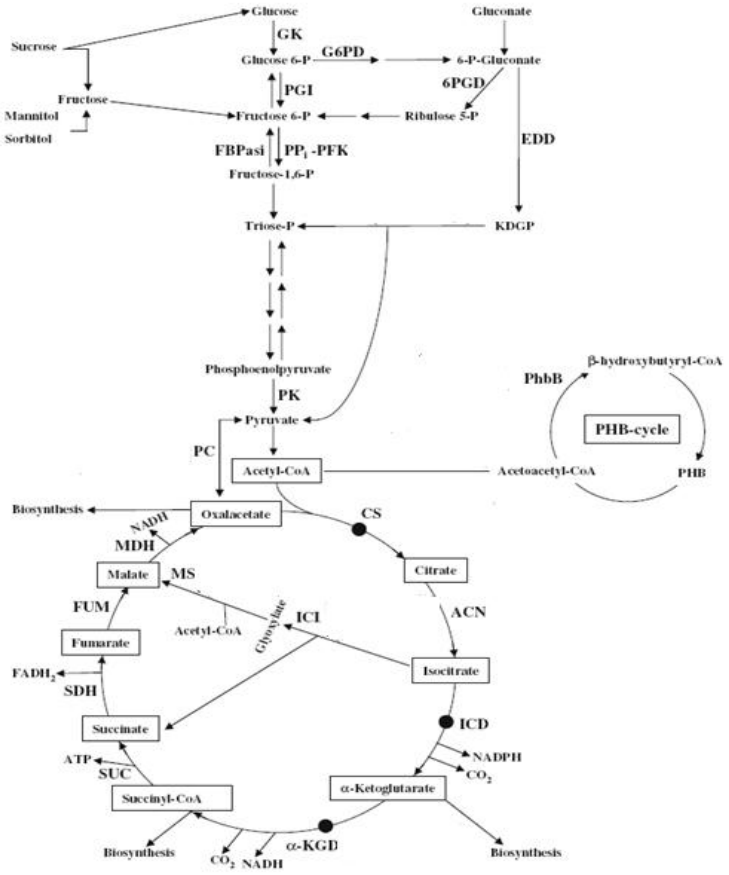


Fig.1.20: Carbohydrate metabolism in *S. meliloti* (Imperlini et al., 2009). MS malate synthase; PhbA β-ketothiolase; PhbB acetoacetyl-CoA reductase.

S. meliloti converted glucose to gluconate, which then entered metabolism (Stowers, 1985; Portais et al., 1999). Pyruvate carboxylase was assumed to be the anaplerotic reaction by analogy to *A. tumefaciens* (Dunn et al., 2001). Both the PP and ED pathways were present, and glycolysis was absent, in both rhizobia (Arthur et al., 1979; Stowers, 1985) In rhizobia, the ED pathway was basically the exclusive pathway of glucose degradation, while the pentose-5-P precursors for biomass were generated through the oxidative and nonoxidative branches of the PP pathway **(Fig. 1.21).** With a flux well above 100% relative to the glucose uptake rate high TCA cycle flux is seen. ^{13}C- and ^{31}P-NMR profiles of bacteroids presented quantitative and qualitative differences from that of their vegetative state, which reflect some physiological adaptations of rhizobia to the plant host environment**.**

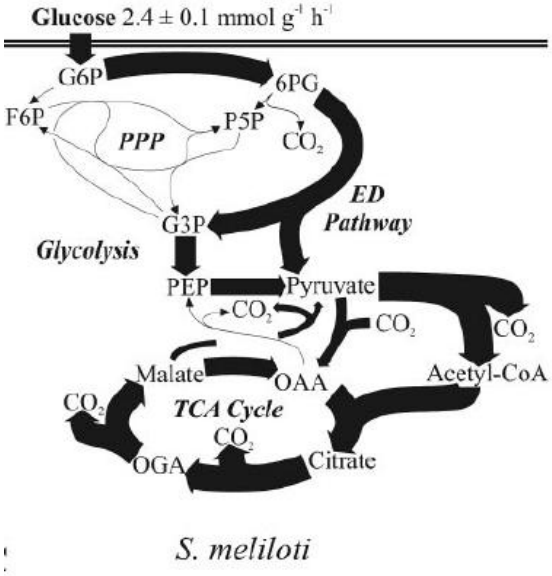


Fig. 1.21: *In vivo* carbon flux distribution in *S. meliloti* (Fuhrer et al., 2005). All fluxes normalized to the glucose uptake rate that is given at the top of each panel, and the widths of the arrows are scaled to the relative percentages of flux. Fluxes below 2.6% of the glucose uptake rate are represented by nonscaled hairlines.

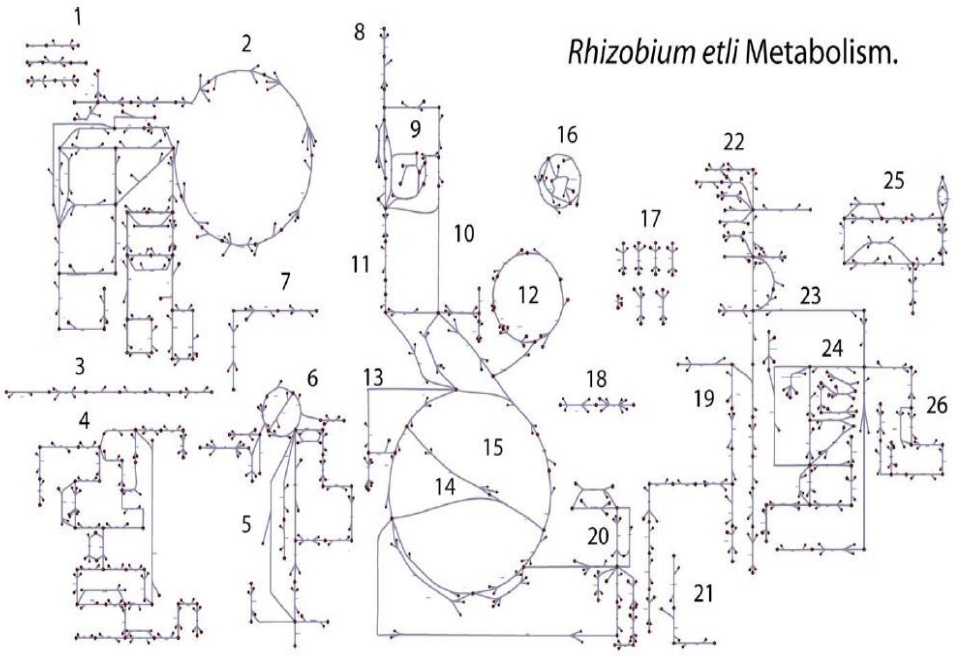


Fig. 1.22: Metabolic pathways for *Rhizobium etli* (Resendis -Antonio et al., 2007).

The metabolic reconstruction for *R. etli* includes 26 metabolic pathways involving 363 genes and 383 metabolic reactions. (**Fig. 1.22**)There are some major differences between free living *B. japonicum USDA110*and bacteroids **as depicted in Fig. 1.23.** (Vauclare et al., 2013).

1. Pentose and glucuronate.
2. Purine metabolism.
3. Histidine metabolism.
4. Pyrimidine metabolism.
5. Arginine and proline metabolism.
6. Amino group/ Urea.
7. Inositol metabolism.
8. Glycogen metabolism.
9. Penthose phosphate.
10. Entner-Doudoroff.
11. Glycolysis.
12. PHB cycle.
13. Aspartate metabolism
14. Glyoxylate shunt.
15. TCA cycle.
16. Oxidative phosphorylation.
17. Aminoacids tRNA.
18. Nitrogen fixation.
19. Valine, leucine and isoleucine.
20. Glutamate-glutamine metabolism.
21. Glutathione metabolism.
22. Glycine, serine and threonine metabolism.
23. Methionine metabolism.
24. Sulfur assimilation and cysteine metabolism.
25. Nicotine/nicotinamide.
26. Lysine.

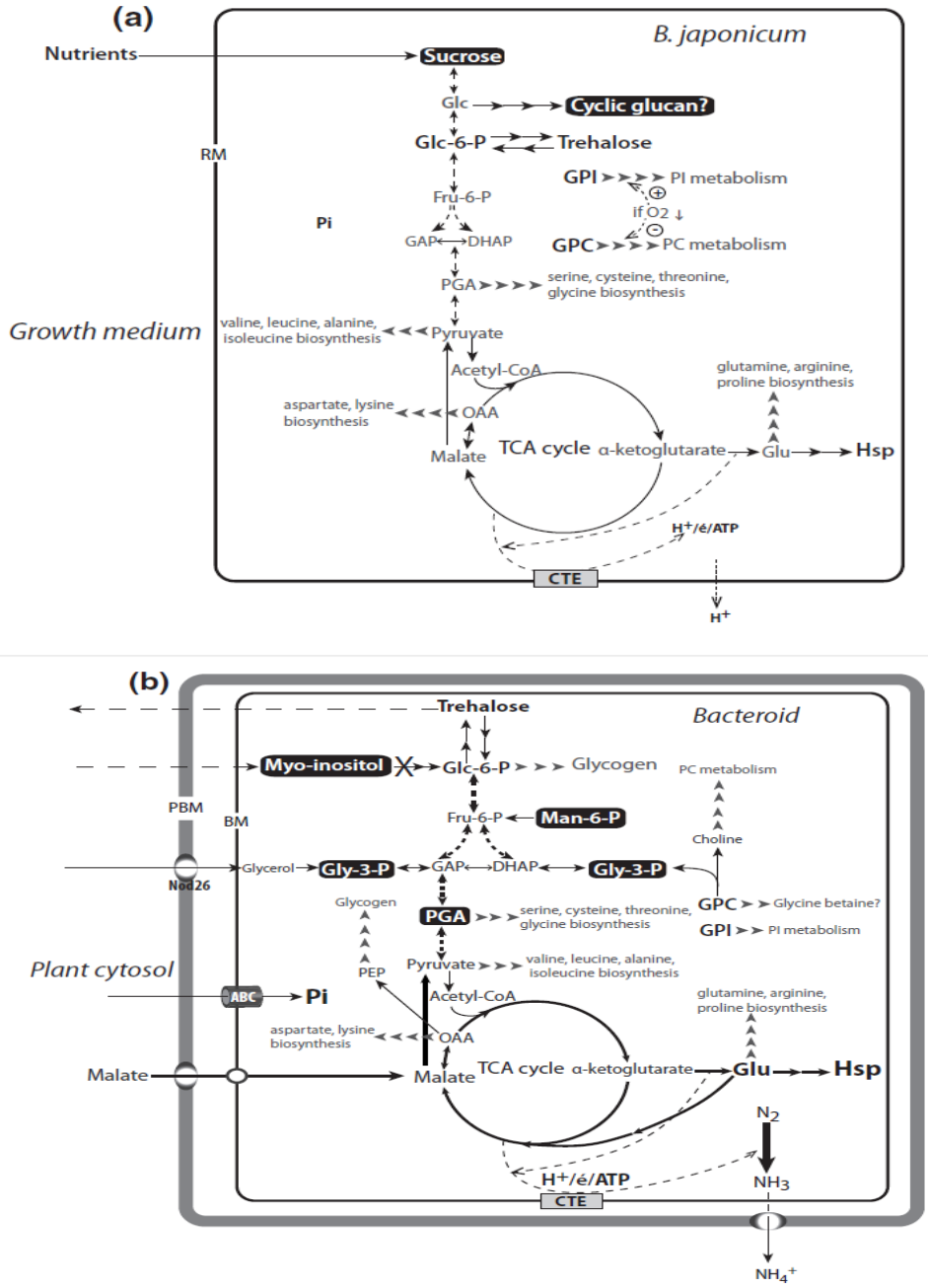


Fig. 1.23: Schematic representation of major differences between(a) free living *B. japonicum USDA110*and (b) bacteroids (Vauclare et al., 2013).

1.2.5: Importance of PEP-Pyruvate-OAA branch point in the cellular metabolism

In most chemotrophic, aerobic and facultatively anaerobic bacteria, the Embden–Meyerhof–Parnas pathway (glycolysis) or the Entner–Doudoroff pathway and the tricarboxylic acid (TCA) cycle are the main pathways of central metabolism. The former two are the primary routes for carbohydrate breakdown to phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA,there by providing energy and building blocks for the synthesis of cellular components. The TCA cycle also serves a dual role in catabolism and anabolism by catalyzing complete oxidation of acetyl-CoA to CO_2 for respiratory ATP formation and by providing carbon precursor metabolites and NADPH for biosynthetic processes. Upon growth on TCA cycle intermediates or on substrates that enter central metabolism via acetyl- CoA (e.g. acetate, fatty acids and ethanol), the cycle intermediates malate or oxaloacetate must be converted to pyruvate and PEP for the synthesis of glycolytic intermediates.

This gluconeogenic formation of sugar phosphates from PEP is accomplished by the reversible reactions of glycolysis and one further enzyme, fructose-1,6-bisphosphatase.The metabolic link between glycolysis/gluconeogenesis and the TCA cycle is represented by the PEP–pyruvate–oxaloacetate node, also referred to as the anaplerotic node **(Fig. 1.24).**

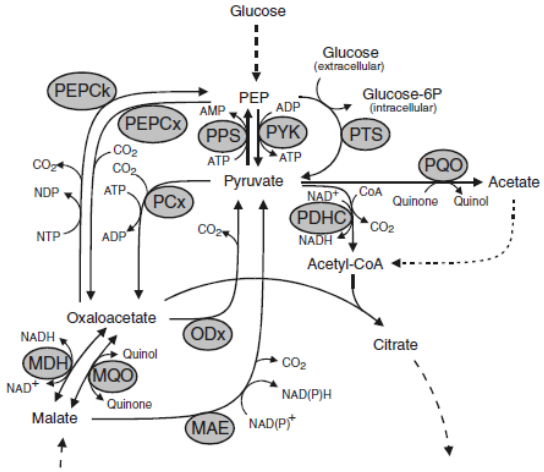


Fig. 1.24: The enzymes at the PEP–pyruvate–oxaloacetate node in aerobic bacteria.

The carbon flux control at the PEP–pyruvate–oxaloacetate node is often more complex than simple on/off regulation under a given condition and in some bacteria, two C3-carboxylating and up to three C4-decarboxylating enzymes are simultaneously active, even during growth on glucose as sole carbon and energy source (Dauner et al., 2001; Fischer et al., 2003) One enzyme, although operating in the same direction, fulfills a different function under certain conditions (Petersen et al., 2000).In other organisms or under different conditions, the same enzyme can operate in the reverse direction and thus contribute to a third function. These examples show that there is a complex interplay of the enzymes at the PEP–pyruvate–oxaloacetate node and it is obvious that sophisticated control is realized to ensure an optimal carbon and energy flow within central metabolism.

In most aerobic and facultatively anaerobic bacteria the chief metabolic pathways are the EMP pathway (glycolysis), ED pathway and the TCA cycle. The former two are involved in breakdown of carbohydrate to PEP and pyruvate which serve as precursors for biosynthesis of several cellular components. The pyruvate dehydrogenase (PDH) complex links the glycolytic/ED pathway and TCA cycle by further breakdown of pyruvate to acetyl-CoA which enters directly into TCA cycle. TCA cycle also performs dual functions of complete catabolism of acetyl-CoA for respiratory ATP formation as well as supplying the precursors for anabolism. On the other hand, when grown on TCA cycle intermediates or substrates that enter the central metabolism via acetyl-CoA, the cell diverts the metabolism towards gluconeogenic pathways for synthesis of the PEP and pyruvate to synthesize the essential sugar phosphates. Hence, the balance in the cellular physiology is highly dependent on the interactions between the catabolic and anabolic pathways.

The crucial metabolic link between the glycolytic / gluconeogenic / ED pathway and TCA cycle is the PEP-Pyruvate-OAA node often referred to as the anaplerotic node (Sauer and Eikmanns, 2005). The set of reactions operating at this node decide the carbon flux in a particular direction depending on the growth condition, thus acting as a key

switch governing the overall cellular metabolism. Under glycolytic conditions, PEP and pyruvate enter the TCA cycle by two routes, one by oxidative decarboxylation forming acetyl-CoA and second by C_3 carboxylation to form OAA which together energize the first reaction of TCA cycle. The formation of OAA by carboxylation of PEP or pyruvate is defined as anaplerosis, a process that replenishes the TCA intermediates utilized for anabolic purposes. Commonly, enzymes involved in C_3 carboxylation are PEP carboxylase (PPC) and pyruvate carboxylase (PYC). C_4 decarboxylation is irreversibly catalyzed by OAA decarboxylase (ODx). PEP carboxykinase (PEPCk), PEP carboxytransphosphorylase and malic enzyme perform the same function in a reversible manner.The differential occurrence of these enzymes in some of the commonly known bacteria is as listed in **Table 1.6.**

Table 1.6: Distribution of enzymes acting at PEP-pyruvate-OAA node in different bacteria. The numbers indicate the number of isozymes present in a given organism. Zero means that the organism has been tested for the enzyme or the respective gene however no activity is found so far. Empty space means that there is lack of evidence for the enzyme or the functional gene (Sauer and Eikmanns, 2005).

Organism	PEPCK	PPC	PYC	ODx	MAE	PPS	PDHC
E. coli	1(ATP)	1	0	0	1(NAD),1(NADP) 1(NADP)	1	1
C. glutamicum	1(ATP)	1	1	1	1 (NADP)	0	1
B. subtilis	1(ATP)	0	1	0	2(NAD) 1(NADP)	0	1
Rhizobium etli	1(ATP)	1	1		1(NAD) 1(NADP)	1	1
S. meliloti	1(ATP)	0	1		1(NAD) 1(NADP)	1	1
Rhododpseudomonas palustris	1(ATP)	1			1 (NAD)	1	1
P. citronellolis	0	1	1	1		1	1
P.fluorescens	1	1	1		1 (NADP)	1	1
Zymomonas mobilis		1			1		1

PEP–pyruvate–oxaloacetate node is very critical, still this node is quite flexible with respect to the metabolite pool and the prevailing biomolecular regulatory circuits. In certain organisms like *E. coli*, the regulation at this node is very simple, mediated by catabolite repression that does not allow gluconeogenic enzymes to express in presence of sugars. But in certain organisms like *Bacillus, Corynebacterium* and specific strains of *Pseudomonas,* more than one enzyme is responsible for C_3 carboxylation and C_4 decarboxylation, As a result, much more complex regulation is required at the anaplerotic node (Sauer and Eikmanns, 2005).

1.2.5.1: PEP-Pyruvate-OAA node in *E. coli*

The carbon flux at this node in *E. coli* is much more stringently regulated because PEP is involved in three major metabolic processes (i) PTS mediated sugar transport, (ii) PPC mediated anaplerotic reaction and (iii) as a precursor in the biosynthesis of amino acids (Clark, 1989; Gokarn et al., 2001). The enzymes participating in the PEP-Pyruvate-OAA interconversion in *E. coli* are as shown in **(Fig. 1.25).** PPC is the exclusive C_3 carboxylating enzyme while ATP-dependent PEPCk is primarily involved in C_4 decarboxylation and gluconeogenesis (Yang et al., 2003). Other options for C_4 decarboxylation are NADP dependent malic enzyme (ME) *mae*A and *mae*B whereas *sfc*A encodes NAD dependent ME which convert malate to pyruvate under physiological conditions but upon pyruvate accumulation can also act in reverse but thermodynamically favorable direction (Stols and Donnelly, 1997). However, malic enzymes are dispensable because PEP formation can be mediated by malate dehydrogenase and PEPCk. Interconversion of PEP and pyruvate is mediated by PYK and PEP synthetase (*pps*A, especially during growth on C3 acids like lactate and pyruvate). When grown on acetate, glyoxylate shunt also contributes to anaplerosis replenishing the essential C_4 intermediates of TCA cycle. Pyruvate apart from being converted to acetyl-CoA by PDH complex is acted upon by pyruvate oxidase to form acetate in the stationary phase (Dittrich et al., 2005). Hence, more than one metabolic reactions or enzymes are competing for the same metabolite to regulate this node.

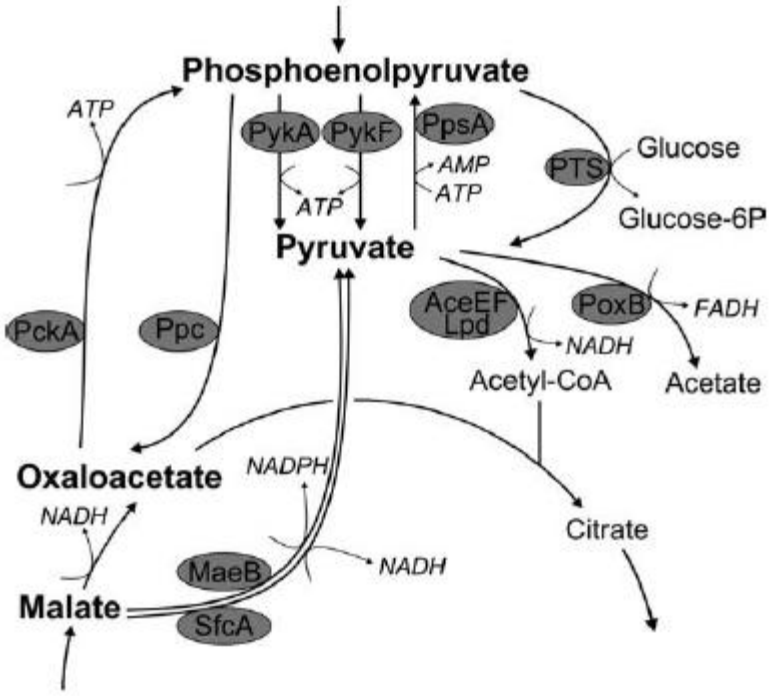
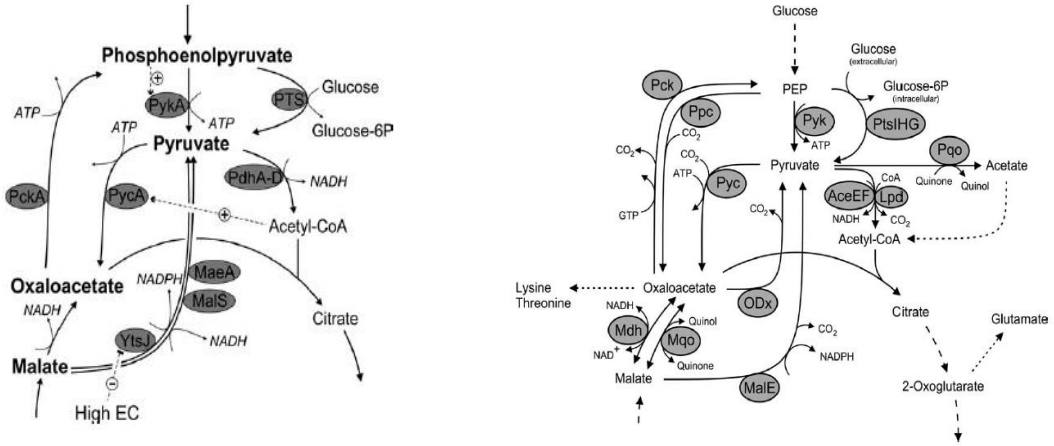


Fig. 1.25: The PEP-Pyruvate-OAA node in aerobic *E. coli*.

1.2.5.2: PEP-Pyruvate-OAA node in *B. subtilis and Corynebacterium glutamicum*

Bacillus is different from *E. coli* in being aerobic Gram-positive organism. The variations in the enzymes catalyzing the metabolic activity at this node in *B. subtilis* are as evident in **Fig. 1.26a**.The major difference is that instead of PPC, PYC acts as the sole anaplerotic enzyme to synthesize OAA. Due to absence of glyoxylate shunt, the organism fails to utilize carbon source that are metabolized via acetyl-CoA. PEPCk serves dual functions, primarily being involved in gluconeogenesis while performs a minor catabolic role in PYK and certain other mutants, by acting in reverse direction, despite being thermodynamically unfavorable (Sauer and Eikmanns, 2005). *B. subtilis* contains four paralogues *mle*A, *yts*J, *mal*S and *mae*A encoding putative ME, of which *yts*J encodes the major NADP-ME which is expressed constitutively on either glucose or malate (**Fig. 1.26a**). PYK and ME(s) constitute *pyruvate shunt* that substantially contributes to glucose uptake rate on carbon-limited conditions. Under these conditions, PEPCk flux is high which along with PYC and PYK constitutes an ATP dissipating futile cycle. Under gluconeogenic conditions PEPCk and MEs play a major role in redirecting the flux

through PEP Pyruvate-OAA node. Major regulation at this point is brought about by allosteric mechanisms and not by transcriptional control, unlike *E. coli*.



(a) ***B. subtilis*** **(b)** ***C. glutamicum***

Fig. 1.26: The PEP–pyruvate–OAA node in (a) *B. subtilis* and (b) *C. glutamicum*. Abbreviations denote the gene products that catalyze a given reaction: AceEF, subunits E1 and E2 of the pyruvate dehydrogenase complex; Lpd, subunit E3 of the pyruvate dehydrogenase complex; MalE, malic enzyme; Mdh, malate dehydrogenase; Mqo, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase (gene not annotated); Pck, PEP carboxykinase; PtsIHG, phosphotransferase system; Pqo, pyruvate: quinone oxidoreductase; Pyc, pyruvate carboxylase; Pyk, pyruvate kinase

C. glutamicum shows some of the major differences in the type of enzyme occurring at this node as compared to *E. coli* and *B. subtilis* (**Fig. 1.26b**). It has both PPC and PYC as anaplerotic enzymes for C3 carboxylation, which are regulated by different allosteric effectors. Additionally, PEPCk, ME or ODx function for C4 decarboxylation converting OAA or malate to PEP or pyruvate (**Fig. 1.26b; Table 1.6**) of which PEPCk (GTP dependent) is the main enzyme with no anaplerotic functions. Unlike *E. coli* and *B. subtilis*, there is only one NADP dependent ME which acts for malate decarboxylation rather than for the reverse pyruvate carboxylation. In glucose grown cultures of *C. glutamicum*, the major anaplerotic role is played by PPC while PYC is the main enzyme for glutamate and lysine production (Shirai et al., 2007). Under glycolytic conditions, PYC, PEPCk and PYK are responsible for an energy (ATP/GTP) consuming (futile) cycle (**Fig. 1.26b**) but its physiological significance is unclear. The PDH complex is

exclusively involved in oxidative decarboxylation of pyruvate to acetyl-CoA . The reaction of the PDH complex may be bypassed by the combined activities of pyruvate:quinone oxidoreductase (catalyzing the oxidative decarboxylation of pyruvate with a naphtoquinone as electron acceptor), acetate kinase and phosphotransacetylase (both constitutively expressed and function to form acetyl-CoA from acetate), but the bypass will be thermodynamically unfavorable due to ATP requirement of acetate kinase. On acetate, the glyoxylate cycle was found to be the essential anaplerotic pathway (Reinscheid et al., 1994; Gerstmeir, R., 2003).

Several other check points are also functional in the cellular metabolism, apart from the PEP-Pyruvate-OAA branch point, which might be influenced by altered flux distribution at the anaplerotic node. The direct involvement of the anaplerotic node in the TCA cycle is evident because the interplay of enzymatic reactions utilizing PEP and pyruvate at this node supplies the substrates OAA and acetyl-CoA for citrate synthase.CS is non-redundant enzyme for catalyzing the first step of TCA cycle to form citrate and it catalyzes a crucial step at the branch-point of oxidative, lipogenic, and anaplerotic pathways (Walsh and Koshland, 1985a; 1985b). CS activity is regulated in *E. coli* at the transcriptional and allosteric level depending on the nature of the available carbon source (Park et al., 1994). Because of its key position as the first enzyme of the TCA cycle, CS had been assumed to be an important control point for determining the metabolic rate of the cell and the carbon flux at the anaplerotic node (PEP levels) which could directly determine the flux through TCA cycle (Peng et al., 2004).

Another branch point in the central metabolism occurs between TCA cycle and the glyoxylate shunt which is mainly governed by the two enzymes NADP dependent ICDH and ICL competing for the common metabolite isocitrate. ICL in *E. coli* is mainly regulated at the level of expression depending on the growth conditions (acetate or glucose as carbon source) while ICDH is regulated by phosphorylation/ dephosphorylation (Walsh and Koshland, 1985b). Low PEP levels increase flux via glyoxylate cycle (Yang et al., 2003; Peng et al., 2004) and PEP *in vitro* inhibits ICDH as well as ICL; however its physiological significance is yet unclear (Ogawa et al., 2007).

1.2.6: Comparison of Central Carbon metabolism

The ED pathway and the TCA cycle were the almost exclusive pathways in *P. fluorescens* and the overall TCA flux was higher in pseudomonads along with very low acetate overflow metabolism as compared to *E. coli*, (Fuhrer et al., 2005). The ICL and ICDH activities at TCA-glyoxylate branch-point in *P. fluorescens* as well the activities of malate synthase and CS alter significantly in response to aluminum stress (Hamel and Appanna, 2001). **T**he gram-positive and gram-negative model bacteria *E. coli* and *B. subtilis* relied primarily on the EMP pathway for glucose catabolism and the relative TCA cycle flux was much lower than that in the other species because secretion of the incompletely oxidized overflow product acetate was extensive. **(Fig. 1.27).** At 38%, the PP pathway flux in *B. subtilis* was the highest observed in all species (Fuhrer et al., 2005).

As a fast-growing rhizobium with a generation time of less than 6 h on complex medium, *S. meliloti* converted glucose to gluconate, which then entered metabolism. Pyruvate carboxylase was assumed to be the anaplerotic reaction by analogy to *A. tumefaciens* (Fischer and Sauer, 2003) Both the PP and ED pathways were present, and glycolysis was absent, in both rhizobia (Arthur et al., 1979; Stowers, 1985), which was confirmed by METAFoR analysis. In both rhizobia, the ED pathway was basically the exclusive pathway of glucose degradation, while the pentose-5-P precursors for biomass were generated through the oxidative and nonoxidative branches of the PP pathway **(Fig. 1.27)**. With a flux well above 100% relative to the glucose uptake rate, *Rhizobium* species exhibited higher TCA cycle fluxes than those of *Agrobacterium tumefaciens*, two pseudomonads, *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*, *Zymomonas mobilis*, and *Paracoccus versutus* investigated here. The *E. coli* and *B. subtilis* PP pathway flux contributed substantially to catabolism (12 and 27%, respectively), Second, the *E. coli* and *B. subtilis* TCA cycle flux was relatively low, metabolism was not fully respiratory, and there was extensive overflow metabolism that is also referred to as aerobic fermentation. In addition to the linear ED pathway, which may be inducible in *E. coli*

during growth on gluconate or constitutive, as it is in *Z. mobilis*, cyclic operation of the ED pathway was reported for organisms that lack phosphofructokinase, e.g., pseudomonads (Lessie et al., 1984) and *S. meliloti* (Irigoyen et al., 1990).

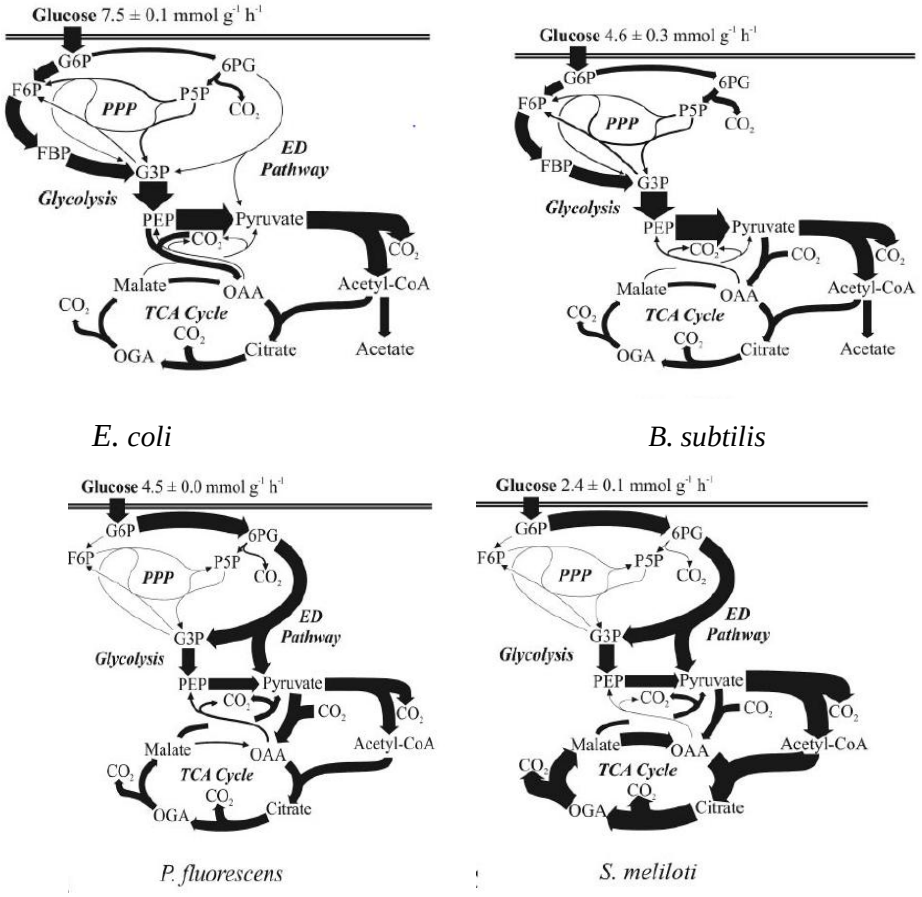


Fig. 1.27: In vivo carbon flux distribution in *E. coli B. subtilis P. fluorescens* and *S. meliloti*. All fluxes were normalized to the glucose uptake rate that is given at the top of each panel, and the widths of the arrows are scaled to the relative percentages of flux. Fluxes below 2.6% of the glucose uptake rate are represented by non-scaled hairlines.

Under any given condition, the metabolic network and its regulatory circuits synchronize to balance the catabolic and anabolic reactions to meet the requirements of energy and biomass. For smooth functioning of the metabolism, there are several check-points existing in the system at the junction of any two metabolic pathways. These branch points are sufficiently flexible so as to maintain a tight control over the carbon flux through a particular path under a particular condition. The following few sections discuss the importance of these check-points in the metabolism of several organisms and their role as potential new targets for metabolic engineering.

1.3: Metabolic engineering

Metabolic engineering is referred to as the "directed improvement of cellular properties through the modification of specific biochemical reaction(s) or the introduction of new ones, with the use of recombinant DNA technology" (Stephanopoulos, 1999). It is explained as the targeted and purposeful alteration of metabolic pathways found in an organism in order to understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard, 1996). This multidisciplinary field involves implementation of principles from chemical engineering, biochemistry, mathematical and computational sciences, and owes its existence to fast developing molecular biology techniques.

Earlier strategies for genetic modifications were based on random chemical mutagenesis and selection of strains exhibiting desired phenotypes but its success relied heavily on mutagens and creative selection techniques (Koffas et al., 1999). Studies by Bailey (1991) and Stephanopoulos and Vallino (1991) pioneered the major transition of this classical approach to a more systematic and rational approach called *Genetic Engineering* involving the use of recombinant DNA technology. The technical manifestation of genetic engineering involving manipulation of enzymatic, transport and regulatory functions of the cell by using recombinant DNA technology was better referred to as "**Metabolic Engineering**" (Stephanopoulos and Vallino, 1991; Koffas et al., 1999).

An integrated approach towards engineering of microorganisms into 'microbial chemical factories' (MCFs) that can be used in a 'biorefinery' for the conversion of biomass into both fuels and value added biochemicals. Biosynthesis of high value chemical compounds from biomass using natural or engineered pathways in microorganisms also serves as a promising alternative to chemical synthesis processes that employ expensive, hazardous and non-renewable raw materials and reagents as well as harsh processing conditions. The evolution of the field of metabolic engineering has

developed principles and tools that enable construction and optimization of MCFs by tapping into naturally occurring pathways in specific host organisms, heterologous expression of non-native pathways in well characterized hosts, or engineering de novo biosynthetic pathways for synthesis of various natural and non-natural products. De novo pathway engineering refers to the design and construction of novel pathways (hitherto unknown in nature in any single organism) by assembling multiple existing partial pathways from different organisms or using promiscuous or engineered enzymes as biocatalysts to catalyze a series of biotransformations with non-natural substrates (Prather and Martin 2008).

The steps involved in the design of novel pathways for MCFs and various tools and approaches used for their optimization are outlined in **Fig. 1.28**. Once a pathway has been designed and selected for experimental exploration (Martin et al., 2009), suitable pathway enzymes are selected to catalyze the reaction steps. Pathway enzymes are the tireless machines of the MCFs that sequentially process raw materials into desired value added products and govern the pathway rates, selectivity, yield and overall productivity. Whenever possible, enzymes known to specifically catalyze reactions with the respective pathway intermediates are selected. The selected enzymes are then expressed in a suitable organism grown in a culture medium supplemented with the required starting materials for the microbial synthesis of the desired value added product.

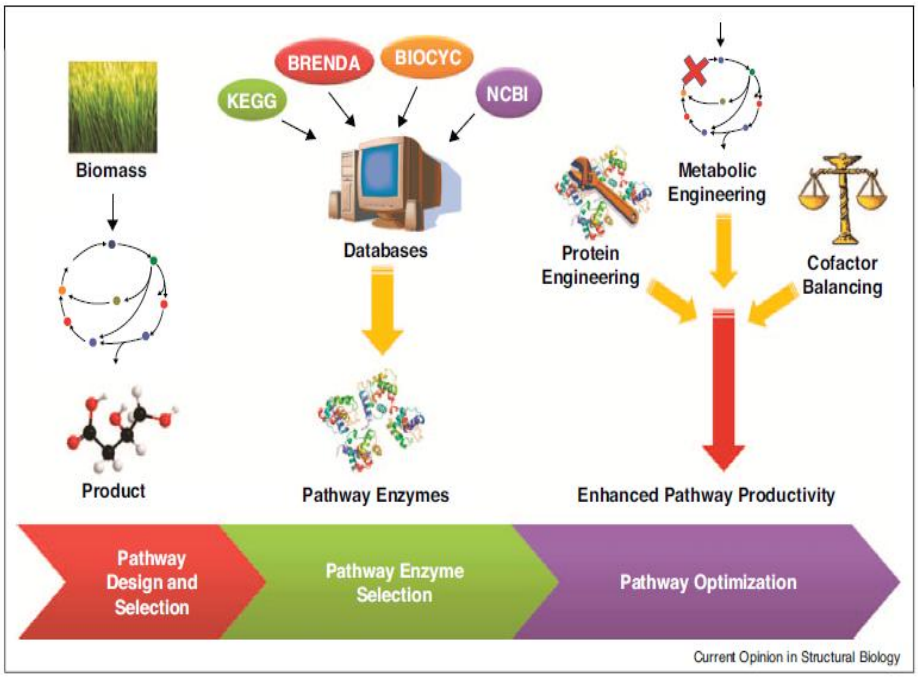


Fig. 1.28: Design and engineering of pathways for microbial chemical factories (MCFs) (Dhamankar et al., 2011).

The first step in engineering novel or natural pathways for MCFs is to identify potential natural cell metabolites or biomass derived feedstocks that can serve as starting materials and the series of biochemical reactions required to convert these into the desired product. Some of the computational tools available for identifying and selecting from the multiple possible pathways connecting different starting materials to a product of interest are studied (Martin et al., 2009). Once a pathway is selected, appropriate natural enzymes expected to catalyze pathway reactions need to be selected using enzyme information from various databases. *In silico* approaches such as protein BLAST searches and molecular docking may help in such enzyme selection. Further pathway optimization to enhance product titers relies on an integrated approach composed of metabolic engineering to enhance precursor metabolite availability using gene knockouts and enzyme expression level manipulation, Protein engineering to enhance pathway enzyme specificity and activity and cofactor balancing via effective cofactor recycling(Prather and Martin 2008; Martin et al., 2009).Protein engineering can be used for improving the selectivity and activity of the pathway enzymes and can effectively complement

conventional metabolic engineering approaches such as increasing the precursor supply by varying pathway enzyme expression levels or knocking out competing pathways to enhance productivity.

This has resulted in the metabolic engineering 'toolbox' greatly expanding from conventional approaches such as rationally deleting and/or over-expressing endogenous genes and introducing heterologous genes to now include tools capable of controlling gene expression and modulating regulatory networks throughout the cell (**Fig. 1.29**) (Klein-Marcuschamer et al., 2007; Tyo et al., 2007 and 2010; Blazeck and Alper, 2010).

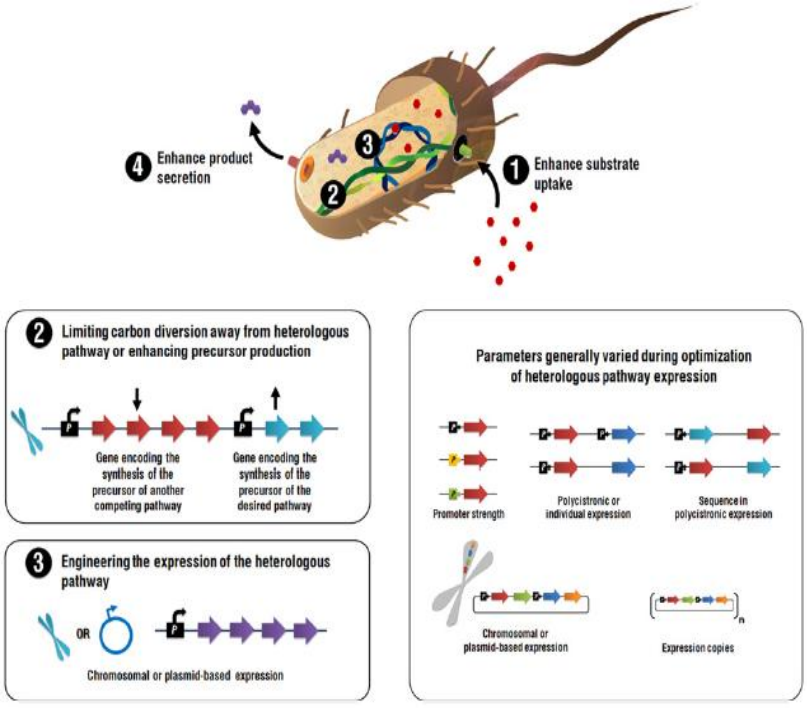


Fig. 1.29: A holistic view of metabolic and cellular engineering (Yadav et al., 2012) Methods for manipulating the flux from a substrate towards the product can be grouped into four categories: (1) enhancement in the rate of substrate uptake, (2)reduction of flux to undesirable by-products and enhancement of precursor and cofactor flux,(3)introduction of the heterologous pathway and optimization of the activity of its constituent enzymes and (4)export of the product to the extracellular medium in order to shift equilibrium towards product formation.

1.3.1 Metabolic engineering of organic acids for P solubilization.

Genetic manipulation of PS bacteria can be done to improve their ability to improve plant growth may include cloning genes involved in both mps and organic P-solubilization (ops),followed by their expression in selected rhizobacterial strains. For example, have shown that Mps activity is genetically coded in a gene cluster on plasmids of microbes endowed with PS activity (Goldstein and Liu, 1987 Rodriguez et al., 2006).They further transferred this gene cluster to an *E. coli* strain that did not previously possess PS activity but could demonstrate that the transferred gene was expressed in the transgenic *E. coli* strain. They have also found that the gene expression and mps activity of bacteria is affected by the presence of soluble P in the medium (feed-back regulation).Chromosomal insertion of these genes under appropriate promoters is another interesting approach (Rodriguez and Fraga 1999) (**Table 1.7**).

Table 1.7 Cloning of genes involved in mineral phosphate solubilization (Zaidi et al., 2009)

Microorganisms	**Gene or plasmid**	**Features**	**Reference**
Serratia Marcescens	pKG3791	Produces gluconic acid and solubilizes P	Krishnaraj and Goldstein, 2001
Rahnella aquatilis	pKIM10	produces gluconic acid and Solubilizes P in E. coli DH5a	Kim et al., 1998
Enterobacter agglomerans	pKKY	Solubilizes P in E. coli 109; Does not lower pH	Kim et al., 1997
Pseudomonas Cepacia	Gab Y	Produces gluconic acid and solubilizes mineral P in E. coli JM109; No homology with PQQ genes	Babu-Khan et al., 1995
Erwinia herbicola	Mps	Produces gluconic acid and solubilizes mineral P in E. coli HB101; Probably involved in PQQ synthesis	Goldstein and Liu, 1987
			Buch et al., 2009
			Buch et al., 2010
E. asburae PSI3	*gad*	Produces gluconic acid and 2-KGA solubilizes RP	Kumar et al., 2012
	Yf citC	Produces gluconic acid and citric	Adhikary, 2012

		solubilizes RP	
Citrobacter DHRSS	Yf citC and *vgb*	Produces citric acid and solubilizes RP	Yadav 2013
Citrobacter DHRSS	////	Produces oxalic acid and solubilizes P	Yadav 2013
***Herbaspirillum seropedicae* Z67**	Yf citC	Produces citric acid and solubilizes P	Wagh, 2013
***Herbaspirillum seropedicae* Z67**	*Pqq,gad*	Produces gluconic and 2-KGA and solubilizes P	Wagh, 2013

Gene technologies can be used to enhance specific traits that may increase an organism's capacity to mobilize soil P directly, enhance its ability to colonize the rhizosphere (ie, rhizosphere competence, Lugtenberg *et al*., 2001), or even to form specific associations with plant roots (Bowen and Rovia, 1999). Alternatively, microorganisms may provide a novel source of genes for directly modifying plants. For example, it has been reported that, when expressed in roots, a bacterial citrate synthase gene increases the exudation of organic acids and significantly improves plant access to soil P (López-Bucio *et al*., 2000).

1.3.2: Genetic manipulations at the anaplerotic node in *E. coli, B. subtilis* and *C. glutamicum*

Anaplerotic node has been manipulated not only for understanding the regulatory network but also for altering molecular fluxes for improving the bioprocesses. One of the most successful examples is the case of fermentative and aerobic succinate overproduction in *E. coli* (detailed in Section 1.3). Pyruvate overproduction was achieved in various *E. coli* mutants having block in conversion of PEP to OAA and pyruvate to acetyl-CoA, PEP, acetate, lactate and ethanol by deletion of the genes coding for the PDH complex (*ace*EF), pyruvate formate lyase (*pfl*B), PEP synthetase (*pps*), pyruvate: quinone oxidoreductase (*pox*B), acetate kinase, lactate dehydrogenase (*ldh*A), PPC and alcohol dehydrogenase (Wendisch et al., 2006). Anaplerotic node in *E. coli* has been successfully engineered for optimizing the amino acid production as PEP forms a key precursor molecule (Bongaerts et al., 2001; Kramer et al., 2003). These strategies include avoiding the drain of PEP to pyruvate by mutation in gene encoding PYK; a non-PTS

sugar uptake and increasing the gluconeogenic fluxes to PEP (e.g. overexpression of PEP synthetase), coupled with overexpression of transketolase which increases erythrose-4P level (Patnaik and Liao, 1994; Flores et al., 1996).

Several mutations in the enzymes acting at this node affect the physiological state of other relevant enzymes and *in vivo* pathway fluxes. Deletion of PYK gene developed a local catabolic loop involving PPC and ME which jointly function for both anapleriosis and catabolism thereby highlighting a newer function of the two enzymes (Emmerling et al., 2002) in *E. coli* but not in *B. subtilis*. *E. coli ppc* mutant exhibits auxotrophy for TCA cycle intermediates like succinate when grown on glucose because glyoxylate bypass that can theoretically substitute the PPC reaction is inactive due to catabolite repression as well the competition of isocitrate dehydogenase (ICDH) and isocitrtae lyase (ICL) for the common substrate isocitrate. The anaplerotic function in *ppc* mutant could be fully restored by overexpressing pyruvate carboxylase (*pyc*) which is otherwise absent in *E. coli* (Gokarn et al., 2000; 2001). Similarly *ppc* gene overexpression in *E. coli* under aerobic conditions reduced acetate formation and a significantly increased biosynthetic efficiency (Farmer and Liao, 1997). Collectively, these results suggest that the anaplerotic reaction in *E. coli* is not optimized for unhampered growth on glucose and that some of the enzymes apart from their classically recognized functions in catabolism, anaplerosis and gluconeogenesis play novel roles in the metabolism of some bacteria.

Although extensive information on the regulatory mechanisms operating at the anaplerotic node is available for *B. subtilis* (Sauer and Eikmanns, 2005), no genetic manipulations are reported at this node. *C. glutamicum* is an aerobe industrially important for production of L-Lysine and L-glutamate (Kiefer et al., 2004). The fact that PEP-Pyruvate-OAA node is crucial for the supply of precursors for amino acid biosynthesis, a lot of focus has been there on the enzymes and their regulations involved at this node. Increase in PYC activity and abolition of PEPCk activity in *C. glutamicum* independently resulted in increased production of TCA cycle-derived amino acids like glutamate and lysine (Sauer and Eikmanns, 2005). The levels of ME activity affected the growth pattern of *C. glutamicum* on lactate but not on glucose or acetate (Gourdon et al., 2000). *C. glutamicum* overexpressing ME accumulated high levels of pyruvate in the medium. *C.*

glutamicum ppc mutant grown under biotin limitation accumulated pyruvate due to which ME functioned in the reverse direction by utilizing pyruvate to replenish the TCA cycle intermediates (Gourdon et al., 2000).

All these bacteria, discussed above, metabolize glucose via the traditional EMP pathway yet exhibit such a diversified anaplerotic node. Pseudomonads, as discussed in earlier sections, are metabolically distinct with respect to non-PTS glucose uptake, two glucose catabolic routes ultimately following the ED pathway and exhibiting strain specific variations in the occurrence of enzymes of central carbon metabolism and hence are likely to display a completely different interplay between the enzymes at the anaplerotic node. On account of the agricultural importance of pseudomonads and metabolic versatility, detailed analysis of prevailing regulations at the PEP-Pyruvate-OAA node by various genetic perturbations would not only add to fundamental knowledge but also might discover novel targets for metabolic engineering.

1.3.3: Metabolic engineering of organic acids for P solubilization.

1.3.3.1: Role of citric acid in P solubilization

The secretion of gluconic acid is the major mechanism of P-solubilization by gram negative bacteria (Goldstein 1995; Kim et al., 1998). The acidification of soil by organic acids depends on both the nature and quantity of the organic acid for e.g. acetic, lactic and succinic at 100 mM bring about a drop in pH of a soil solution from around 9.0 to about 6.0; a similar drop is brought about by only 20 mM of gluconic acid, 10 mM of oxalic acid and even lesser amount of citric and tartaric acids (Gyaneshwar et al. 1998; Srivastava *et al.*, 2006) (**Table 1.8**). Addition of organic acids decreases the pH of the alkaline vertisol soil solution in the order Acetic = Succinic = Lactic < <Gluconic < <Oxalic < Tartaric = Citric and results in P release in a similar order. *Penicillium billai* secretes 10 mM each of citric and oxalic acids (Cunningham and Kuiack, 1992) and has been shown to be effective in releasing P in the field conditions (Asea et al., 1988). On the other hand, *C. koseri* and *B. coagulans* were found to secrete various organic acids in

the range 1-5 mM whereas as the concentration of these acids required to reduce the pH of the soil was 20-50 times more.

Table 1.8: Organic acids for phosphate solubilisation in different soil types (Srivastava et al., 2006).

Organic acid	Alkaline Vertisol pH >7	Acidic alfisol supplemented With RP pH < 7
Citric acid	10 mM	10-20 mM
Oxalic acid	10 mM	5-10 mM
Gluconic acid	20 mM	50 mM
Tartaric acid	10 mM	20 mM

Citric acid has better chelation properties due to presence of its three –COOH group‟ s having pKa values of 3.15, 4.77, and 6.40, respectively. Hence, *Rhizobium* strains producing citric acid could be effective as P biofertilizers in alkaline soils

1.3.3.2: Metabolic engineering of rhizobacteria for citric acid secretion.

Rhizobia are well known for nitrogen fixing ability and other PGPR effects. Genome sequence of several bacteria and metabolic data reveals that there are lot of interspecies diversity in terms of occurrence and regulation of enzymes at the central metabolism and PEP-Pyruvate-OAA node. Also *Rhizobium* has shown P solubilization ability. This study is an effort to genetically engineer a stable system for phosphate biofertilizer and to examine its applicability amongst *Rhizobium* spp. *Bradyrhizobium* is very efficient is nitrogen fixation which is more than the plant requirement and releases ammonia which can be used by other crops. Thus could be useful in intercropping systems *B. japonicum USDA110*USDA110 can nodulate several legumes and it has PQQ but not sufficient to function as cofactor for GDH..*M. loti* MAFF303099 nodulates lotus plants and it lacks PQQ. *S. fredii* NGR 234 is a broad host range *Rhizobium* .Thus these three *Rhizobium* strains were selected for the study. Present study describes improvement in citric acid secretion in *B. japonicum USDA110*USDA110, *M. loti* MAFF303099 and to the required amount for P release from soils. Additionally, the genetic manipulations

need be directed to the chromosomal integration as it would lead not only to increased stability but also decrease the metabolic load caused by the presence of the plasmids in the bacterial cell (Buch et al., 2010; Sharma et al., 2011).

P-solubilization was improved by overexpression of PEP carboxylase (*ppc*) and citrate synthase (*cs*) genes in fluorescent pseudomonads. Introduction of genetic perturbations in the glucose metabolic pathway at the anaplerotic node of *Pseudomonas* and at citric acid formation from OAA and acetyl-CoA by the overexpression of *ppc* and *cs* genes, respectively, demonstrated a direct correlation of increased CS activity with citrate accumulation (Buch et al 2010). The resultant ~2-fold increase in CS activity in the *gltA*-overexpressing strain *Pf* (pAB7) enhanced the intracellular and extracellular citric acid yields. Low extracellular citrate levels as compared to the intracellular levels in Pf(pAB7) suggested a probable limitation of efficient citrate transport (Buch et al 2009). Studies done in our laboratory in which overexpression of NADH insensitive *cs* gene in *P. fluorescens Pf* O-1 strains harbouring pY145F led to simultaneous increase in intracellular and extracellular citric acid level. However, the intracellular increase was not proportional to the extracellular increase in citric acid. The reason may be the inefficiency of the native proton dependent citrate transporter.(Adhikary, 2012)

Herbaspirilum seropidecae Z67 (pAB7) and (pJNK3) transformants increased CS activity but citric acid secretion was not significant. *Hs* (pJNK3) secreted 45 mM acetic acid while *Hs* (pJNK4) secreted 2.7mM and 51 mM citric and acetic acids, respectively. *Hs* (pJNK3) and (pJNK4) transformants, released ~80 µM and ~110 µM amount of P, respectively, in buffered medium in both aerobic and microaerobic conditions. These transformants also showed better growth and colonization parameters (Wagh, 2013).

The 2-hydroxycarboxylate transporter (2HCT) family of secondary transporters are transporters for citrate, malate and lactate. Na^+ coupled citrate transporters like CitS of *Klebsiella pneumoniae* and CitC of *Salmonella enterica* are highly specific for citrate (Lolkema, 2006).Earlier studies in the lab have shown that, among them, *Salmonella typhimurium* Na^+ dependant citrate transporter is a better citrate transporter (Adhikary, 2012).

Currently there is more interest in modifying the chromosome itself than in plasmid-based gene manipulation, which necessitates selection pressures such as antibiotic supplementation and is not feasible in agriculture practices. Plasmids can be lost from the host cell more easily than genes found on the chromosome by a variety of mechanisms.

1.4: RATIONALE

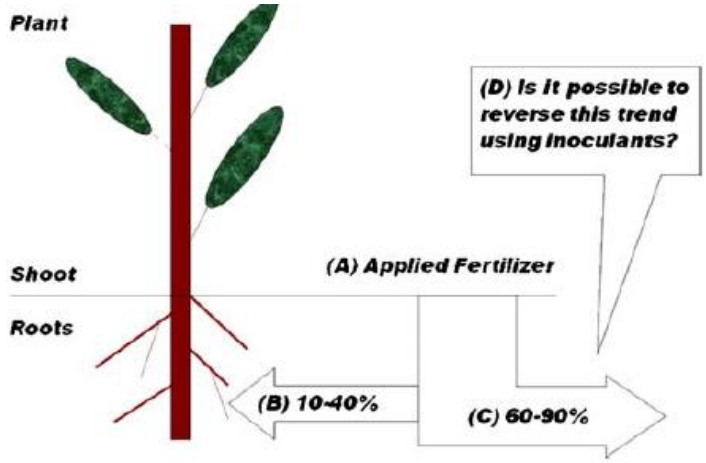


Fig. 1.30: Model for improved plant nutrient use efficiency with inoculants (Adesemoye et al., 2009).

The part of the applied fertilizer that is lost could be in the range of 60% to 90% of the original amount of fertilizer or manure applied (Hardy and Eaglesham 1995; Rowarth 1997; Hood et al. 1999; Gyneshwar et al. 2002; Barlog and Grzebisz 2004; Kleinman et al. 2005). Examples of the route of nutrient loss include N leaching, P fixation, and nutrient run-off among others. Then, the question being asked is whether it is possible to reverse the trend of (i) loosing high percentage of applied fertilizer and (ii) applying large amounts of fertilizers by supplementing reduced fertilizer with inoculants while maintaining plant growth and high yield comparative to the use of full recommended fertilizer rates?

Under any given condition, the metabolic network and its regulatory circuits synchronize in order to balance the catabolic and anabolic reactions to meet the requirements of energy and biomass. For smooth functioning of the metabolism, there are

several check-points existing in the system at the junction of any two metabolic pathways. These branch points are sufficiently flexible so as to maintain a tight control over the carbon flux through a particular path under a particular condition .CS had been assumed to be an important control point for determining the metabolic rate of the cell and the carbon flux at the anaplerotic node (PEP levels) which could directly determine the flux through TCA cycle (Peng et al., 2004).

Under diverse soil and agro-climatic conditions, the organisms with phosphate solubilizing abilities have proved to be an economically sound alternative to the more expensive superphosphates and possesses a greater agronomic utility (Khan et al., 2007; Xiao et al., 2009). Emphasis is therefore, being placed onto the possibility of greater utilization of unavailable phosphorus forms wherein the phosphate solubilizing microbes could play a pivotal role in making soluble phosphorus available to plants (Khan et al., 2010).

Key questions –

1. What would be the effect of incorporation of *ppc*, *cs* and *citrate transporter* genes on the biosynthesis and secretion of citrate in Rhizobia?
2. If citric acid is secreted, whether the amount is sufficient for solubilization of rock phosphate in media conditions mimicking alkaline vertisols?
3. Can citric acid secretion promote the plant growth promotion and nodulation efficiencies of Rhizobia?
4. Can metabolic engineering strategy for citric acid be applied to different Rhizobia?

Thus the present study dealt with monitoring the effect of overexpressing *ppc* gene of *Synechococcus elongatus* PCC 6301, *E. coli cs*, NADH insensitive Y145F *cs* gene and NADH insensitive Y145F *cs* along with Na^+ dependant citrate transporter genes on production of organic acid and mineral phosphate solubilizing ability in two different *Rhizobium* strains: *Bradyrhizobium japonicum* USDA 110 and *Mesorhizobium loti* MAFF303099. This transgenic *Rhizobium* having P solubilizing activity could be

effective as N and P biofertilizers. Part of the study also dealt with monitoring the effect of genomic integration of *yc* operon containing *E. coli* NADH insensitive *cs* along with *S. typhimurium citC*, *vgb* and *egfp* gene on production of organic acid and mineral phosphate solubilizing ability in *Bradyrhizobium japonicum* USDA 110, *Mesorhizobium loti* MAFF303099 and *Sinorhizobium fredii* (NGR 234). Preliminary plant experiment was done to see the effect of *S. fredii* NGR 234 genomic integrant on growth promotion of Mung bean plants. The overall strategy of the present study is depicted in **Fig. 1.31**.

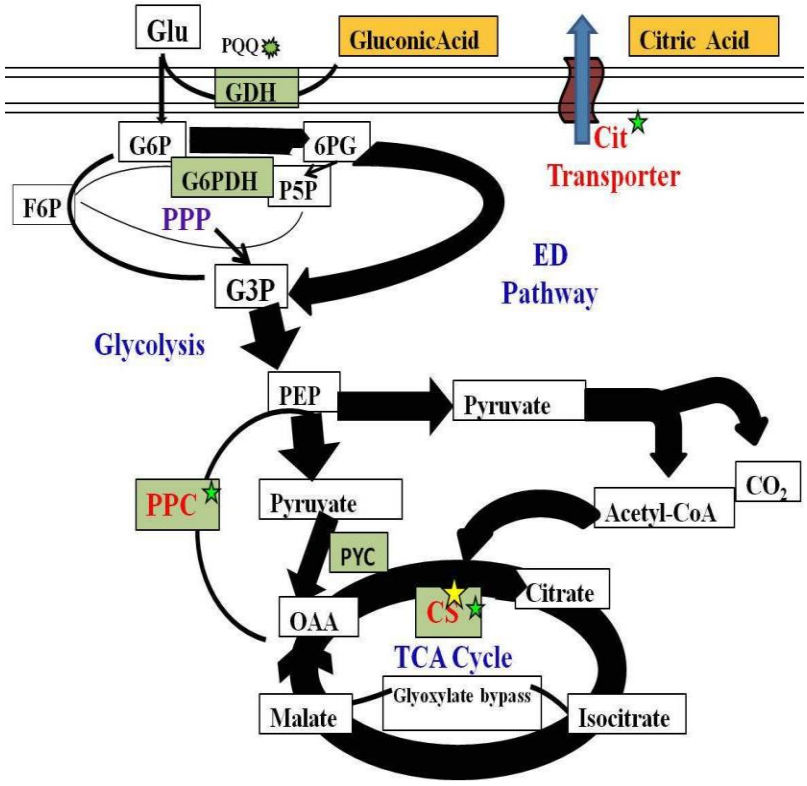


Fig. 1.31: Metabolic basis for designing the genetic modifications in *Rhizobium* strains. - Presence of PQQ is species dependent; .

- Shows overexpression of *ppc*,*cs* and *citC* genes
- Shows overexpression of NADH insensitive *cs* gene

1.5: Objectives of the present study

The objectives of the present study were defined as follows-

1. Effect of constitutive overexpression of *ppc* gene of *Synechococcus elongatus* PCC 6301 on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

2. Effect of constitutive overexpression of *E. coli cs* gene on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

3. Effect of overexpression of *E. coli* NADH insensitive Y145F *cs* gene on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

4. Effect of overexpression of *E. coli* NADH insensitive Y145F *cs* and *Salmonella typhimurium* Na^+ dependant citrate transporter on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

5. Effect of genomic integration of *E. coli* NADH insensitive *cs* along with *Salmonella typhimurium* Na^+ dependent citrate transporter with *vgb, egfp* on production of organic acid in *B. japonicum* USDA110, *M. loti* MAFF030669 and *S.fredii* NGR 234.

6. Effect of *Sinorhizobium fredii* NGR 234 genomic integrant containing *E. coli* NADH insensitive *cs* along with *S. typhimurium citC, vgb* and *egfp* gene cluster on growth promotion of Mung bean plants.

Chapter 2

Materials and methods

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* MAFF303099

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**).

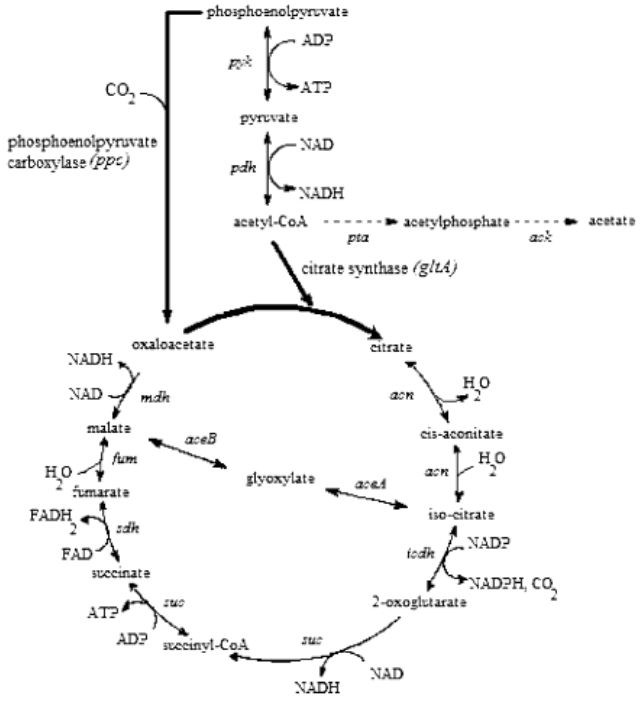


Fig. 3.1: Summary of the effects of overexpression of TCA cycle genes in *E. coli*. Over-expressed enzymes are indicated with bold arrows (De Maeseneire et al., 2006)

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**.

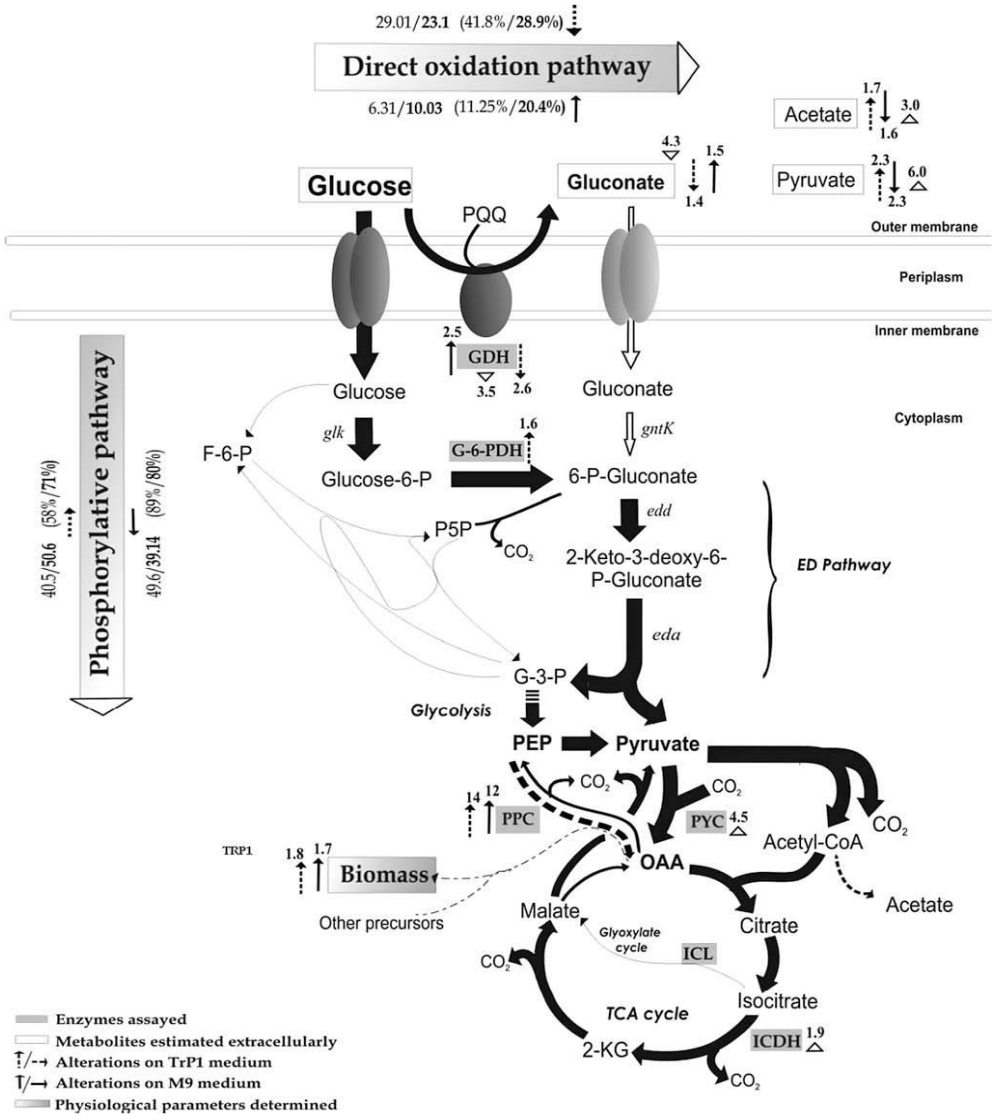


Fig. 3.2: Glucose metabolism of *P. fluorescens* and media dependant alterations due to *S. elongatus* PCC 6301 *ppc* overexpression. Numbers depicted on the arrows represent the fold change in response to ppc overexpression on M9 and TRP1 minimal media. Numbers on the arrow heads (D) depict the fold 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 37ºC and 30ºC 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.

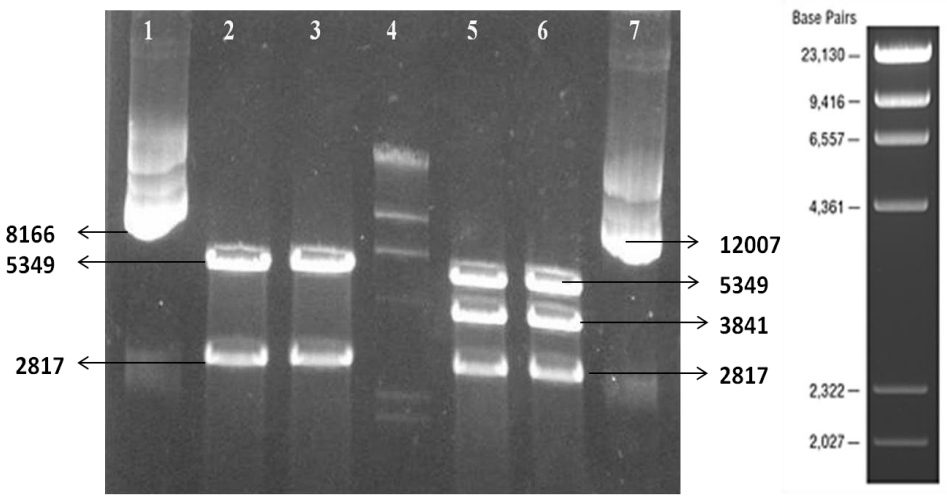


Fig. 3.3: Restriction digestion pattern of plasmids containing *ppc* gene isolated from transformants of *Bradyrhizobium japonicum USDA110* and *M. loti MAFF030669.* Lane 1: pAB4 plasmid Undigested (8166 bp); Lane 2 and 3: pAB4 plasmid digested with BamHI and HindIII (5349 bp and 2817 bp); Lane 4 : MWM-Lambda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

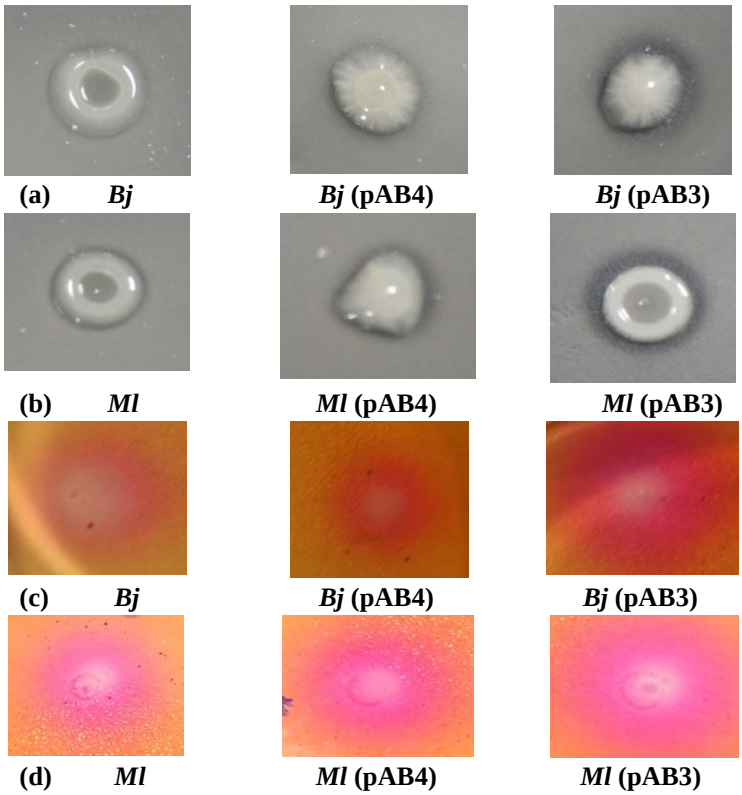


Fig. 3.4: MPS phenotype of *Bradyrhizobium japonicum* USDA110 and *M. loti* MAFF030669 strains harboring pAB3 plasmid. (A) and (B) on Pikovskaya's agar and **(C) and (D)** Tris rock phosphate agar containing 50 mM glucose and 100 mM Tris HCl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 ºC. 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 ºC 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**).

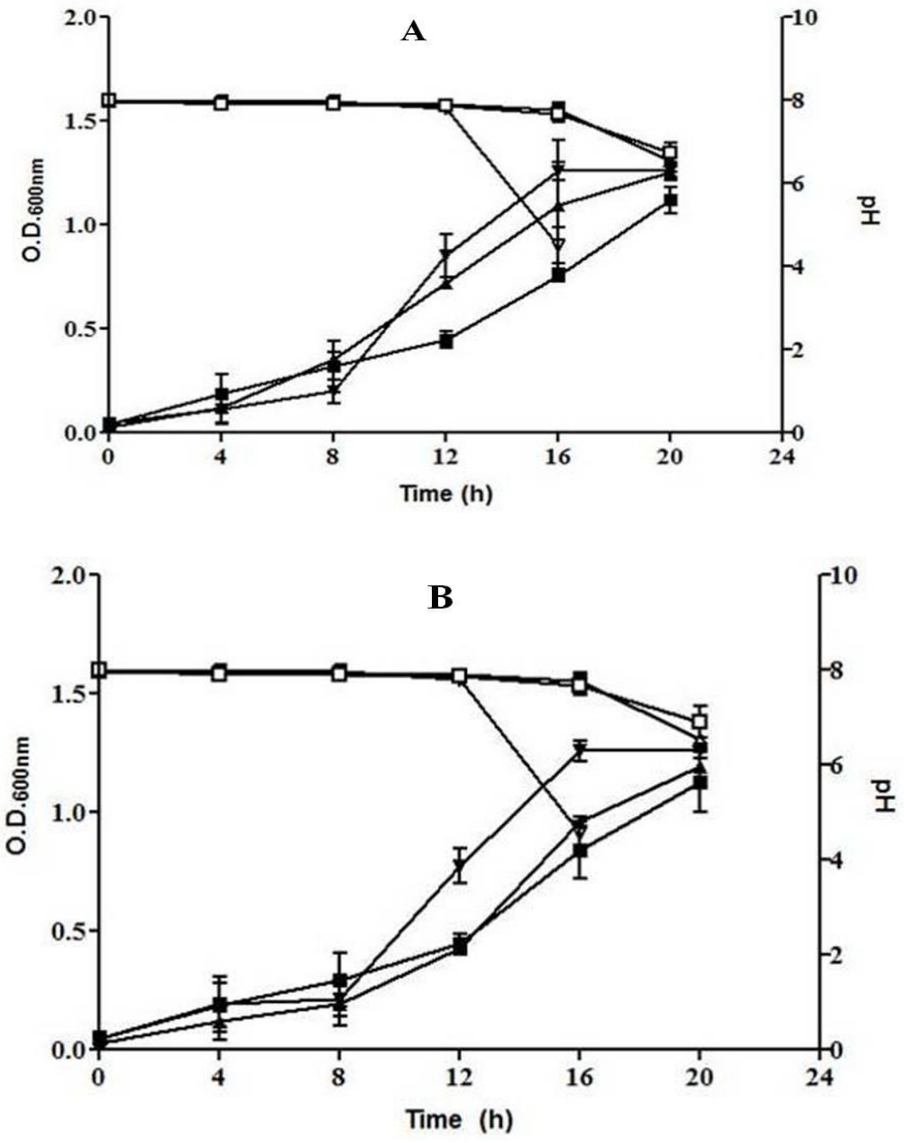


Fig. 3.5 Effect of *ppc* overexpression on extracellular pH (□, Δ, ▽,) and growth profile (■, ▲, ▼) of (A) *Bradyrhizobium japonicum* USDA110 and (B) *M. loti* MAFF030669, with 50 mM glucose and 100 mM Tris-Cl pH 8, 1mg/ml of rock phosphate medium. (□, ■, *Bj*, *Ml* wild type); {Δ, ▲, *Bj* (pAB4), *Ml* (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***

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

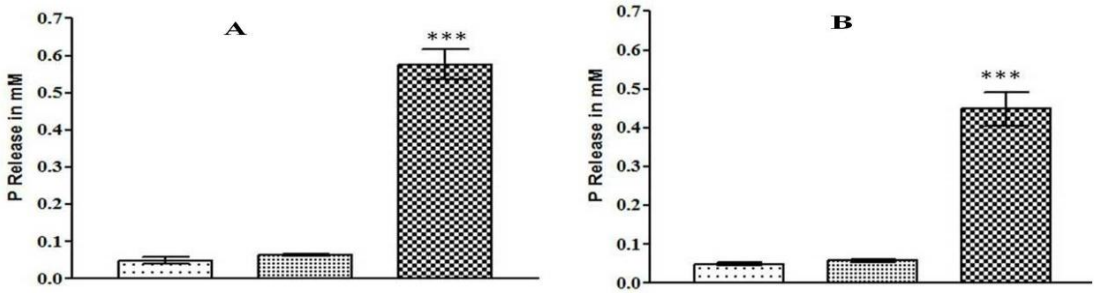


Fig. 3.6: P solubilization by (A) *Bradyrhizobium japonicum* USDA110 and (B) *M. loti* MAFF030669 transformants on TRP medium.

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

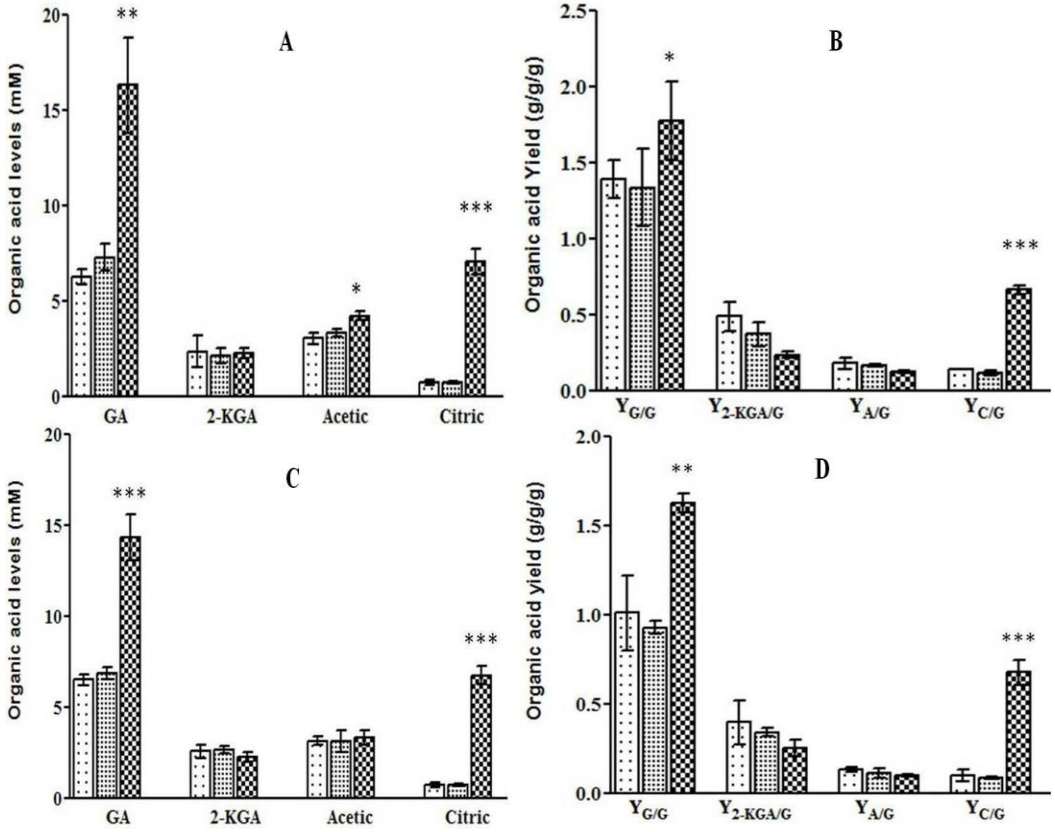


Fig. 3.7: Organic acid production from *Bradyrhizobium japonicum* USDA110 *and M. loti* MAFF030669 *ppc* gene: A and C : Gluconic, 2-keto gluconic, acetic and citric acids levels and B **and D: Organic acid Yields** ($Y_{G/G}$ $Y_{2\text{-}KGA/G}$, $Y_{A/G}$ and $Y_{C/G}$ in *Bj*, *Bj* (pAB4), *Bj* (pAB3),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

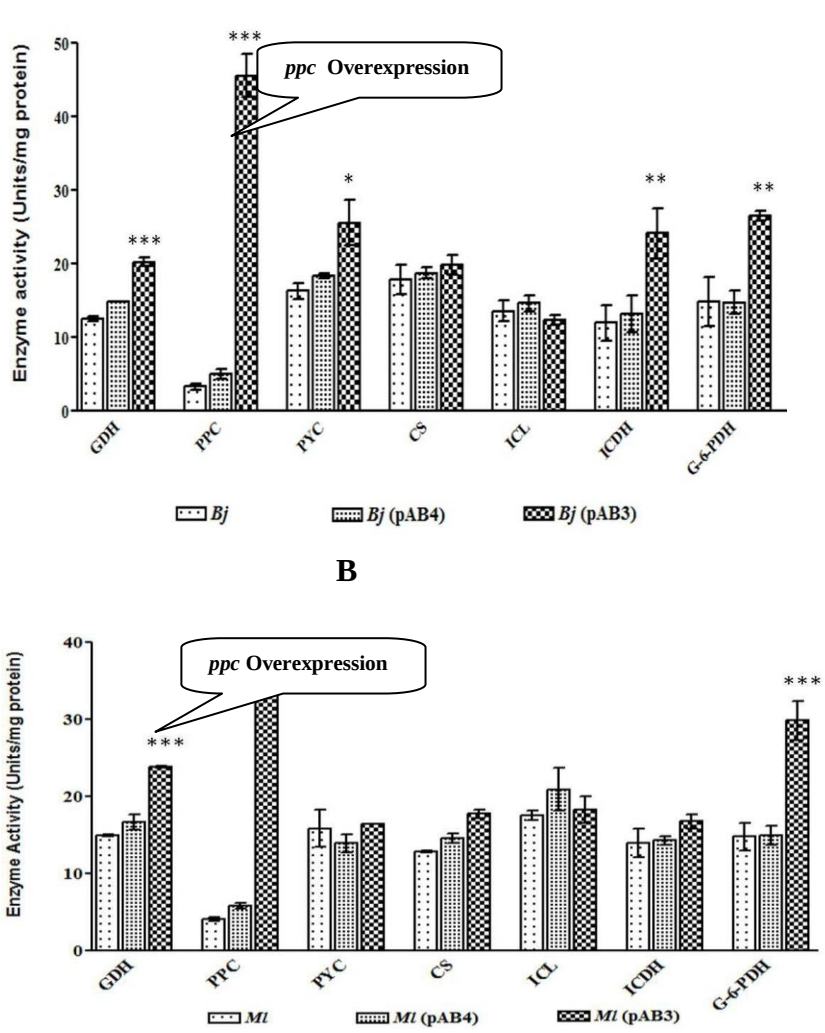


Fig. 3.8: **Activities of enzymes PPC, PYC, GDH, G-6-PDH, ICDH and ICL in (A) *Bradyrhizobium japonicum* USDA110 and (B) *M. loti* MAFF030669 *ppc* transformant.** The activities have been estimated using cultures grown on TRP medium with 50mM glucose. All the enzyme activities were estimated from mid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

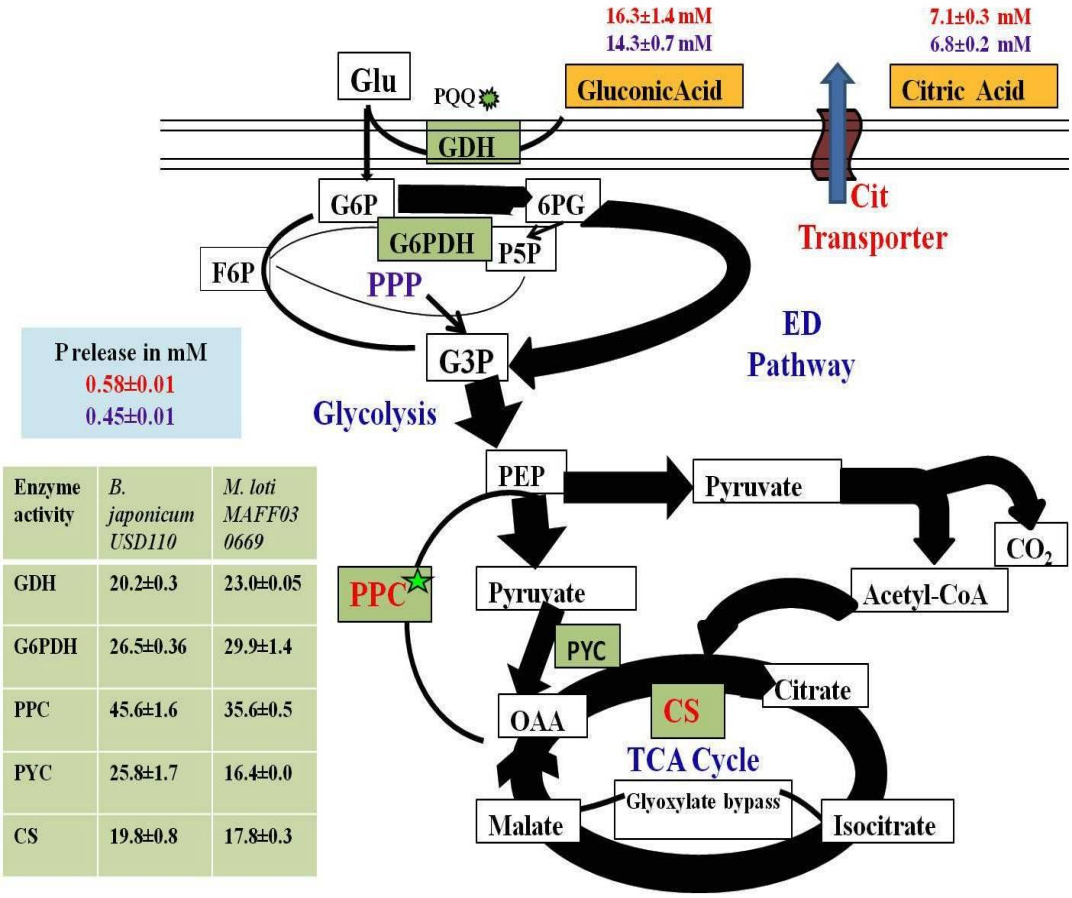
Biofilm formation in *Rhizobium*–legume N_2-fixing symbiosis contributes to effective root colonization by rhizobia and provides an effective mode for defense and helping rhizobia to survive under harsh and nutrient-limiting environments (Jebara et al., 2006). Diverse forms of the fungal bacterial biofilms (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 USD110*	*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.

Chapter 4

Effect of overexpression of *E. coli cs* gene on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF303099

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).

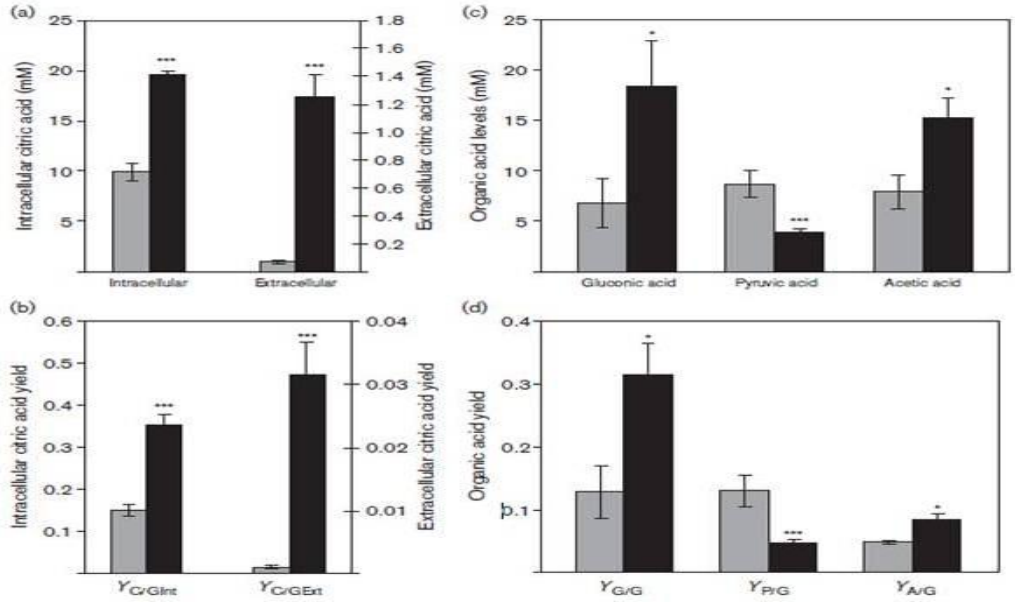


Fig. 4.1: Effect of *E. coli gltA* gene overexpression on organic acid secretion by *P. fluorescens* ATCC 13525. Yields are expressed as g citric or other organic acid (g glucose)$^{-1}$ (g dcw)$^{-1}$ (Buch et al., 2009).

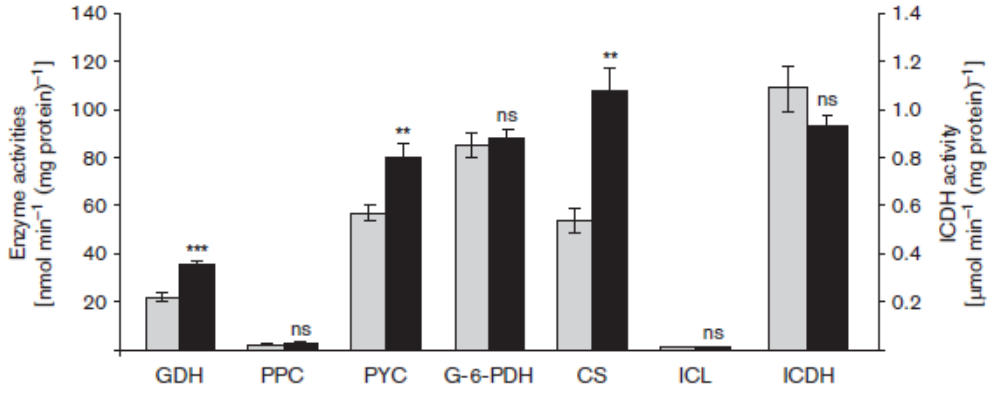


Fig. 4.2: Activities of key carbon utilization enzymes in *P. fluorescens* ATCC 13525 overexpressing *E. coli gltA* gene (Buch et al., 2009).

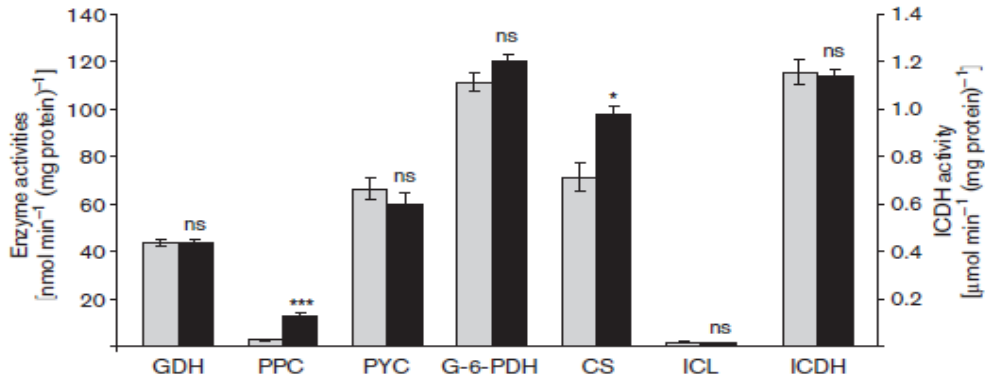


Fig.4.3: Activities of key enzymes of carbon utilization in *P. fluorescens* 13525 co-expressing the *ppc* and *gltA* genes (Buch et al., 2009).

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).

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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 37ºC and 30ºC 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).

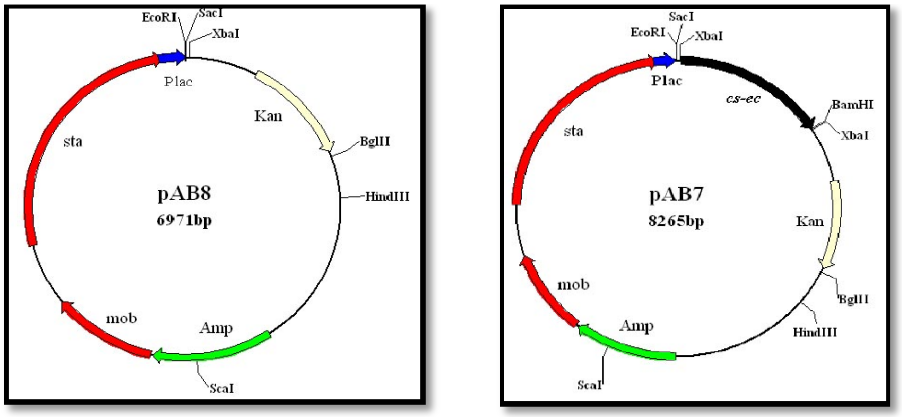


Fig. 4.4: Restriction maps of the plasmids used in this chapter (Buch et al., 2008).

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.

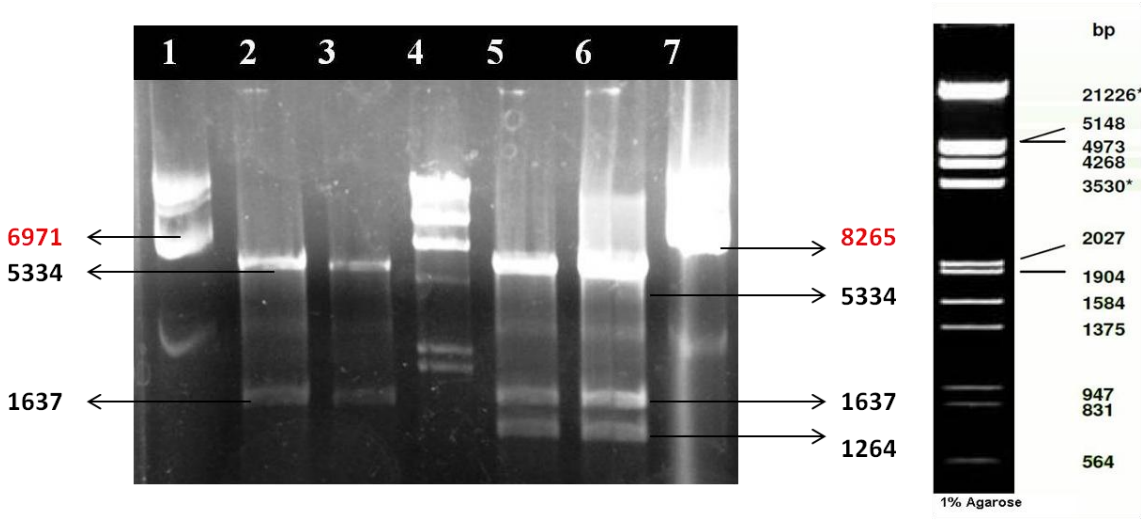


Fig. 4.5: Restriction Digestion pattern of plasmids containing *cs* gene isolated from transformants of *B. japonicum* USDA110 and *M. loti* MAFF030669*:* Lane 1: pAB8 plasmid Undigested (6971 bp); Lane 2 and 3:pAB8 plasmid digested with XbaI and HindIII (5334 bp and 1637 bp); Lane 4 : MWM-Lambda DNA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

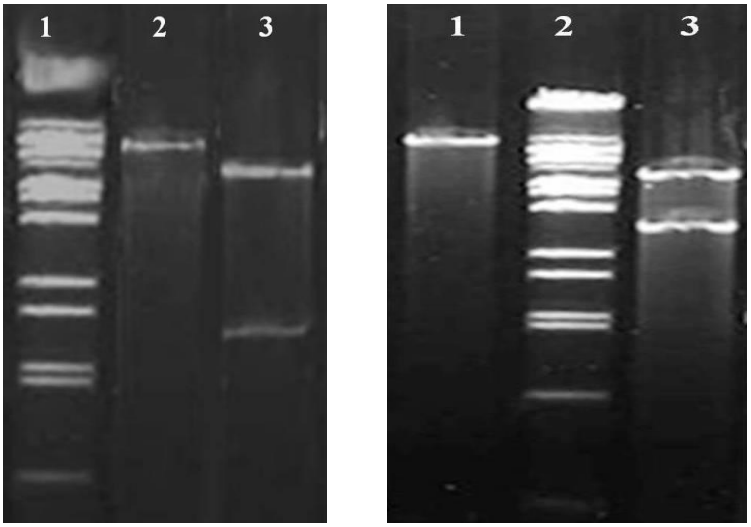


Fig. 4.6: Restriction digestion pattern of plasmids containing *cs* gene isolated from transformants of analysis of *M. loti* MAFF030669: Lane 1: Molecular Weight Marker (MWM)- Lambda DNA cut with BstEII; Lane 2: pAB8 linearised with EcoR I(8.26 kbp); Lane 3: pAB8 digested with EcoRI-HindIII (5,298bp,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

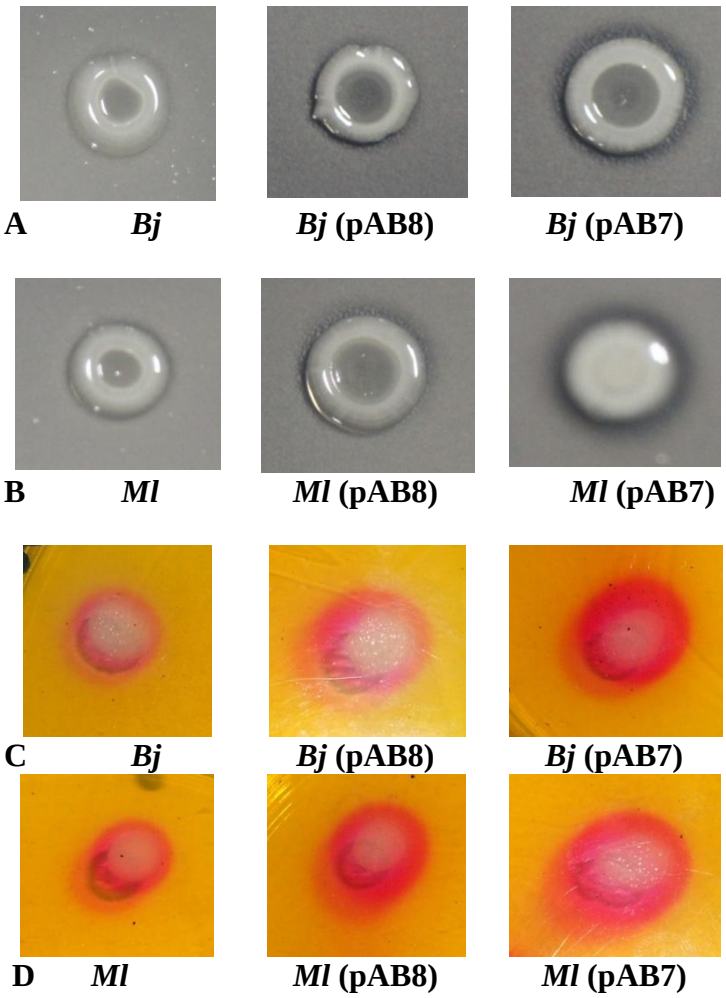


Fig. 4.7: MPS phenotype of (A),(C) *B. japonicum* USDA110 and (B),(D) *M. loti* MAFF030669 harboring pAB7 plasmid expressing *E. coli cs* gene. Zone of clearance formed by the transformants on Pikovskyas agar and TRP agar containing 50mM glucose was monitored noted after an incubation of 3 days at 30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

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 ºC 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).**

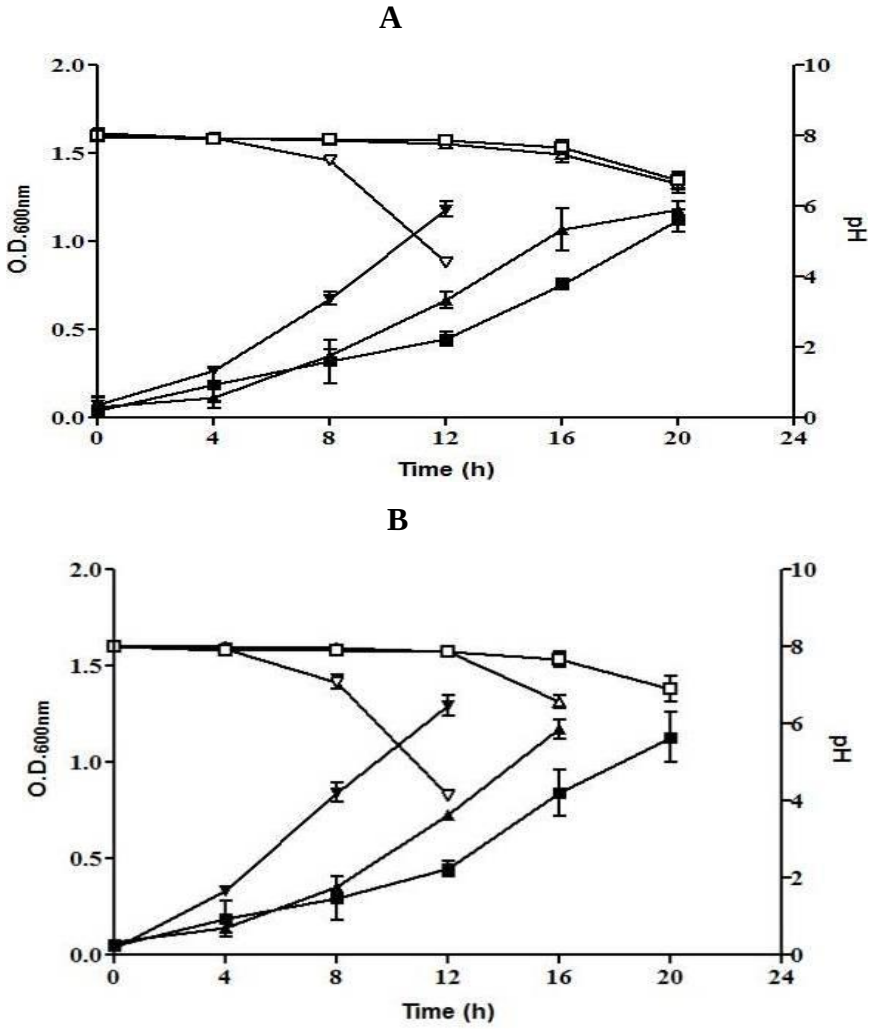


Fig. 4.8: Effect of *cs* gene overexpression on extracellular pH (□, Δ, ▽,) and growth profile (■, ▲, ▼) of (A) *B. japonicum* USDA110 and (B) *M. loti* MAFF030669, with 50 mM glucose and 100 mM Tris-Cl pH 8, 1mg/ml of rock phosphate medium. (□, ■, *Bj, Ml* wild type); {Δ, ▲, *Bj* (pAB8), *Ml* (pAB8)}; {▽, ▼, *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)**.

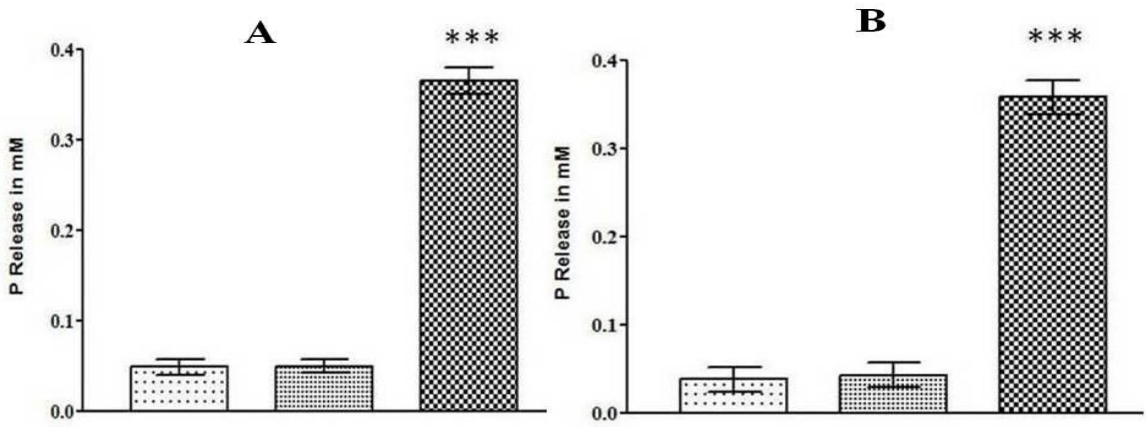


Fig. 4.9: P solubilization by (A) *B. japonicum* USDA110 , (B) *M. loti* MAFF030669 transformants on TRP medium. (▭, *Bj*, *Ml* wild type); {▭, *Bj* (pAB8), *Ml* (pAB8)}; {▭, *Bj* (pAB7), *Ml* (pAB7)}; the values are depicted as Mean ± S.E.M of 7-10 independent observations. ******* P<0.001.

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**).

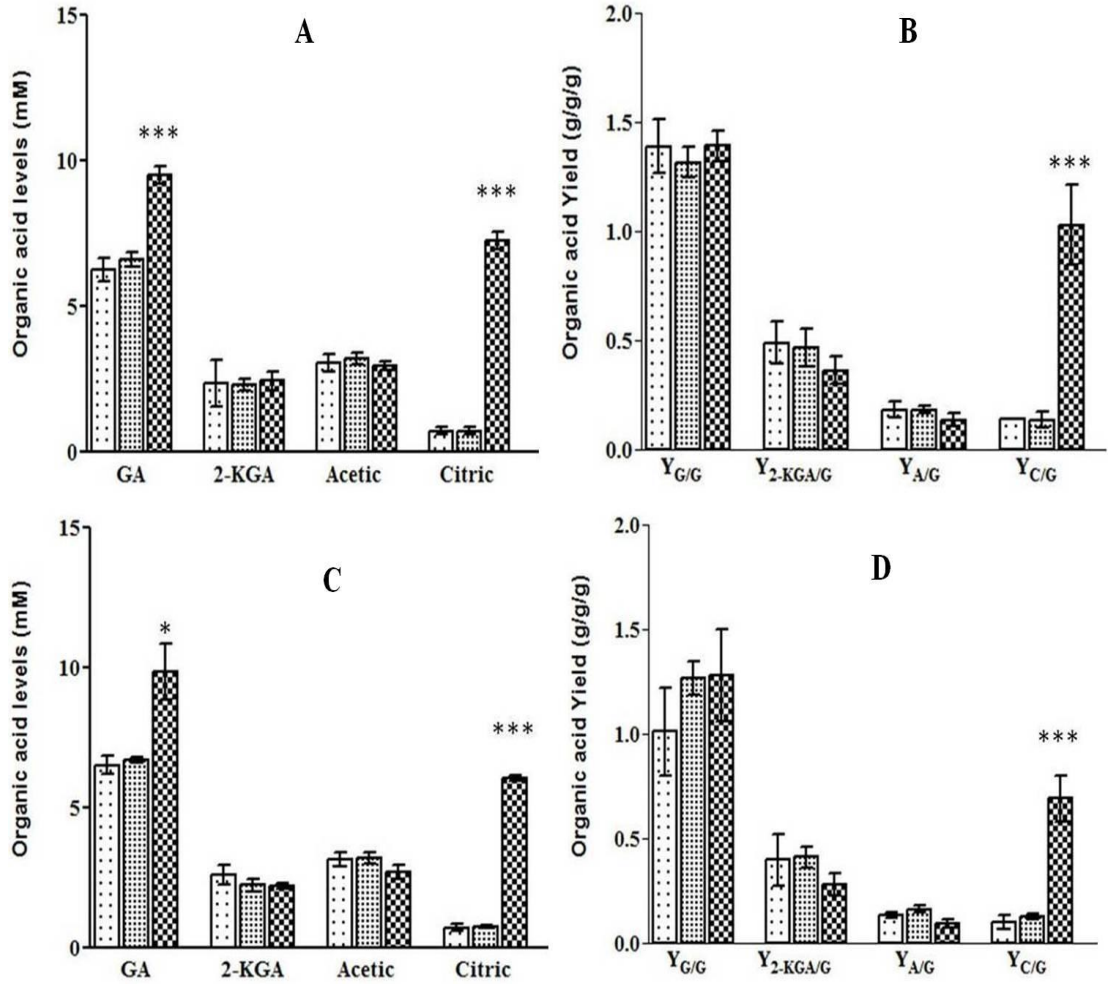


Fig. 4.10 : Organic acid production from *B. japonicum* USDA110 *and M. loti* MAFF030669 *cs* gene (A), (C) organic acids in mM (Gluconic, 2-keto gluconic, acetic and citric acids); (B), (D) Organic acid Yields ($Y_{G/G}$ $Y_{2\text{-}KGA/G}$, $Y_{A/G}$ and $Y_{C/G}$ in *Bj, Bj* (pAB8), *Bj* (pAB7), *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**).

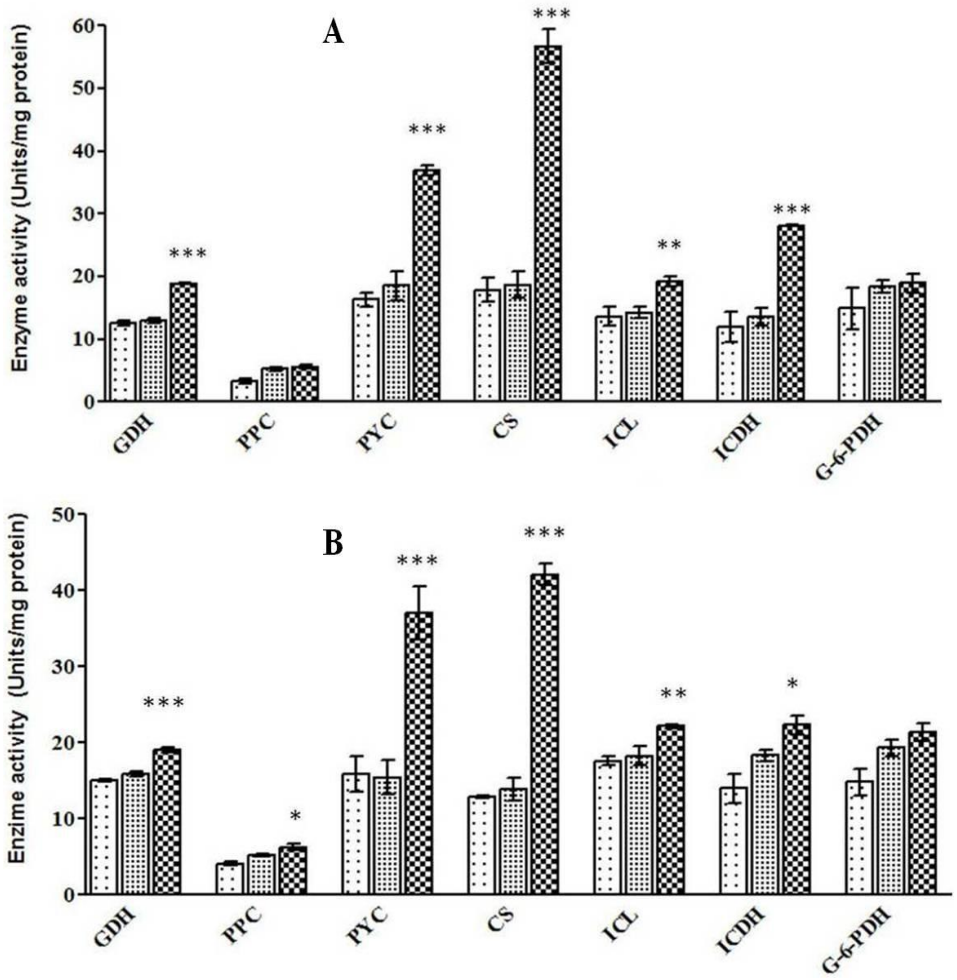


Fig. 4.11: Activities of enzymes. A- *B. japonicum* USDA110; B- *M. loti* MAFF030669. PPC, PYC, GDH, G-6-PDH, ICDH and ICL in and *cs* transformant. (A): *B. japonicum* USDA110 and (B): *M. loti* MAFF030669: The activities have been estimated using cultures grown on TRP medium with 50mM glucose. All 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.

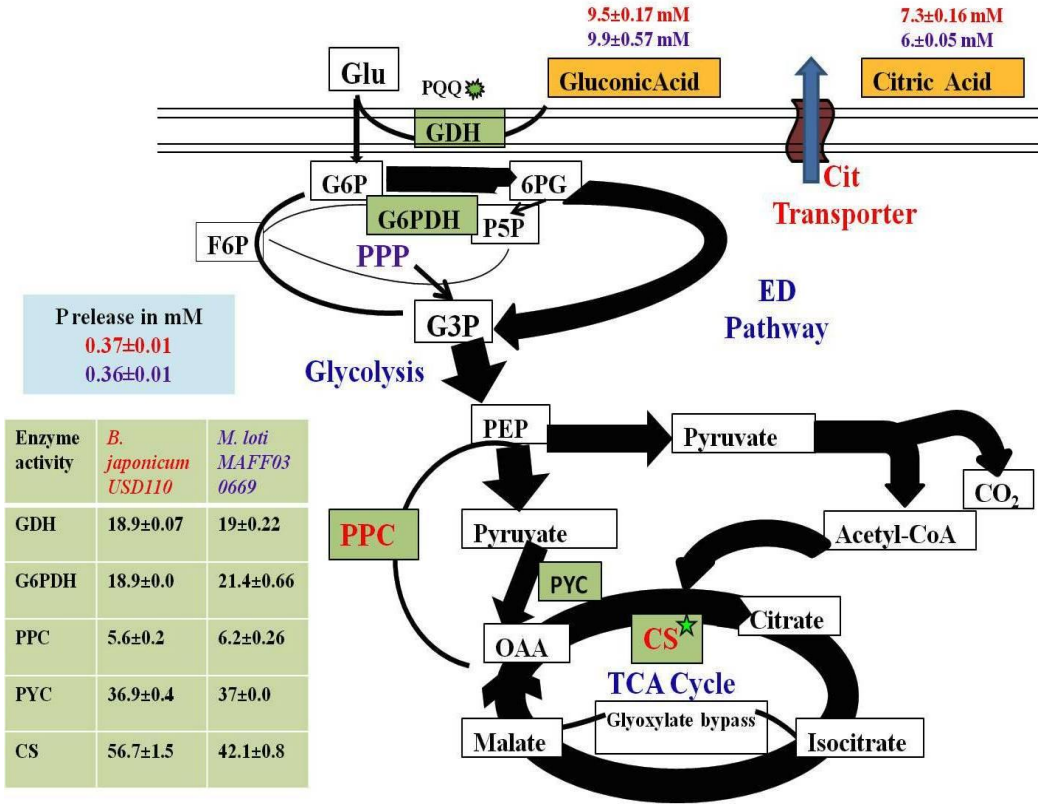
Increase in gluconic acid by *Bj* (pAB7) and *Ml* (pAB7) was ~1.4 and ~1.5 fold, respectively which was comparatively lower than the ~2.7 fold increase in gluconic acid by *Pseudomonas* transformants. This indicates that direct oxidative pathway showed an enhancement in *Pseudomonas* while remains unaltered 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 MAFF03 0669*
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.

Chapter 5

Effect of overexpression of *E. coli* NADH insensitive Y145F *cs* gene on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF303099

5.1 INTRODUCTION

Citrate synthase (CS) is a ubiquitous enzyme that catalyzes the first committed step of tricarboxylic acid (TCA) cycle involving condensation of oxaloacetate (OAA) and acetyl-CoA to form citrate. 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 (Park et al., 1994).

5.1.1 CS and NADH sensitivity

E. coli CS and the CS from other Gram-negative bacteria are allosteric enzymes was recognized almost 50 years ago. The CS of Gram-negative bacteria differ from those of Gram-positive bacteria and eukaryotes in that they are subjected to allosteric inhibition by NADH. NADH inhibition of *E. coli* CS is weakened by conditions, such as high salt or alkaline pH, which favor activity (Weitzman, 1966; Weitzman and Danson, 1976). Gram negative bacteria produce a 'large' homo-hexamer of identical subunits with monomer size of ~48kDa, contains 427 amino acid encoded by *gltA* gene, and is strongly and allosterically inhibited by NADH and, in the facultative anaerobes such as *E. coli*, also by 2-oxoglutarate (Weitzman 1981; Nguyen et al., 2001). On the other hand, gram-positive bacteria and all eukaryotes produce a 'small' (dimeric) also known as type I CS which is insensitive to NADH.

Pseudomonas CS is also allosteric and its kinetic properties suggest as an intermediate between *E. coli* and *Acinetobacter anitratum* enzymes (Massarini et al., 1975; Higa et al., 1978). Two forms of CS (EC 4.1.3.7) have been found in several species of *Pseudomonas,* a large form (Mr ~ 1: 250000) which is generally inhibited by NADH and a small form (Mr ~ 1: 100000) which is insensitive to these nucleotide effectors. Hence, the NADH sensitivity of gram negative bacterial CS is attributed to subunit size. A mutant of *Pseudomonas aeruginosa* PAC514 has been found to contain both a large (CSI) and a small (CSII) isozyme (Solomon and Weitzman, 1983; Mitchell

et al., 1995). The CS of gram-negative bacteria is an allosteric enzyme designated as type II. *E. coli* and *.A. anitratum* CS are strongly homologous in amino acid sequence and more distantly resemble the non-allosteric Type I CS of eukaryotes (Bhayana et al., 1984; Donald et al., 1987; Francois et al., 2006).

5.1.2: NADH insensitive CS

The distinctness of the NADH binding sites from the active sites is established by structural studies on a CS-NADH complex. *E. coli* CS exists in two conformational states, one of which (the R state) binds acetyl-CoA selectively, whereas the other (the T state) binds NADH selectively. NADH inhibition is competitive with respect to acetyl-CoA and noncompetitive with respect to the other substrate, OAA (Wright and Sanwal, 1971). NADH binding is competitively inhibited by acetyl-CoA (Duckworth and Tong 1976).

Type I dimers were altered by a series of mutations to generate NADH sites. Site-directed mutagenesis was used to prepare variant CS proteins in which the nine putative hydrogen-bonding residues in the NADH site, identified through the structural studies, were replaced by nonbonding residues (Maurus et al ., 2003). In addition, one particularly intriguing variant has been crystallized in the presence and absence of NADH, and the three-dimensional structures have been determined using x-ray diffraction methods **(**Duckworth et al., 2003.). The structure of the CS-NADH complex allows the prediction of a number of hydrogen bonds between NADH and the protein, including nine involving amino acid side chains. These variants are listed in **Table 5.1**, along with the effects of the amino acid substitutions on NADH binding and inhibition. Each variant protein was active and appeared to be stable. These three variants, Y145F, R163L, and K167A, all involve residues that are believed to take part in a complex hydrogen bonding network with the pyrophosphate moiety of NADH (**Fig.5.1**). All of the variants show some changes in NADH binding and inhibition and small but significant changes in kinetic parameters for catalysis. In three cases, Y145F, R163L, and K167A, NADH inhibition has become extremely weak.

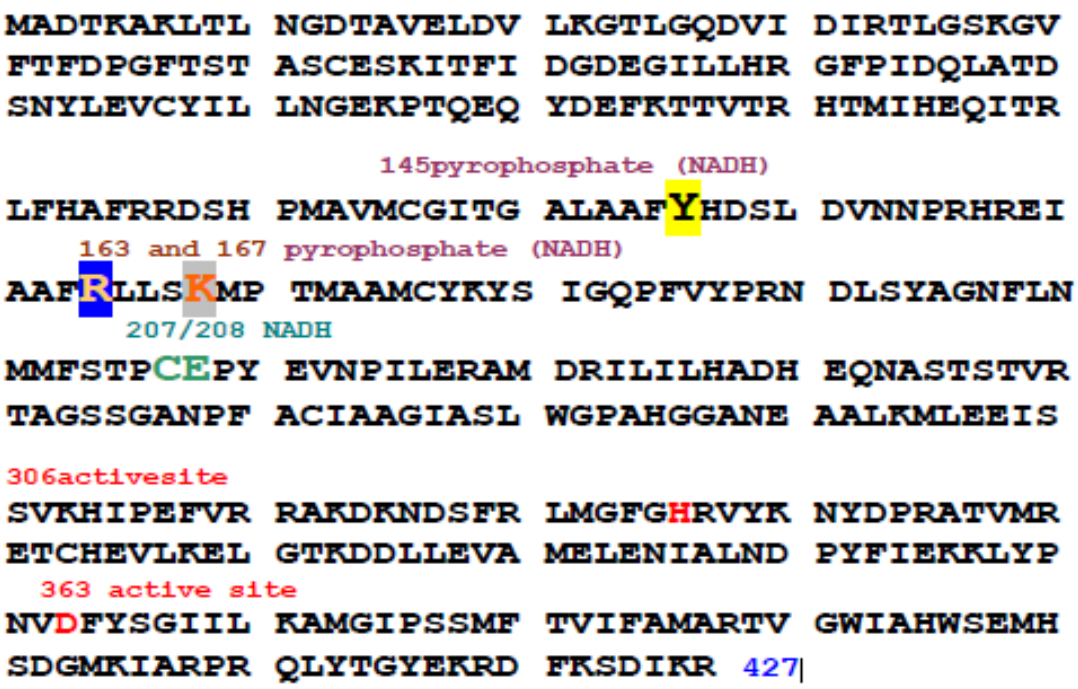


Fig. 5.1: *E. coli* CS protein sequence showing the regulatory variant (Duckworth et al., 2003.).

Table 5.1: NADH binding and inhibition by variant CSs (Stokell et al., 2003)

Variant	Kd (µM)	Ki (µM)	Maximum inhibition(%)
Wild type	1.6±0.1	2.8±0.4	100±10
R109L	1.16±.04	1.7±0.2	96±6
H110A	5.2±0.2	121±11	97±3
T111A	6.6±0.2	80±15	87±6
Y145F	>100	790±210	100±3
R163L	5.81±.04	400±80	77±8
K167A	4.1±0.2	630±130	100±10
Q128A	6.1±0.5	18±3	100±7
N189A	6.9±0.8	242±26	96±5
T204A	10.2±0.4	165±36	100±7

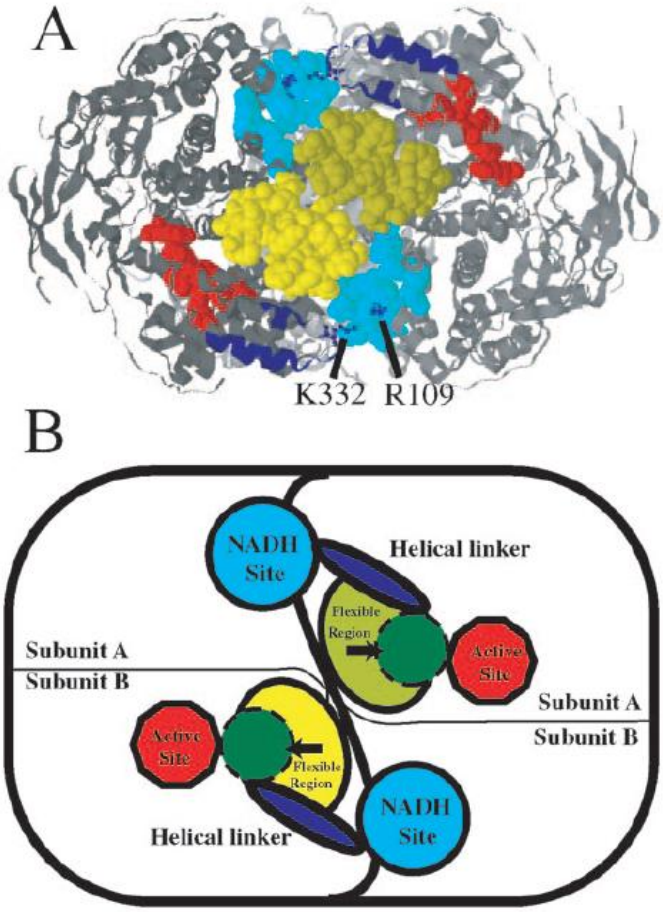


Fig. 5.2: **Route of communication between NADH binding sites and active sites in wild type hexameric *E. coli* CS** (Stokell et al., 2003).

Examination of the hexameric structure of *E. coli* CS as a whole showed that the helical linker region (residues 316–342) structurally links the refolded active site region polypeptide chain segment (residues 262–298) to an NADH binding site. However, the NADH binding site involved is not from the same subunit, instead being located across the dimer-dimer interface on an adjacent subunit (**Fig. 5.2**. **A and B)** (Stokell et al., 2003).

5.1.3: Increase in production of Citric acid

Isocitrate lyase (*icl*) gene overexpression in *Yarrowia lipolytica* altered citrate/isocitrate ratio towards citric acid (Forster et al., 2007a). Additionally, invertase gene expression enabled *Y. lipolytica* to secrete high levels of citric acid in presence of sucrose. Similarly, *B. subtilis* 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 levels could be attained on glucose and depending on the host metabolism. Disruption of *icd* gene of *E. coli* has been reported to secrete citric acid levels up to 3.4 mM (Aoshima et al., 2003; Kabir and Shimizu, 2004). Although citric acid was increased, the mutant showed glutamate auxotrophy. However, citric acid secretion is difficult to explain as *E. coli* K strain does not possess citrate transporter (Hall, 1982). Citric acid secretion was also found in *Bacillus subtilis icd* and *Streptomyces coelicolor* aconitase mutants (Matsuno et al., 1999; Viollier et al., 2001).

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 *Pseudomonas 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 but the levels were insufficient for releasing P from soils (Gyaneshwar et al., 1998; Srivastava et al., 2006).

Metabolic studies in *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 (Elias, 2009). To increase the flux through the anaplerotic node for increasing oxaloacetate levels, *ppc* gene of *S. elongatus* was over-expressed in *P. fluorescens* 13525. *ppc* gene overexpression enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted

in increased gluconic, pyruvic and acetic acids and intracellular citric acid but citric acid was not secreted. Overexpression of either of *ppc* and *cs* genes enhanced MPS ability of *P. fluorescens* 13525 on Pikovskya's agar; but *ppc-cs* co-expression neither altered *P. fluorescens* ATCC 13525 metabolism nor affected the citrate production (Buch et al., 2010). Overexpression of *E. coli* CS *gltA* gene in *Pseudomonas fluorescens* ATCC 13525 yielded intracellular and extracellular citric acid levels during the stationary phase, respectively (Buch et al., 2009). Hence further strategies are required to increase the citrate level.

Among the three CS variants studied, Y145F showed maximum inhibition, highest Ki and Kd values (Stokell et al., 2003) Also *Pf* (pY145F) showed the highest CS activity amongst all the other variants which is 2 fold,1.7 fold and 1.96 fold higher as compared to the wild type *cs* bearing strain *Pf* (pAB7) and other two NADH insensitive *cs* bearing strain *Pf* (pR163L) and *Pf* (K167A) respectively in the mid log phase, so pY145F was used for further study (Adhikary, 2012).

Partial sequencing of pY145F plasmid

Partial sequencing of the PCR product amplified from pY145F plasmid when analysed using NCBI BLAST (Basic Local Alignment Search Tool) and Ribososmal Database Project (RDP) II, online homology search programs, revealed maximum identity (99%) to *E. coli* CS (GenBank Accession number AAA23892) (**Fig. 5.3 and 5.4**).

>YFPCRPRODUCT_YFFOR_S702

CNCNNANCGGCACCCTGAACGGGGATACAGCTGTTGAACTGGATGTGCTGAA
AGGCACGC TGGGTCAAGATGTTATTGATATCCGTACTCTCGGTTCAAAAGGT
GTGTTCACCTTTGACCCAGGCTTCACTTCAACCGCATCCTGCGAATCTAAAAT
TACTTTTATTGATGGTGATGAAGGTATTTTGCTGCACCGCGGTTTCCCGATCG
ATCAGCTGGCGACCGATTCTAACTACCTGGAAGTTTGTTACATCCTGCTGAAT
GGTGAAAAACCGACTCAGGAACAGTATGACGAATTTAAAACTACGGTGACCC
GTCATACCATGATCCACGAGCAGATTACCCGTCTGTTCCATGCTTTCCGTCGC
GACTCGCATCCAATGGCAGTCATGTGTGGTATTACCGGCGCGCTGGCGGCGT
TCTTTCACGACTCGCTGGATGTTAACAATCCTCGTCACCGTGAAATTGCCGCG
TTCCGCCTGCTGTCGAAAATGCCGACCATGGCCGCGATGTGTTACAAGTATTC
CATTGGTCAGCCATTTGTTTACCAGCGCAACGATCTCTCCTACGCCGGTAACT
TCCTGAATATGATGTTCTCCACGCCGTGCGAACCGTATGAAGTTAATCCGATT
CTGGAACGTGCTATGGACCGTATTCTGATCCTGCACGCTGACCATGAACAGA
ACGCCTCTACCTCCACCGTGCGTACCGCTGGCTCTTCGGGTGCGAACCCGTTT
GCCTGTATCGCAGCAGGTATTGCTTCACTGTGGGGACCTGCGCACGGCGGTG
CTAACGAAGCGGCGCTGAAAATGCTGGAAGAAATCAGCTCCGTTAAACACA
TTCCGGAATTTGTTCGTCGTGCGAAAGACAAAAATGATTCTTTCCGCCTGATG
GGCTTCGGTCACCGCGTGTACAAAATTACGACCCGCGCGCCACCGTAATGCG
TGAAACCTGCCATGAAGTGCTGAAAGAGCTGGGCACGAANNTGACCTGCTGN
AGT

Fig. 5.3: Partial sequence of *E. coli* NADH insensitive *cs* gene.

```
>gb|U00096.2|  Escherichia coli str. K-12 substr. MG1655, complete genome
Length=4639675

 Features in this part of subject sequence:
   citrate synthase

 Score = 1777 bits (962),  Expect = 0.0
 Identities = 971/975 (99%), Gaps = 1/975 (0%)
 Strand=Plus/Minus

Query  4       CACCCTGAACGGGGATACAGCTGTTGAACTGGATGTGCTGAAAGGCACGCTGGGTCAAGA  63
               ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753668  CACCCTCAACGGGGATACAGCTGTTGAACTGGATGTGCTGAAAGGCACGCTGGGTCAAGA  753609

Query  64      TGTTATTGATATCCGTACTCTCGGTTCAAAAGGTGTGTTCACCTTTGACCCAGGCTTCAC  123
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753608  TGTTATTGATATCCGTACTCTCGGTTCAAAAGGTGTGTTCACCTTTGACCCAGGCTTCAC  753549

Query  124     TTCAACCGCATCCTGCGAATCTAAAATTACTTTTATTGATGGTGATGAAGGTATTTTGCT  183
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753548  TTCAACCGCATCCTGCGAATCTAAAATTACTTTTATTGATGGTGATGAAGGTATTTTGCT  753489

Query  184     GCACCGCGGTTTCCCGATCGATCAGCTGGCGACCGATTCTAACTACCTGGAAGTTTGTTA  243
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Query  244     CATCCTGCTGAATGGTGAAAAACCGACTCAGGAACAGTATGACGAATTTAAAACTACGGT  303
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Sbjct  753428  CATCCTGCTGAATGGTGAAAAACCGACTCAGGAACAGTATGACGAATTTAAAACTACGGT  753369

Query  304     GACCCGTCATACCATGATCCACGAGCAGATTACCCGTCTGTTCCATGCTTTCCGTCGCGA  363
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Query  364     CTCGCATCCAATGGCAGTCATGTGTGGTATTACCGGCGCGCTGGCGGCGTTCTTTCACGA  423
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Query  424     CTCGCTGGATGTTAACAATCCTCGTCACCGTGAAATTGCCGCGTTCCGCCTGCTGTCGAA  483
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Query  484     AATGCCGACCATGGCCGCGATGTGTTACAAGTATTCCATTGGTCAGCCATTTGTTTACCA  543
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Sbjct  753188  AATGCCGACCATGGCCGCGATGTGTTACAAGTATTCCATTGGTCAGCCATTTGTTTACCC  753129

Query  544     GCGCAACGATCTCTCCTACGCCGGTAACTTCCTGAATATGATGTTCTCCACGCCGTGCGA  603
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Query  604     ACCGTATGAAGTTAATCCGATTCTGGAACGTGCTATGGACCGTATTCTGATCCTGCACGC  663
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753068  ACCGTATGAAGTTAATCCGATTCTGGAACGTGCTATGGACCGTATTCTGATCCTGCACGC  753009
```

Fig. 5.4: NCBI BLAST analysis of partial *cs* sequence.

Pair wise alignment of the sequence with original *E. coli* K12 NADH sensitive *cs* sequence from database using EBI pair wise alignment tool, revealed a mutation of tyrosine residues in 146 amino acid position to phenylalanine (**Fig. 5.5**).

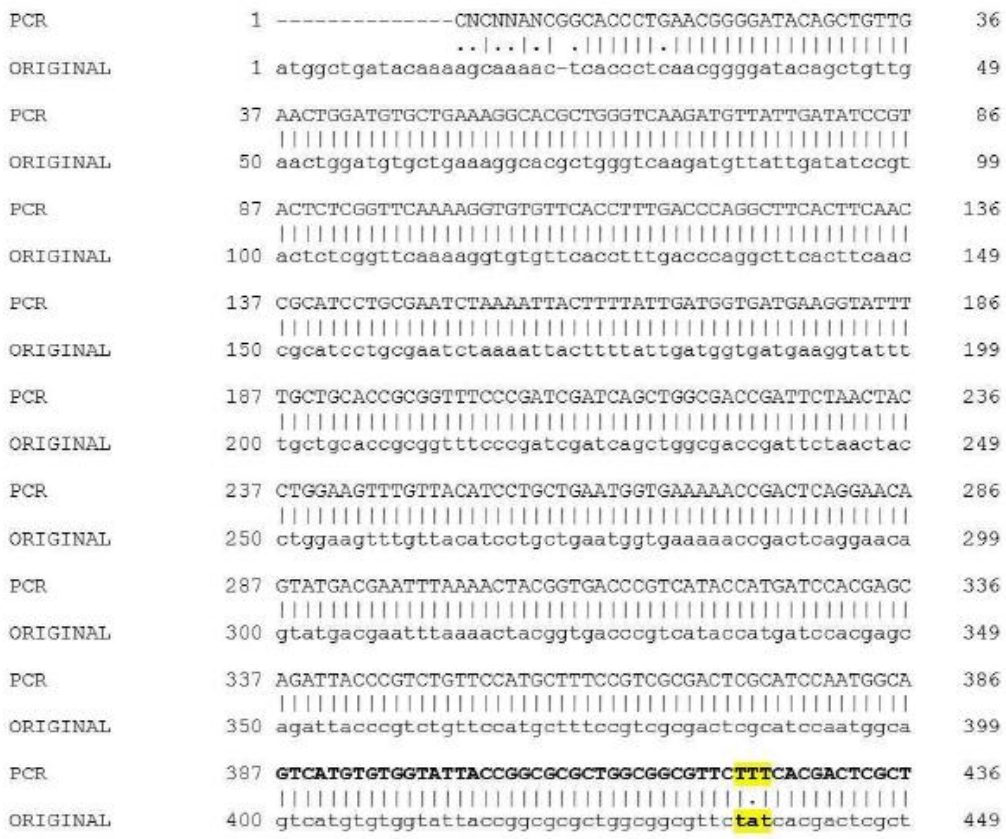


Fig. 5.5: EBI pair wise alignment of NADH insensitive and wild type *cs* gene showing the position of mutation.

To increase the flux through the anaplerotic node for increasing oxaloacetate levels, NADH insensitive *cs* gene was over-expressed in fluorescent *P. fluorescens* PfO-1 which resulted in significant increase in CS activity and citric acid levels as compared to the wild type NADH sensitive CS *P. fluorescens* PfO-1 harboring pY145F showed maximum CS activity of 333.4±8.5U on M9 minimal medium in the presence of 100 mM glucose which is ~ 5.6 fold higher than that in the control *Pf* (pAB8) (Adhikary, 2012). The NADH insensitive *cs* gene overexpression caused significant alterations in both intracellular and extracellular citric acid levels and yields. Intracellular citric acid levels in *Pf* (pY145F) increased by 5.7, 6.9 and 2.6 fold while there is a 5, 7.9 and 2.9 fold increase in intracellular citrate yield as compared to wild type strain, *Pf* (pAB8)and *Pf* (pAB7), respectively. Corresponding extracellular citrate levels increased by 57, 51.6 and

1.9 fold with an increase of extracellular citrate yield by 39.8, 29.9 and 2.39 fold compared to respective controls (**Fig. 5.6;** Adhikary et al., 2012).

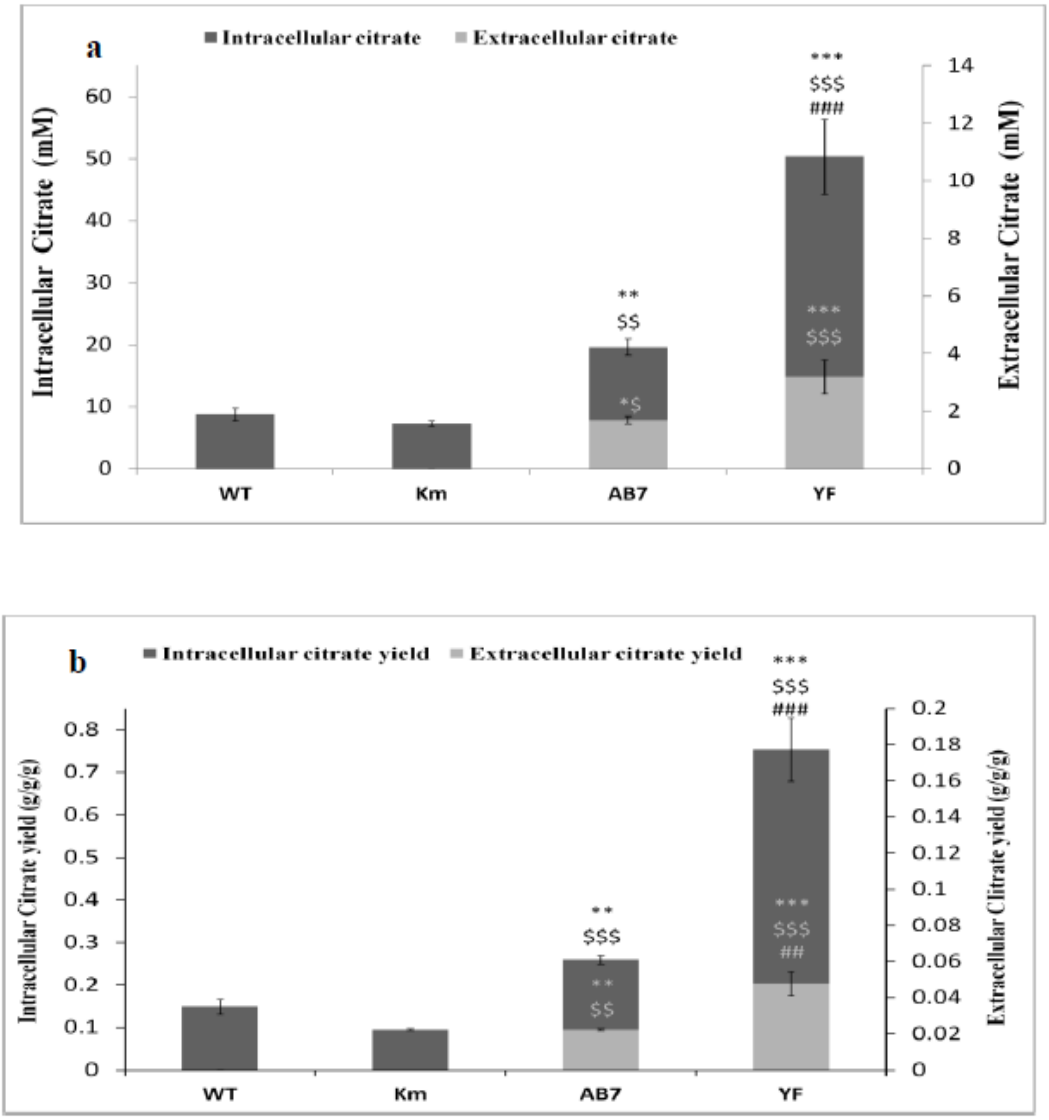


Fig. 5.6: Citric acid levels and yields in *P. fluorescens* PfO-1 wild type and plasmid bearing strains Km, AB7 and YF. Intracellular citrate levels (a) and yields (b) are represented in black bars and extracellular citrate levels (a) and yields (b) are represented in grey bars.

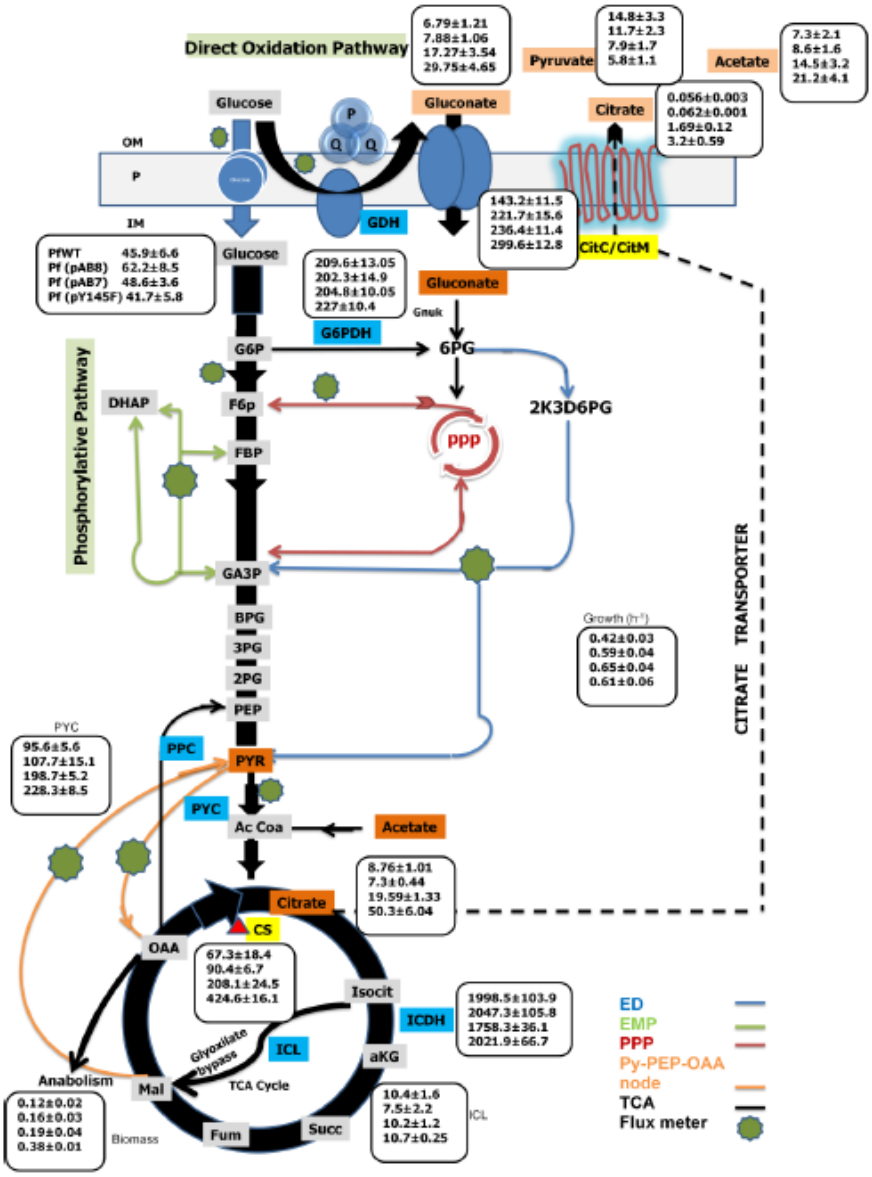


Fig. 5.7: **Key metabolic fluctuations in *P. fluorescens* PfO-1 overexpressing NADH insensitive *E. coli* CS.**

5.1.4 Rational of the present study

Overexpression of *ppc* gene of *S. elongatus* in *B. japonicum USDA110* and *M. loti MAFF030669* enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increased gluconic and citric acids secretion, this high citric acid was due to increased activities of both PYC and PPC. Similarly,

overexpression of *gltA* gene of *E. coli* in the last chapter also resulted in significant increase in citric acid secretion upto 7 mM due to significant increase in the activity of CS. However, P solubilization was better in *ppc* gene overexpression as compared to *glt* gene overexpression which could be attributed to the lower levels of gluconic acid in the latter case. In order to further increase citric acid secretion, this chapter dealt with developing *Rhizobium* strains expressing *E. coli* NADH insensitive *cs* gene and monitoring its effects on citric acid accumulation and secretion and glucose metabolism.

5.2: Experimental design

The experimental plan of work includes the following-

5.2.1: List of bacterial strains used

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

Table 5.2 list of bacterial strains

Bacterial strains	Characteristics	Source/Reference
***E. coli* strains**		
E. coli JM101	Used for molecular biology experiments	Sambrook and Russell, 2001
E. coli W620	*cs* mutant strain exhibiting glutamate auxotrophy, CGSC 4278 - *glnV44 gltA6 galK30 LAM-pyrD36 relA1 rpsL129 thi-1*; Str^r	*E. coli* Genetic Stock Center
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	NC_002678.2	NCBI

MAFF030669		
Bj (pJNK3)	*B. japonicum* USDA110 with pJNK3 plasmid; Ap^r, Km^r (*E. coli* NADH insensitive *cs*)	This study
Bj (pAB8)	*B. japonicum* USDA110 with pAB8 plasmid; Ap^r, Km^r (control vector)	This study
Ml (pJNK3)	*M. loti* MAFF030669 with pJNK3 plasmid; Ap^r, Km^r (*E. coli* NADH insensitive *cs*)	This study
Ml (pAB8)	*M. loti* MAFF030669 with pAB8 plasmid; Ap^r, Km^r (control vector)	This study

5.2.2: Details of Plasmid used

pUCPM18 with *E. coli* NADH insensitive *cs** gene under *Plac* , Ap^r and km^r gene, named as pJNK3 was used in this chapter.

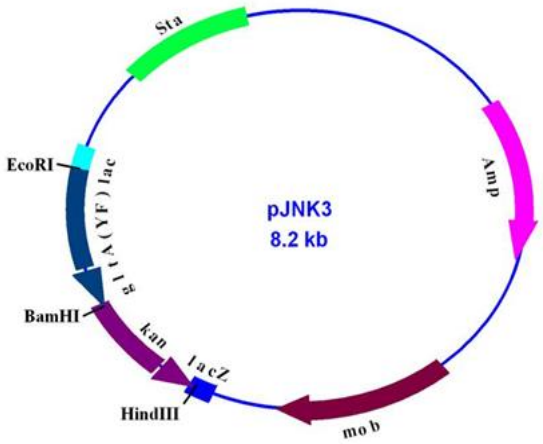


Fig. 5.8: Restriction map of the plasmid used in this chapter (Wagh et al., 2013).

5.3: RESULTS

5.3.1: Heterologous overexpression of *E. coli* NADH insensitive *cs** gene in *Rhizobium* spp.

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 before studying the effect of overexpression of *E. coli* NADH insensitive *cs** gene (**Fig. 5.9)**.

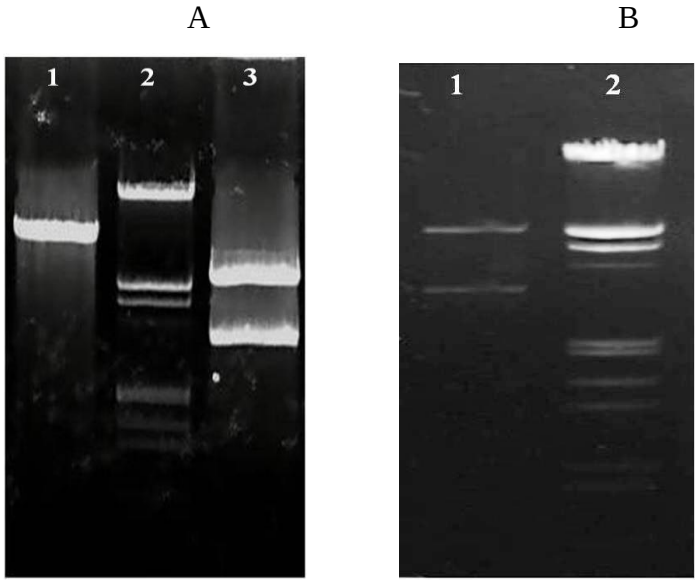


Fig. 5.9 : Restriction Digestion pattern of plasmids containing pJNK3 isolated from transformants of (A) *B. japonicum* USDA110 and (B) *M. loti* MAFF030669 Lane 1: pJNK3 linear with HindIII (8.26 kbp); Lane 2: Molecular Weight Marker (MWM)-Lambda DNA cut with ECORV/ HIND III; Lane 3: pJNK3 digested with EcoRI-HindIII (5,298bp, 2,967bp); Lane 1: pJNK3 digested with EcoRI-HindIII (5,298bp, 2,967bp); Lane 2: Molecular Weight Marker (MWM)-Lambda DNA cut with EcoRV/ Hind III.

The CS activity estimated in *B. japonicum* USDA110 and *M. loti* MAFF030669. harboring *E. coli* NADH insensitive *cs* gene {*Bj* (pJNK3)} and{ *Ml* (pJNK3)} grown on TRP medium with 50mM glucose, was 3.4 and 4.8 fold higher (62.41 ±.0.48 U and 67.02

± 1.98 U) in both the strains compared to control which possessed very low 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* (pJNK3) and *Ml* (pJNK3) 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* (p JNK3) = *Ml* (p JNK3) > *Bj* (pAB8) = *Ml* (pAB8) >*Bj*=*Ml* on PVK medium after 3 days of incubation at 30°C (**Fig. 5.10**).

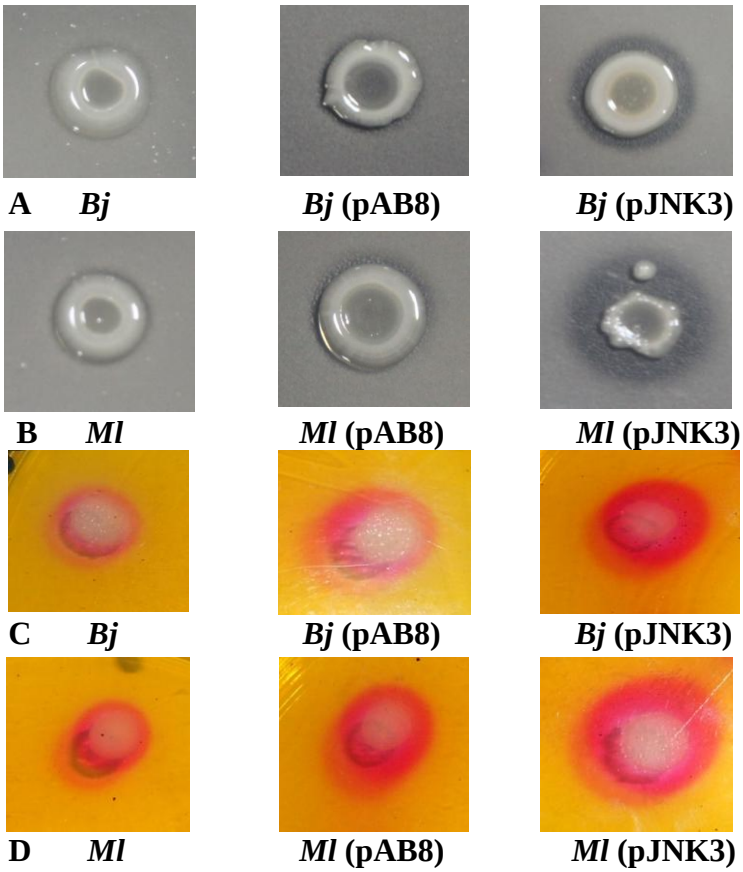


Fig. 5.10: MPS phenotype of *B.japonicum* USDA110(a), (c) and *M. loti* MAFF030669 (b),(d) harboring pJNK3 plasmid expressing *cs* gene. Zone of clearance formed by *Rhizobium* transformants on Pikovskaya's agar and Tris rock phosphate agar containing

50mM glucose The results were noted after an incubation of 3days at 30 ºC. Media composition and other experimental details are as described in Sections 2.2.4 and 2.7.

Table 5.3: 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): *B.japonicum* with vector control and *Bj* (pJNK3) **:** *B.japonicum* with NADH insensitive *cs* gene. The results were noted after an incubation of 3 days at 30 ºC 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* (pJNK3)**	14.50 ± 0.50	10.17 ± 0.29	1.44
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
***Ml* (pAB8)**	12.17 ± 0.29	10.17 ± 0.29	1.22
***Ml* (pJNK3)**	16.83 ± 0.29	10.17 ± 0.29	1.60

The pJNK3 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* (pJNK3) and *Ml* (pJNK3) (**Table 5.3).**

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

The growth profiles and organic acid secretion of *Bj* (pAB8) , *Bj* (pJNK3), *Ml* (pJNK3) and *Ml* (pAB8) along with native, on TRP medium with 50 mM glucose demonstrated that maximum O.D. was reached faster in transformants (12h compared to 20h of the control *Bj* (pAB8) and *Ml* (pAB8). Acid production was monitored and it was

found that there was slight pH drop within 20 h in the native and control vector while pH drop to 4.3 and 4.2 was seen in *Bj* (pJNK3) and *Ml* (pJNK3). Significant media acidification was seen within 12 h in both the cases. Both *Bj* (pJNK3) and *Ml* (pJNK3) acidified the medium when grown on TRP medium (**Fig. 5.11).**

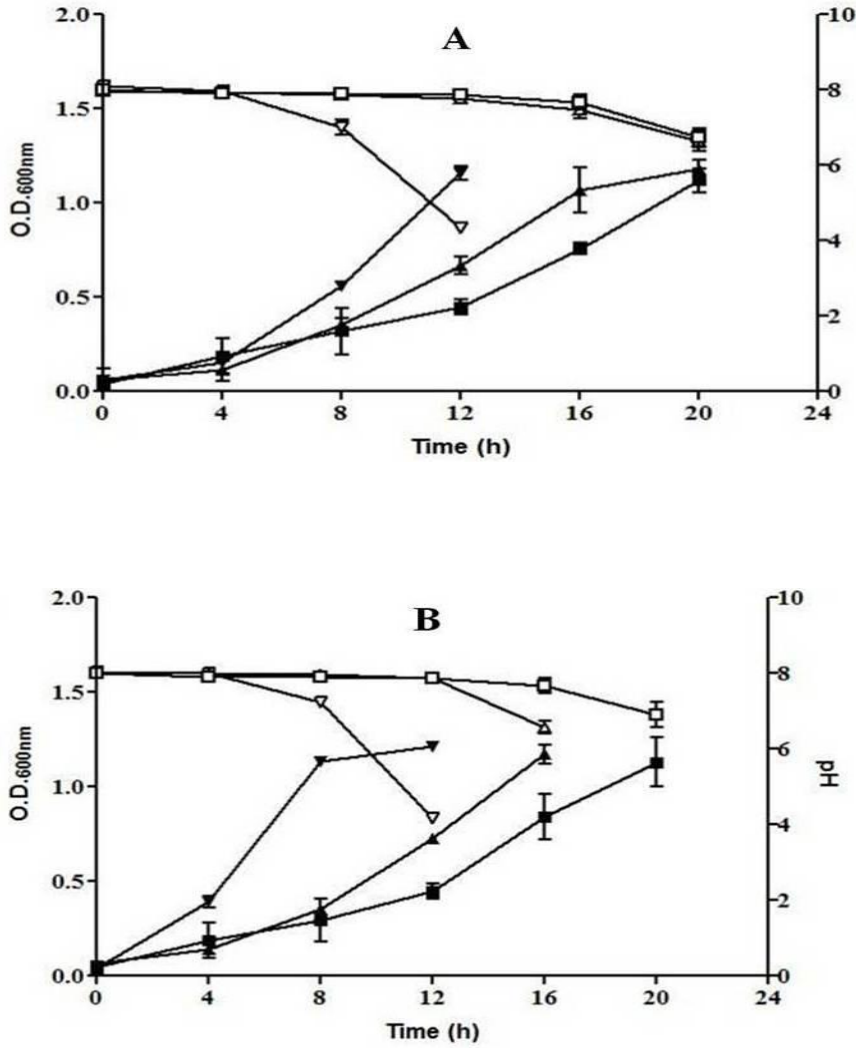


Fig. 5.11: Effect of *E. coli* NADH insensitive *cs* gene overexpression on extracellular pH (□, Δ, ▽,) and growth profile (■, ▲, ▼) of (A) *B. japonicum* USDA110 and (B) *M. loti* MAFF030669, on TRP medium with 50 mM glucose .(□, ■, *Bj*, *Ml* wild type); {Δ, ▲, *Bj* (pAB8), *Ml* (pAB8)}; {▽, ▼, *Bj* (pJNK3), *Ml* (pJNK3)}. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

5.3.7: Physiological effects of *E. coli* NADH insensitive *cs* gene overexpression on TRP medium with 50 mM glucose.

In presence of 50 mM glucose, increase in CS activity significantly affected growth profile in transformants of both the strains (**Fig. 5.11**). The total glucose utilization rate showed negligible increase and the total amount of glucose consumed showed ~1.9 fold and ~1.8 fold decrease in *Bj* (pJNK3) and *Ml* (pJNK3) respectively at the time of pH drop. The increase in CS activity increased biomass yield by ~1.6 fold and also ~1.7 fold decrease was seen in specific glucose utilization rate in the transformants of both the *Rhizobium* strains (**Table 5.4**).

Table 5.4: 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<0.001.

***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 ±0.2	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* (pJNK3)**	0.259±0.01	48.07 ±0.10	19.61±0.48***	2.54 ±0.11***	0.10 ±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* (pJNK3)**	0.381±0.03	48.15 ±0.30	20.91±1.50***	1.73 ±0.09***	0.14 ±0.01***

5.3.6: Biofilm, exopolysaccharide and indole acetic acid production by *Bj* (pJNK3) and *Ml* (pJNK3) transformants in TRP medium.

Biofilm showed significant increase by ~2.2 and ~2.75 fold in *Bj* (pJNK3) and *Ml* (pJNK3) respectively, compared to control, exopolysaccharide production was increased by ~1.6 fold in both the transformants compared to control vectors. Indole acetic acid production was increased by ~1.5 fold in *Bj* (pJNK3) and ~1.3 fold in *Ml* (pJNK3). This increase helped in phosphorus release by overexpression of *cs* gene when grown in TRP medium 100mM Tris-Cl Buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml in comparison to control (**Table 5.5**).

Table 5.5: Biofilm, exopolysaccharide and indole acetic acid production by *Bj* (pJNK3) and *Ml* (pJNK3) transformants in TRP medium.The results are expressed as Mean ± S.E.M of 6-10 independent observations *** 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* (pJNK3)**	4.53 ±0.22***	21.95 ±0.71***	38.03 ±1.06***
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* (pJNK3)**	4.24 ±0.1***	24.31 ± 1.83***	34.66 ±0.55***

5.3.5: P Solubilization and Organic acid secretion by *Bj* (pJNK3) and *Ml* (pJNK3) transformants in TRP medium.

There was significant increase in release of P by ~13 fold in both the transformants compared to control vectors when grown in TRP medium containing 50 mM glucose (**Fig. 5.12)**.

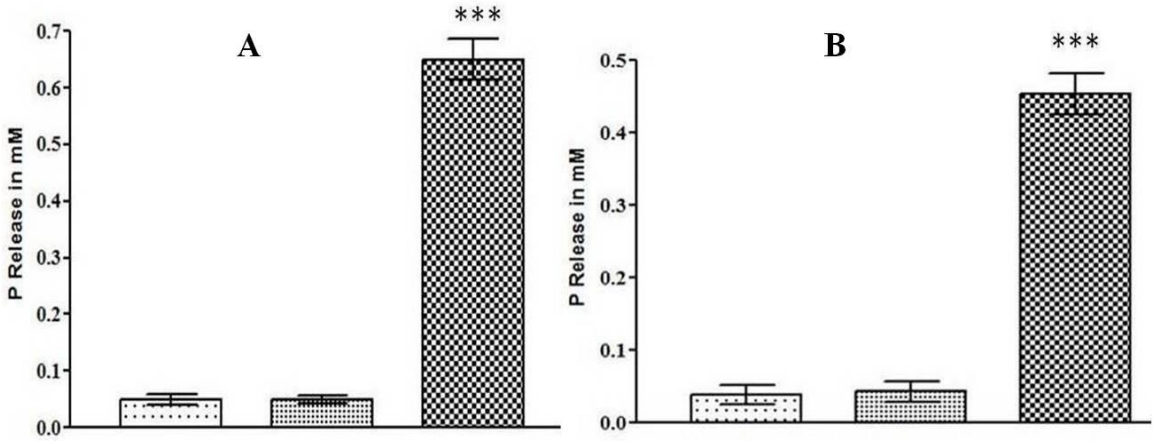


Fig. 5.12: P solubilization by (A) *B. japonicum* USDA110 , (B) *M. loti* MAFF030669 transformants on TRP medium. (▭, *Bj*, *Ml* wild type); {▭, *Bj* (pAB8), *Ml* (pAB8)}; {▭, *Bj* (pJNK3), *Ml* (pJNK3)}; the values are depicted as Mean ± S.E.M of 7-10 independent observations. *** P<0.001.

On TRP medium in presence of 50 mM glucose, the organic acids identified were mainly gluconic, 2-ketogluconic, acetic and citric acids. As a result of *E. coli* NADH insensitive *cs* gene overexpression, there was only quantitative change in two organic acids secreted. Due to overexpression of *cs** gene, there was ~10.8 and~11.1 fold increase in citric acid with their corresponding increase in yield ($Y_{C/G}$) by ~5.7 and ~4.9 fold in *B. japonicum* USDA110 and *M. loti* MAFF030669 *E. coli* NADH insensitive *cs* gene transformants, respectively. Also extracellular medium of *Bj* (pJNK3) and *Ml* (pJNK3) contained ~4 and ~3.7 fold higher amount of gluconic acid, and~2.3 and~1.8 fold increase in its yield was seen respectively as compared to *Bj* (pAB8) and *Ml* (pAB8) (**Fig. 5.13)**.Intracellular citric acid levels remained unchanged **(Table 5.6).**

Table 5.6: Intracellular citric acid production by *Bj* (pJNK3) and *Ml* (pJNK3) 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* (pJNK3)**	0.73 ± 0.06	***Ml* (pJNK3)**	0.81 ± 0.03

Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

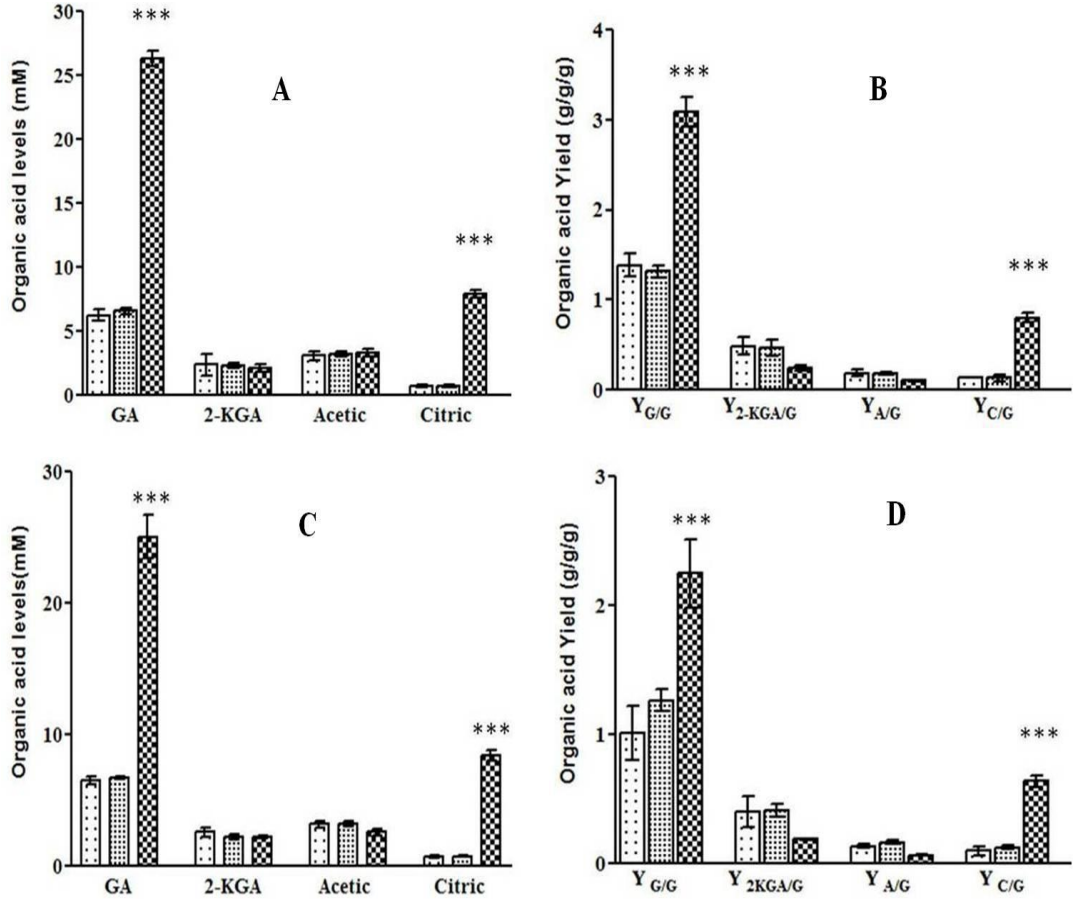


Fig. 5.13: Organic acid production {(a) and (c)} and Yield {(b) and (d)} from *B. japonicum* USDA110 *and M. loti* MAFF030669 *cs* gene transformants, respectively. All organic acids are estimated from stationary phase cultures (at the time of pH drop) grown on TRP medium with 50mM glucose. Results are expressed as Mean ± S.E.M of 4-6 independent observations *** P<0.001.**(ii) Alterations in enzyme activities in *Bj* (pAB3) and *Ml* (pJNK3)**

In order to correlate the alterations in physiological variables and organic acid profile, enzymes involved periplasmic direct oxidation and intracellular phosphorylative were estimated. In response to *cs** gene overexpression, about ~ 3.4 and ~ 4.8 fold increase is seen in CS activity in *Bj* (pJNK3) and *Ml* (pJNK3), respectively. GDH activity increased by about ~1.5 and ~1.3 fold, respectively, as compared to the control, PYC showed ~2.3 and ~3.2 fold increase, respectively, in the transformants. The activity of G-6-PDH showed ~1.5 and ~1.4 fold increase in the transformants and also there was ~1.5 and ~1.9 fold increase in ICDH activity. The activity of PPC in *Bj* (pJNK3) and *Ml* (pJNK3) did not alter significantly as compared to the control. Glyoxylate pathway enzyme ICL also remained unaltered in *Bj* (pJNK3) and *Ml* (pJNK3) (**Fig. 5.14**).

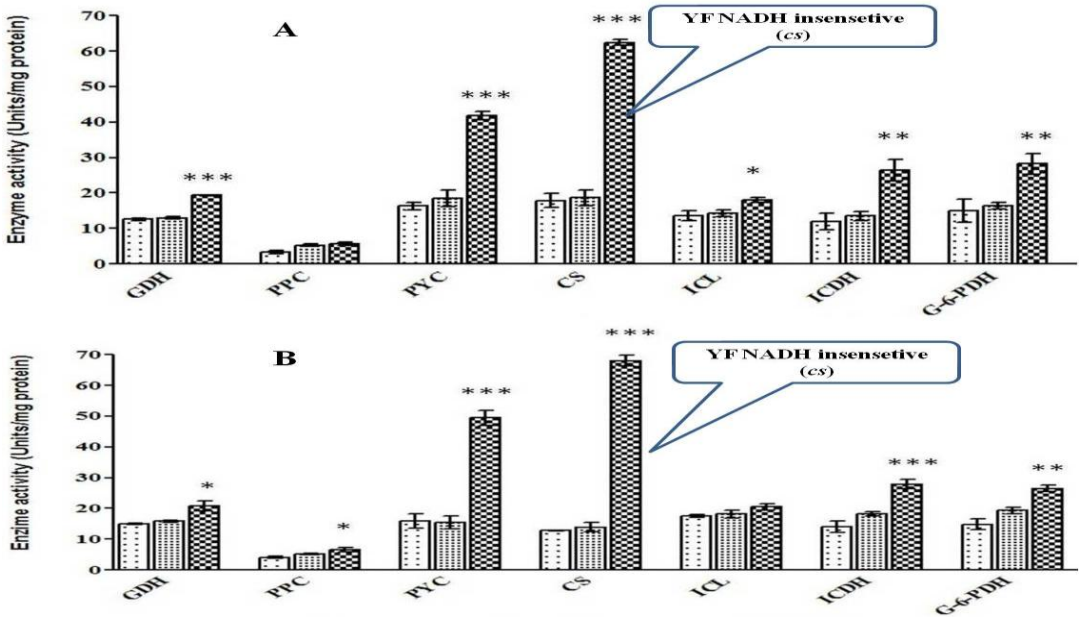


Fig. 5.14: Activities of enzymes PPC, PYC, GDH, G-6-PDH, ICDH and ICL in *B. japonicum* USDA110 and *M. loti* MAFF030669 *ppc* transformants. The activities have been estimated using cultures grown on TRP medium with 50mM glucose. All the enzyme activities were estimated from mid log phase to late log 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.

5.4: DISCUSSION

Present study demonstrates the effect of overexpression of NADH insensitive *E. coli cs* gene in *B. japonicum* USDA110 and *M. loti* MAFF030669. CS activity showed ~3.4 fold and ~4.8 fold increase in *Bj* (pJNK3) and *Ml* (pJNK3), respectively, as compared to the control and 1.1 and ~1.6 fold increase compared to the strain bearing the wild type *cs* gene. In earlier work when NADH insensitive *E. coli cs* was constitutively overexpressed under *lac* promoter in *Pf* O1 a maximum of 5.6 fold and 2 fold overexpression was obtained in *Pf* (Y145F) as compared to the control and strain bearing the wild type *cs* gene (Adhikary., 2012). In other earlier work when *E. coli* wild type *cs* gene was constitutively overexpressed under *lac* promoter in *P. fluorescens* ATCC 13525, 2 fold enhanced activity was observed compared to the control strain (Buch et al., 2009).

In the present study, as a consequence of increase in CS activity in *Bj* (pJNK3) and *Ml* (pJNK3) there is a ~11 fold elevated extracellular citrate level in both and ~5.7 and 4.9 fold increase, respectively in the yield .But there is no change in the intracellular levels of citrate. While an increase of ~6.9 fold and ~2 fold in intracellular citrate and ~51.6 fold increase and ~26 fold increase in extracellular citrate was seen in *Pf* O1 overexpressing NADH insensitive *E. coli cs* gene and *P. fluorescens* ATCC13525 overexpressing *E. coli* wild type *cs* gene, respectively (Buch et al 2009; Adhikary 2012). In another study *icd* mutant of *E. coli* K and B strains resulted in an increase of ~3.8 and 2.5 fold CS activities and enhanced citrate accumulation but unlike our study, in this case citrate accumulation had a negative effect on growth of the *E. coli* strains (Aoshima et al., 2003). Overexpression of mitochondrial CS genes also resulted in increased citrate efflux in cultured carrot cells (Koyama et al. 1999), *Arabidopsis* (Koyama et al. 2000), and canola plants (Anoop et al. 2003).

Increase in extracellular citrate levels by ~11 fold in both the strains compared to the control suggests an efficient flux system in *Rhizobium*, which is not seen in *P. fluorescens*. Increase in intracellular citrate level and yield by 1.9 and 2.39 fold, respectively, in *Pf* (pY145F) compared to *Pf* (pAB7) does not lead to similar increase in

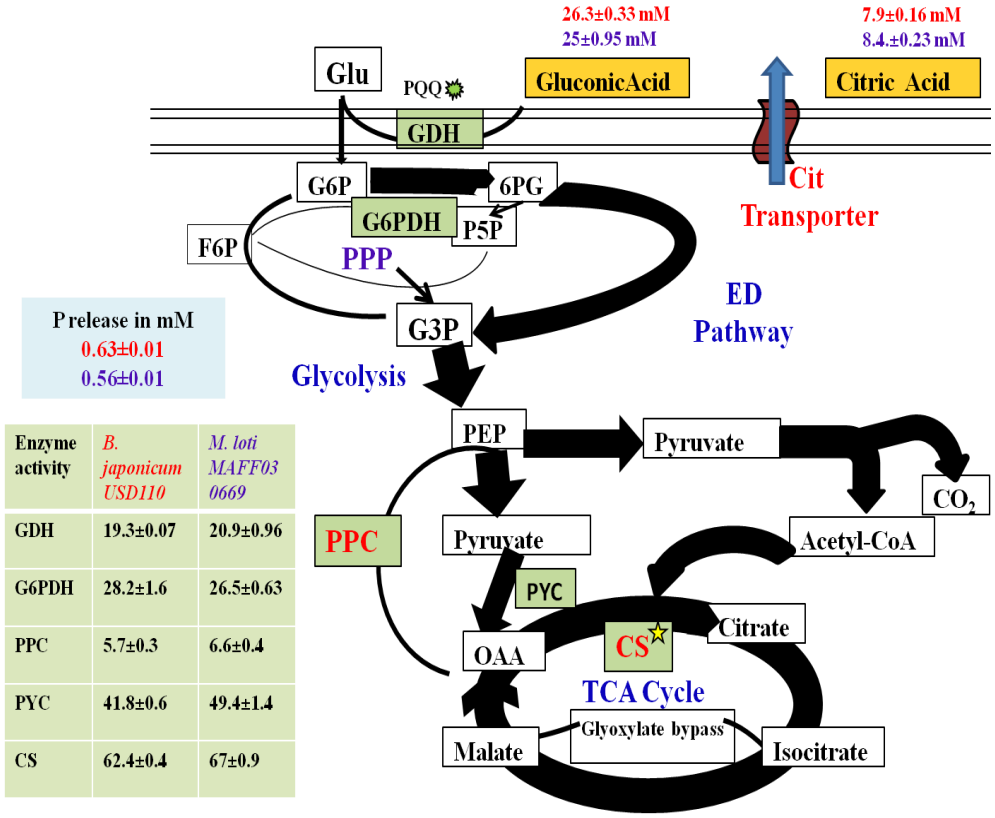
extracellular citrate levels. Citric acid being the substrate of central carbon metabolism must be transported into and out of the cell for efficient bioactivity. Therefore low level of extracellular citrate can be attributed to weak efflux transport mechanism in *P. fluorescens* (Buch et al 2009; Adhikary, 2012).

Increased gluconic acid levels could be explained by increased PYC activity in *Bj* (pJNK3) and *Ml* (pJNK3), which could probably diverts pyruvate flux towards increased OAA biosynthesis to meet the increased CS activity. Even in *A. niger,* the enhancement of anaplerotic reactions replenishing TCA cycle intermediates predisposes the cells to form high amounts of citric acid (Legisa and Mattey, 2007). However, *cs* overexpression in *A. niger* did not affect PYC activity (Ruijter et al., 2000). Enhancement of biosynthetic reactions due to shortage of TCA cycle intermediates was also observed in citric acid accumulating *E. coli* K and B strains in the form of increased glyoxylate pathway (Aoshima et al., 2003; Kabir and Shimizu, 2004).

ICL activity remained unaltered in both the strains, which was similar to studies done in *Pf* O1 overexpressing NADH insensitive *E. coli cs* gene and *P. fluorescens* ATCC13525 overexpressing *E. coli* wild type *cs* gene, respectively (Buch et al 2009; Adhikary 2012). Low ICL activity was consistent with earlier reports in *P. fluorescens* ATCC13525 and *P. indigofera* in which ICL contributed negligibly to glucose metabolism (Buch et al., 2009; Diaz-Perez et al., 2007).

GDH and G-6-PDH activities increased by ~1.5 and ~1.3 fold in *Bj* (pJNK3) and *Ml* (pJNK3), respectively, suggesting an increase in periplasmic glucose oxidation and phosphorylative pathway. Enhanced CS activity in *Pf* (pY145F) also increased the periplasmic glucose oxidation which is reflected by increase in GDH activity and gluconic acid production. Moreover, significant decrease in glucose consumption without affecting the glucose utilization suggested the involvement of direct oxidation pathway for carbon flux distribution in *P. fluorescens.* The increased carbon flow through glycolysis led to increased protein synthesis that is reflected to increased biomass (Adhikary et al., 2012). The citrate induced oligosaccharide synthesis was reported in *Agrobacterium* sp. ATCC 31749 (Ruffing et al., 2011).

The central carbon metabolism network gets to the heterologous overexpression of NADH insensitive *E. coli cs* in *B. japonicum* USDA110 and *M. loti* MAFF030669 (**Fig. 5.15**). The conditions created in the present work include: improvement of glucose uptake, improvement of CS activity and citrate production compared to the earlier report by Buch et al. (2009) and Adhikary et al (2012) (**Fig. 5.16**). increased direct oxidation of glucose leading to more gluconate production and increased phosphorylative pathway.



Enzyme activity	*B. japonicum USD110*	*M. loti MAFF030669*
GDH	19.3±0.07	20.9±0.96
G6PDH	28.2±1.6	26.5±0.63
PPC	5.7±0.3	6.6±0.4
PYC	41.8±0.6	49.4±1.4
CS	62.4±0.4	67±0.9

Fig. 5.15: Key metabolic fluctuations in *B. japonicum* USDA110 and *M. loti* MAFF030669 overexpressing NADH insensitive *E. coli cs* gene.

P solubilization was increased by *B. japonicum USDA110* and *M. loti MAFF030669* strains containing pJNK3 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 (pY145F) as compared to their respective controls (**Fig. 5.16**) (Buch et al., 2010; Adhikary, 2012).

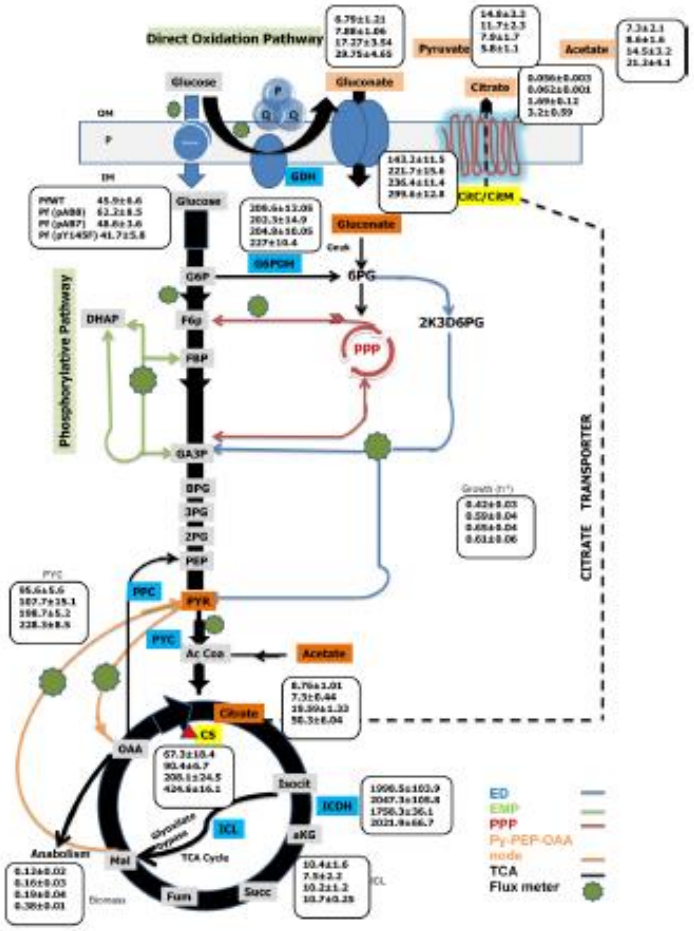


Fig. 5.16: Key metabolic fluctuations in *Pseudomonas* overexpressing NADH insensitive *E. coli cs* gene (Adhikary, 2012

P solubilization by *Rhizobium* transformants was better compared to overexpression of wild type *cs* gene due to increased amount of gluconic acid as well as citric acid levels.

Both *Rhizobium* transformants had enhanced growth promoting activities such as biofilm formation, exopolysaccharide and indole acetic acid production. 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.

Chapter 6

Effect of overexpression of *E. coli* NADH insensitive Y145F *cs* along with Na^+ dependent citrate transporter *citC* gene in *B. japonicum* USDA110 and *M. loti* MAFF303099

6.1 INTRODUCTION

MPS ability of rhizobacteria is mainly due to secretion of low molecular weight organic acids such as citric, oxalic, gluconic, 2-ketogluconic, lactic, malic, succinic, tartaric and acetic (Hazen et al., 1991; Srivastava et al., 2006; Archana et al., 2012). Strategy of increasing the activities of GDH and GAD enzymes has been successful in enhancing the secretion of GA and 2KGA (Zaidi et al., 2009; Kumar et al., 2013). *Citrobacter sp.* DHRSS has been isolated from the rhizosphere of sugar cane which demonstrated MPS ability on a variety of aldosugars and ketosugars like glucose, fructose and sucrose by secreting organic acids such as gluconic, acetic and pyruvic acids (Patel et al., 2008). With a view to increase the flux through the anaplerotic node for increasing oxaloacetate levels, *ppc* gene of *S. elongatus* was over-expressed in fluorescent pseudomonads leading to increase in cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increased gluconic, pyruvic and acetic acids but citric acid was not secreted (Buch et al., 2009). Overexpression of either of *ppc* and *cs* genes enhanced MPS ability of *P. fluorescens* 13525 on Pikovskya's agar; but *ppc-cs* co-expression neither alter *P. fluorescens* ATCC 13525 metabolism nor influenced citrate production (Buch et al., 2010). NADH insensitive *cs* overexpression in *Pf*-O1 increased both intracellular and extracellular citric acid levels upto 52 mM and 3.2 mM, respectively with concomitant increase in gluconic acid secretion (Adhikary, 2012). Similarly, *Citrobacter* DHRSS had intracellular accumulation of citric acid (~26 mM) but lacked secretion (Yadav, 2013). Thus intracellular accumulation of citric acid in spite of these bacteria ability grow on citrate as sole carbon source suggests that the citrate transporter is effective in up take but not in the efflux of citrate.

6.1.1 Citrate transporters in fungi and bacteria

Secretion of microbial metabolites including organic acids across the plasma membrane requires specific transporter proteins. The efflux of organic anions e.g., malate, citrate, or oxalate is an important mechanism for Al resistance in cereal and non-cereal species. Overexpression of gene, encoding a transporter reported to enhance citrate

efflux and Al tolerance in several plant species (**Table 6.1**) (Ryan et al., 2011). Members of the multidrug and toxin compound extrusion (MATE) family of proteins control Al-activated citrate efflux from barley (*Hordeum vulgare*) and sorghum (*Sorghum bicolor*). MATE proteins are widely present in bacteria, fungi, plants, and mammals (Omote et al. 2006), but there is no apparent consensus sequence conserved in all MATE proteins. MATE proteins are proposed to transport small, organic compounds (Omote et al., 2006). In contrast to MATE genes in the bacterial and animal kingdom, plants contain more MATE-type transporters (Furukawa et al., 2007). These proteins are characterized by having 400 to 700 amino acids with 12 transmembrane helices.

Table 6.1: Enhanced organic acid efflux by transporter gene expression (Ryan et al., 2011)

Transporter gene	Transgenic Strategy	Proposed mechanism
Al^{3+} activated malate transporter(Ta LMT1)	*Arabidopsis* gene expressed in *Arabidopsis,* wheat gene gene expressed in wheat and *Arabidopsis*	Enhanced malate efflux
Multidrug and toxic compound efflux gene (MATE) called Frd^3	*Arabidopsis* gene expressed in *Arabidopsis*	Enhanced citrate efflux
Multidrug and toxic compound efflux gene (MATE) HvAACT1	Barley gene expressed in tobacco plant	Enhanced citrate efflux
H+ pyrophosphatase AVP1	Over expression of endogenous in *Arabidopsis, wheat and rice*	Enhanced organic acid efflux
Multidrug and toxic compound efflux gene (SbMATE)	Sorghum gene expressed in Arabidopsis Atalmt mutant	Enhanced citrate efflux
Al^{3+} activated malate transporter(HvALMT1)	Barley gene expressed in barley	Enhanced malate efflux

Multidrug and toxic compound efflux gene (ZmMATE1)	Maize gene expressed in *Arabidopsis*	Enhanced citrate efflux

The excretion of intermediates of the TCA cycle (organic acids; for instance citrate, oxalate or succinate) is a characteristic feature of many anamorphic fungal species, such as *Aspergillus* spp. and *Penicillium* spp. Excretion of organic acids is observed in natural habitats (Gadd, 1999) and during growth on solid/liquid media in the laboratory (Foster,1949). Excretion of citrate by *A. niger* is exploited in biotechnological processes for commercial citric acid production (Roehr et al., 1996). Citrate secretion is a common characteristic feature of many anamorphic fungal species like *Aspergillus* and *Penicillium* (Burgstaller, W., 1993; 2005). Total intracellular citrate level in *A. niger* is between 2 - 30 mM. In *P. simplicissimum,* citrate levels are between 10 - 50 mM during the growth in batch cultures and between 20 mM and 60 mM in chemostat cultures (Gallmetzer and Burgstaller, 2001). More than 1 M citrate secretion is achieved in *A. niger* in improved biotechnological production processes (Netik et al., 1997; Ruijter et al., 2002). Citrate overflow mechanism in *A. niger* is very different from bacteria and is pH dependent (must be < 3). In addition to pH, other factors *viz* carbon source type and concentration, N source and P concentration, excessive aeration and Mn^{2+} limiting condition are contribute towards citrate secretion in fungus (Mlakar and Legisa, 2006). An efflux of protons was postulated as the main charge-balancing ion flow in *Penicillium cyclopium* (Roos and Slavik, 1987). Transport of dicarboxylates plays an important role in cell metabolism. In particular, they are intermediates of the citrate Cycle. Plasmalemmal dicarboxylate transporter is also involved in citrate influx and is modulated by pH and cations. Citrate and succinate influx is mediated by a common plasma membrane transporter in *Saccharomyces cerevisiae.* This is not typical for fungi. **(Fig.6.1, 6.2)** (Aliverdieva et al., 2006, 2008, 2010).

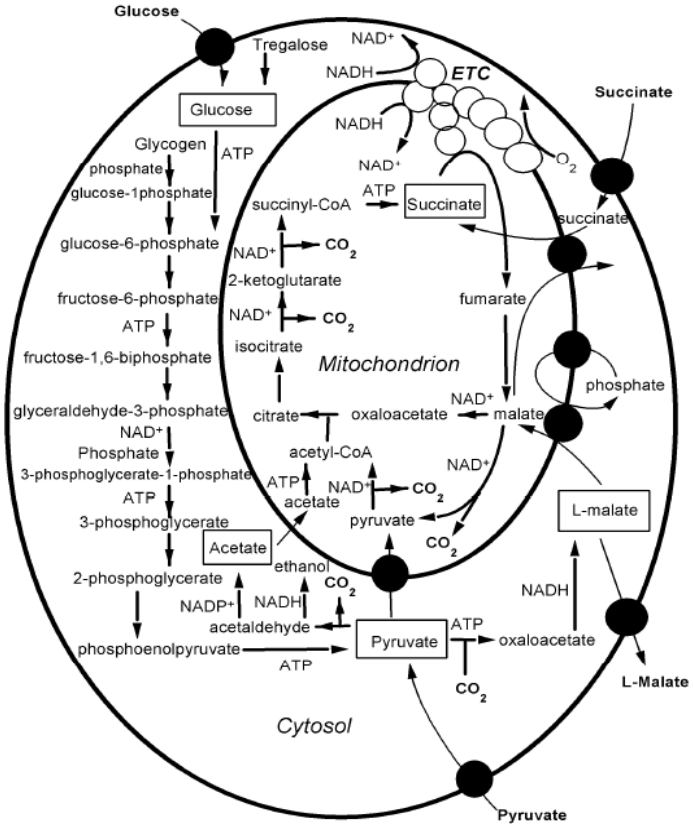


Fig. 6.1: Basic metabolism and the electron transport chain (ETC) in *S. cerevisiae* cells (Aliverdieva et al., 2006)

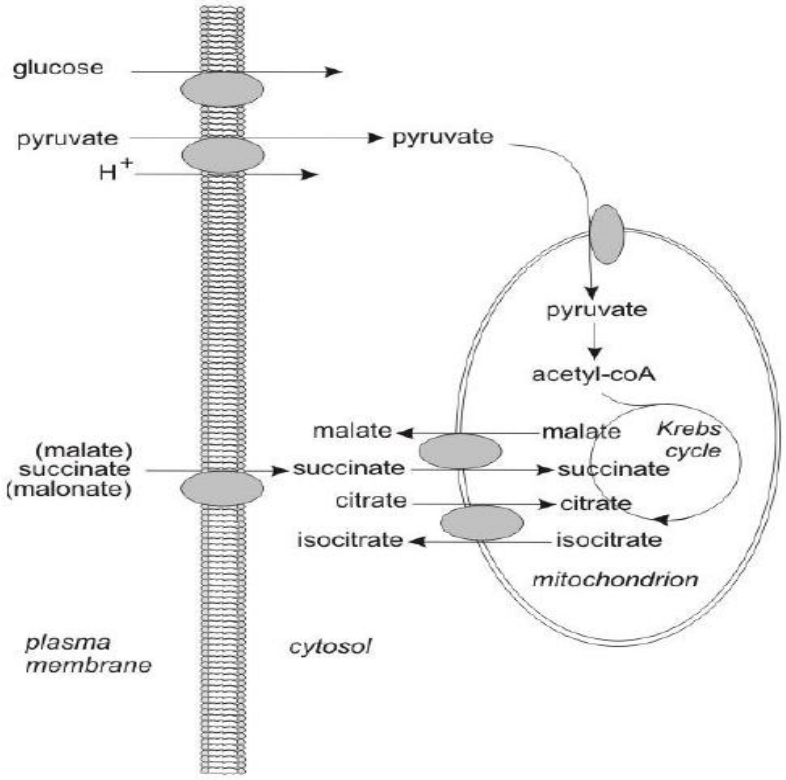


Fig. 6.2 Oxidation of substrates of plasma membrane dicarboxylate transporter in *S. cerevisiae* (Aliverdieva et al., 2008)

Very few bacterial species like *Corynebacterium*, *Arthrobacter*, *Brevibacterium*, *Bacillus sp.*, *Bradyrhizobium japonicum*, and *Citrobacter koseri* are known to secrete or accumulate citrate at levels much lower than fungi (Gyaneshwar et al., 1998; Khan et al., 2006). A proton efflux could either be coupled directly to citrate secretion via a citrate/proton symport similar to the secretion of lactate together with protons in *Escherichia coli* and *Lactobacillus lactis* (van der Rest et al., 1992). The membrane potential generating secondary transporters involved in malolactic (MelP) and citrolactic (citP) fermentation process are well reported in several lactic acid bacteria. The nature of transporters differ from "usual" secondary transporters in two aspects: (i) they translocate net negative charge across the membrane, and (ii) they catalyze efficient heterologous exchange of two structurally related substrates (Bandell et al., 1997). The electrochemical gradient of protons across the cytoplasmic membrane is a major store of free energy in the bacterial cell. Usually, the proton motive force (pmf) is generated by translocation of protons against the gradient across the cell membrane which results in the two

components of the pmf, a membrane potential and a pH gradient. Proton pumping is catalyzed by primary transport systems at the expense of some source of chemical energy or light.

E. coli cannot utilize citrate as a sole source of carbon and energy (Dimroth, 1987; Kastner 2000). On the other hand, the facultative anaerobic bacteria *Klebsiella pneumoniae* and *Salmonella typhimurium* and many other species of the *Enterobacteriacaea* can grow aerobically or anaerobically, utilizing citrate as the sole carbon source. Most of the bacteria have transport proteins in the cytoplasmic membrane that mediate the transport of citrate. The carriers belong to the class of secondary transporters that use the free energy stored in transmembrane electrochemical gradients of ions to drive the transport of the substrates. The citrate transporter CitH of *K. pneumoniae* is driven by the proton motive force (van de Rest et al., 1992)) while the transporters CitS and CitC of *K. pneumoniae* and *Salmonella serovars* are driven by both pmf and sodium, respectively. CitM of *Bacillus subtilis* is driven by magnesium ion motive force (Ishiguro et al., 1992; Lolkema, 1994; Boorsma et al., 1996). *Klebsiella pneumoniae citS* gene is expressed during anaerobic growth on citrate (Bott et al., 1995; Dimroth and Thomer, 1986).

Mechanistically these transporters catalyze coupled translocation of citrate and H^+ and/or Na^+ and Mg^{2+} (symport). A special case is the citrate carriers of lactic acid bacteria that take up citrate by an electrogenic uniport mechanism or by exchange with lactate, a product of citrate metabolism (citrolactic fermentation) (Marty-Teysset et al., 1996, Ramos et al., 1994). These citrate transporters are involved in secondary metabolic energy generation (Konings et al., 1995). In contrast with most citrate transporters, a member of the CitMHS family characterized from the soil bacterium *Bacillus subtilis*, transport citrate in complex with a bivalent metal ion. This facilitates the utilization of citrate which is available in the metal-ion-complexed state. The best-characterized members of the family are BsCitM and BsCitH. The former transports citrate in complex with Mg^{2+} and is the major citrate-uptake system during growth on citrate under aerobic conditions (Yamamoto et al., 2000; Li et al., 2002: Warner et al., 2002).

These Secondary transporters of the bacterial CitMHS family fall under the group of 2-hydroxycarboxylate transporter (2HCT) family. The 2HCT family of secondary transporters contains 54 unique members that are all found in the bacterial kingdom. The well characterized members of the family are transporters for citrate, malate and lactate, substrates that contain the 2-hydroxycarboxylate motif, hence the name of the family. The transporters are either H^+ or Na^+ symporters or they catalyze exchange between two substrates. Na^+ coupled citrate transporters like CitS of *Klebsiella pneumoniae* and CitC of *Salmonella enterica* found in the γ subdivision of the phylum Proteobacteria are involved in the fermentative degradation of citrate to acetate and carbon dioxide yielding ATP. Citrate is cleaved by citrate lyase yielding acetate and oxaloactetate, which is decarboxylated yielding pyruvate. The latter step results in the transmembrane pH gradient. Secondary transporters are widely distributed in nature and they come in a great genetic and structural diversity, probably reflecting many different translocation mechanisms (**Table 6.2)** (Lolkema, 2006).

Table 6.2: Characterized members of the 2HCT family (Lolkema, 2006).

Transporter	Bacterium	Substrates	Transport mode	Function
CitS	*Klebsiella pneumoniae*	citrate	Na+ symport	Citrate fermentation
CitC	*Salmonella typhimurium*	citrate	Na+ symport	Citrate fermentation
CitW	*Klebsiella pneumoniae*	Citrate, acetate	exchange	Citrate fermentation
MleP	*Lactococcus lactis*	Malate, lactate	exchange	Malolactic fermentation
CitP	*Leuconostoc mesenteroides*	Citrate, lactate	exchange	Citrolactic Fermentation
CimH	*Bacillus subtilis*	Citrate, malate	H^+ symport	Unknown
MalP	*Streptococcus bovis*	malate	H^+ symport	Malate Fermentation
MaeN	*Bacillus subtilis*	malate	Na+ symport	Growth on malate

6.1.2 Structural Model of 2-HCT transporters

The transporters in the 2HCT family are integral membrane proteins consisting of about 440 amino acid residues (Lolkema, 2006). The core of the structure is formed by two homologous domains that are connected by a large hydrophilic loop that resides in the cytoplasm. The domains contain 5 transmembrane segments (TMSs) each and they have opposite orientations in the membrane. They are likely to originate from a duplication of an internal gene fragment coding for an odd number of TMSs. In the structural model of the transporters in the 2HCT family, the loops between the 4th and 5th TMSs in each domain fold back in between the TMSs and form so called re-entrant or pore loops (**Fig. 6.3**). The pore loop in the N-terminal domain (region VB) enters the membrane-embedded part from the periplasmic side of the membrane, the one in the C-terminal domain (region XA) from the cytoplasmic side (trans pore loops). The two re-entrant loops are believed to be in close vicinity in the 3D structure and to form the translocation pathway for co-ions and substrates. The binding site is believed to be positioned at the membrane-cytoplasm interface where an arginine residue interacts directly with the bound substrate. Different families may have additional TMSs at the N- or C-termini or in between the two domains. The transporters of the 2HCT family have one additional TMS at the N-terminus locating the latter in the cytoplasm. The odd number of TMSs in each domain forces the orientation of the two domains in the membrane to be opposite; the N-terminus of the N-terminal and C-terminal domains resides in the periplasm and cytoplasm, respectively. The pore loops contain an extraordinarily high fraction of residues with small side chains (glycine, serine, and alanine) which may reflect a compact packing of the loops in between the TMSs. The regions containing the pore loops are among the best conserved regions in the transporter families. The two pore loops would be in close contact in the 3D structure in a single pore that alternately would be opened to either side of the membrane during the catalytic cycle.

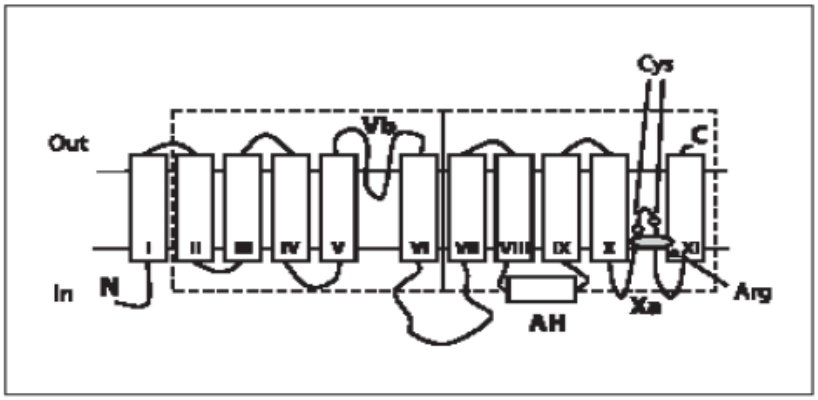


Fig. 6.3: Structural model for 2HCT family transporters (Lolkema, 2006).

The substrate-binding site is located at the membrane-cytoplasmic interface, which positions it deep down in the pore when opened to the external face of the membrane. The cytoplasmic pore loop (XA) extends into the pore beyond the binding site, making cysteine residues in the loop accessible from the periplasmic side even when substrate is bound. Opening and closing of the pore to either site of the membrane would be controlled by binding of the substrate and co-ions. The accessibility of cysteine residues in the cytoplasmic pore loop was shown to be different in different catalytic states of the transporter by experiment.

Citric acid secretion could be stabilized if there were a mechanism whereby the cells could secrete elevated levels (Delhaize et al. 2004). Na^+ dependent citrate transporters are highly specific for citrate. The major species transported across the cell is $HCit^{2-}$. It accumulates citrate at the expense of Na^+ concentration gradient generated by various sodium ion pumps. Mainly these transporters function in citrate uptake inside the cells **(Fig. 6.4).**

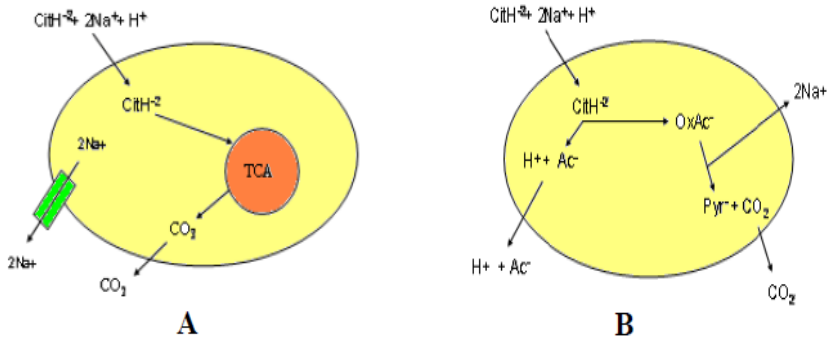


Fig. 6.4: Na^+ efflux mechanisms in bacteria. (A). Aerobic and (B). Anaerobic conditions (Lolkema, 1994).

All bacterial cells although maintain an intracellular Na^+ concentration lower than the extracellular, intracellular concentration above 20 mM is harmful to *E. coli* and in halophiles is above 3 M (Lolkema, 1994). Bacterial cells protect from the adverse effects of Na^+ by primary and secondary Na^+ extrusion system. NhaA, the Na^+/H^+ antiporter is the system responsible for adaptation to Na+ and alkaline pH (**Fig. 6.4 and 6.5)**. All bacterial cells maintain the optimum intracellular Na^+ levels by (i) Symport with metabolites and antiport against H^+ are widely used mechanisms in almost all bacteria for Na^+ influx and efflux; (ii) Decarboxylases and ATPases function in anaerobic bacteria. Decarboxylases act as Na^+ pumps for efflux and ATPases use the energy obtained by influx of Na^+ down its concentration gradient for ATP synthesis; (iii) Marine organisms have respiratory chain mechanism for efflux of Na^+ to maintain sodium motive force (smf) and flagella motors which use energy derived from influx of Na^+ down the concentration gradient.

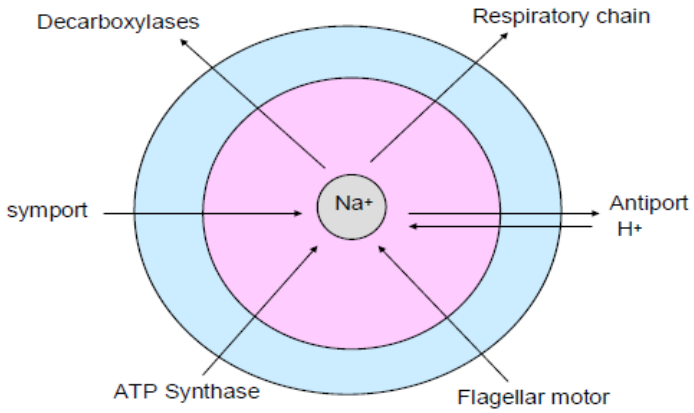


Fig. 6.5: Bacterial stress responses and Na^+ homeostasis (Storz et al., 1996).

Many pseudomonads possess PYC as well as PPC involved at the anaplerotic node. Unlike *E. coli*, pseudomonads possess a citrate transporter which would facilitate the growth on citric acid (Stover et al., 2000; Nelson et al., 2002). Reversibility is recognized as a fundamental feature of coupled vectorial transport systems. Therefore, the decarboxylase systems could also function in reversible manner wherein the direction of operation depends on the cation gradient and free energy change under the conditions of the physiological steady state. Heterologous overexpression of citrate symporter coupled to Na^+ and Mg^{2+} may play an important function as an alternative pump for efflux of Na^+ and/or Mg^{2+} along with citrate (**Fig. 6.4 and 6.5**).

Citrate transport in *Enterobacteriaceae* family is mainly mediated by cation dependent or ATP dependent transporters (Lolkema., 2006). The Na^+-dependent citrate carriers CitS of *Klebsiella pneumoniae* and CitC of *Salmonella typhimurium serovars*, are citrate specific Na^+ coupled symporter, belonging to 2-hydroxycarboxylate family, and are driven by both the proton motive force and sodium ion motive force (Ishiguro et al., 1992; Lolkema., 2006). The citrate transporter CitH of *K. pneumoniae* is driven by the proton motive force (Lolkema., 2006). In other bacteria such as *B. subtilis*, *Streptococcus bovis*, *Lactococcus lactis* the citrate transporters are Mg^{+2} or proton dependent. Heterologous overexpression of NADH insensitive *cs* gene along with *S. typhimurium* Na^+ dependent and *B. subtilis* Mg^{2+} dependent citrate transporter in *P. fluorescens* PfO-1

showed significant changes in citric acid levels and yields. *citM* does not cause any significant difference in citric acid levels and yields when compared to *Pf* (pY145F) without any external citrate transporter (**Fig. 6.6**; Adhikary, 2012).

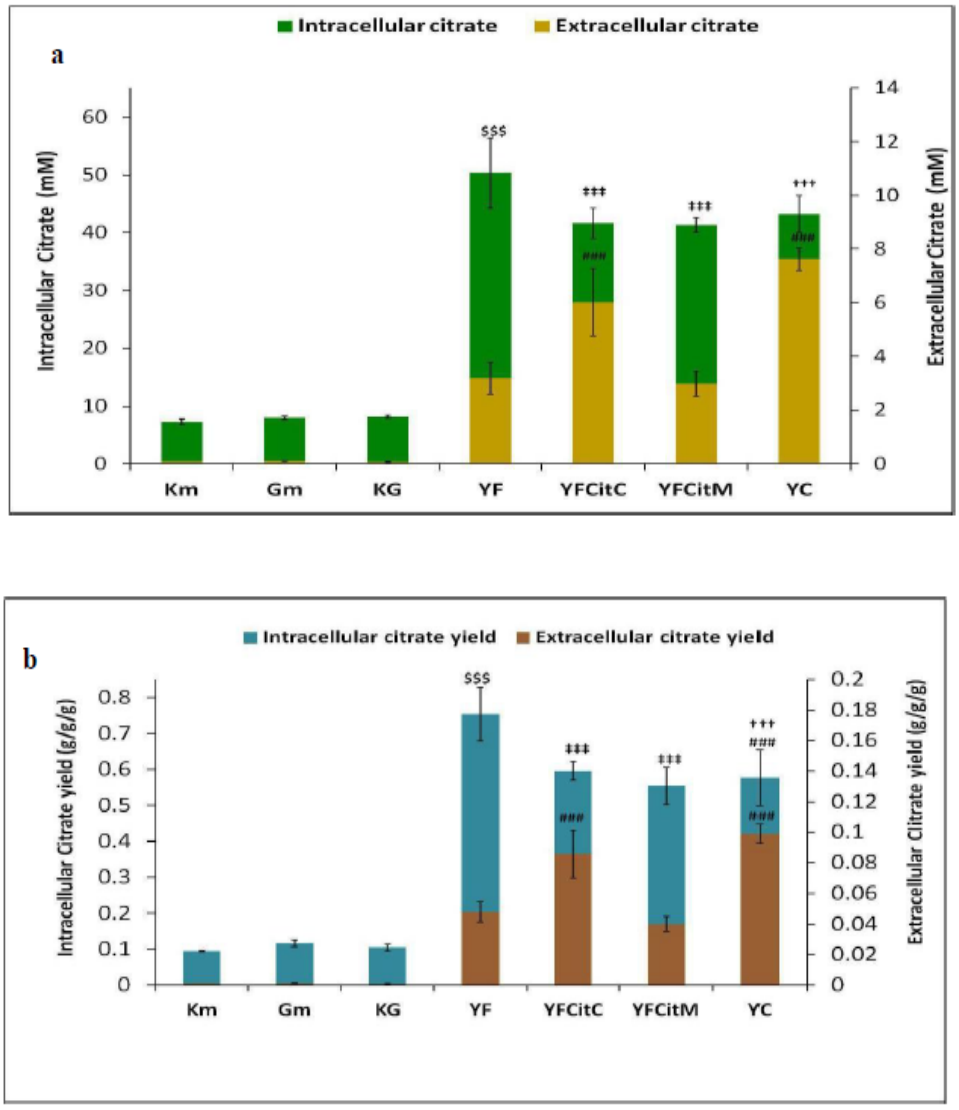


Fig. 6.6: Citric acid levels and yields in *P. fluorescens* PfO-1 overexpressing citrate transporter. Intracellular and extracellular citrate levels (a) are represented in **green** and **orange** bars respectively. Intracellular and extracellular citrate yields are represented in **blue** and **magenta** bars respectively. Organic acid yields were estimated from stationary phase cultures grown on M9 medium with 100mM glucose and are expressed as g/g of glucose utilized/g dry cell mass.

Extracellular citric acid levels in *Pf* (pYFCitC) and *Pf* (pYC) increase by 1.88 and 2.3 fold as compared to *Pf* (pY146F) which is 92.6 and 84.6 fold higher as compared to vector control strain, respectively. Corresponding extracellular citrate yield increased by 1.79 and 2.07 fold with an increase of 103.4 and 76 fold compared to respective vector controls (**Fig. 6.6**). Although there is an approximately 1.26 fold decrease intracellular citrate yield amongst the citrate transporter bearing strain this is not statistically significant. All experiments were further continued with *Pf* (pYC) and *Pf* (pGm) as control.

In *Pf* (pYC) the periplasmic GDH activity increased by 1.46 fold as compared to the *Pf* (pGm) in late log to stationary phase of growth. Similarly a significant increase in G6PDH, PYC and CS activities by 1.46, 4.74 and 4.5 fold, respectively, were observed as compared to the controls. However, ICL and ICDH activities in the stationary phase cultures remained unaltered (**Fig. 6.7**).

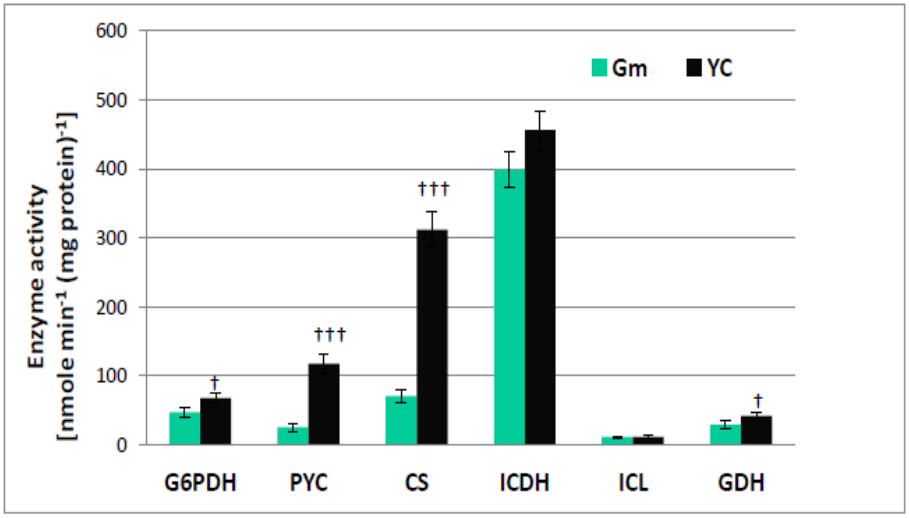


Fig. 6.7: Activities of enzymes G-6-PDH, ICDH, ICL, PYC, and GDH in *P. fluorescens* PfO-1 overexpressing citrate transporter.

Overexpression of NADH insensitive CS on *Citrobacter* sp. DHRSS resulted in intracellular accumulation of 26 mM of citric acid and incorporation of citrate operon containing Na^+-dependent CitC transporter in addition to NADH insensitive CS increased the efflux of citrate up to 7 mM in TRP medium indicating that the native transporter was

the limiting factor for citrate secretion, without affecting gluconic acid secretion which in turn improved phosphate solubilization under aerobic conditions (**Fig. 6.8) (**Yadav (2013)**.**

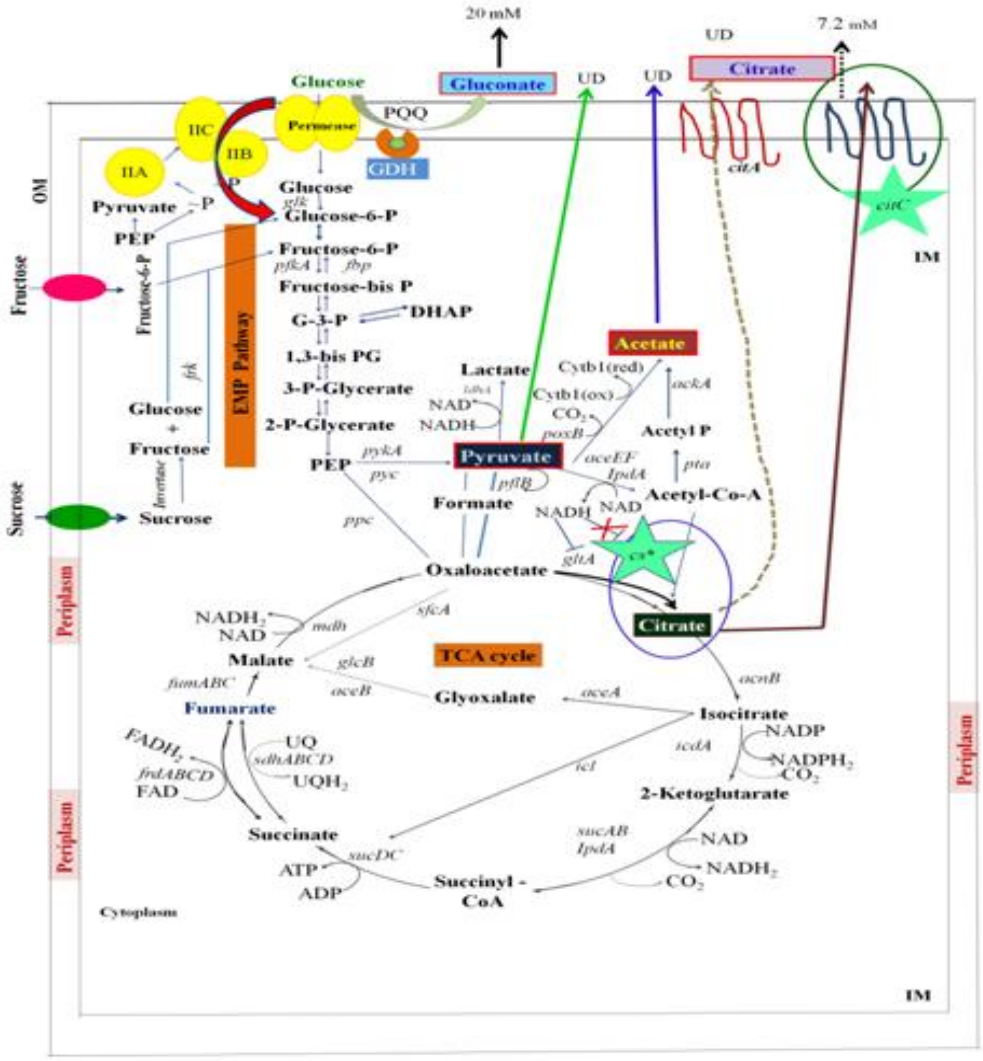


Fig. 6.8: Schematic representation of citric acid secretion in *Citrobacter* sp. DHRSS containing artificial citrate operon (Yadav,2013).

H. seropedicae Z67 and *Hs* (pAB7) had 12.9 U and 14.98 U of CS activity, respectively, in M9 medium while *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.8 and ~1.9 folds increase in CS activity, respectively. Similar extent of increase in CS activity was found in HRP medium. Citric acid secretion was not found in native culture and *Hs*

(pAB7) while 0.39 mM and 2.79 mM citric acid secretion was observed with *Hs* (pJNK3) and *Hs* (pJNK4) (**Fig. 6.9) (Wagh et al ., 2013).**

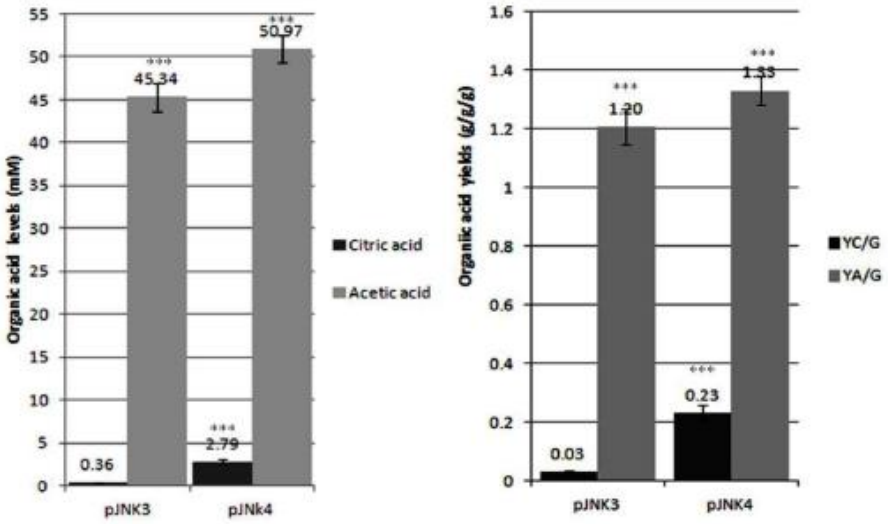


Fig. 6.9: Organic acid production from *H. seropedicae* Z67 transformants (Wagh, 2013).

6.1.3 Rationale of the present work

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Objective of the present study was to secrete high levels of citric acid for efficient MPS ability of *Rhizobium* spp. in field conditions, which is hindered by low availability of carbon sources, high buffering capacity of soils. Previous results in this study showed significant increase in extracellular citrate levels by overexpression of *ppc*, *cs* and *cs** genes. Further improvement in citric acid secretion was investigated by overexpression of NADH insensitive citrate synthase (*cs**) of *E. coli* along with *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene in *B. japonicum* USDA110 and *M. loti* MAFF030669 and monitored its effects on citric acid secretion and glucose metabolism.

6.2 EXPERIMENTAL DESIGN

The experimental plan of work includes the following-

6.2.1: Bacterial strains used in this study

Table 6.3: Bacterial strains used in this study

Bacterial strains	Characteristics	Source/Reference
***E. coli* strains**		
E. coli JM101	Used for molecular biology experiments	Sambrook and Russell, 2001
E. coli W620	*E. coli* W620-deletion mutant of *cs* gene	*E. coli* Genetic Stock Center
Salmonella typhimurium	Sewage isolate	Kumar, 2012
***Rhizobium* strains**		
Bradyrhizobium japonicum USDA110	NC_004463.1	
Mesorhizobium loti MAFF030669	NC_002678.2	
Bj (pAB8)	*B. japonicum* USDA110 with pAB8 plasmid; Ap^r, Km^r (control vector)	This study
Bj (pJNK4)	*B. japonicum* USDA110 with pJNK4 plasmid; Ap^r, Km^r (*cs* NADH insensitive and CitC transporter)	This study
Ml (pAB8)	*M. loti* MAFF030669 with pAB8 plasmid; Ap^r, Km^r (control vector)	This study
Ml (pJNK4)	*M. loti* MAFF030669 with pJNK4 plasmid; Ap^r, Km^r (*cs* NADH insensitive and CitC transporter)	This study

Details of Plasmid used:

pUCPM18 with *E. coli* NADH insensitive *cs** gene along with citrate transporter *citC* gene of *Salmonella typhimorium* under p*lac* having Ap^r and km^r gene, named as pJNK4 of 9.1 kb was used in this chapter.

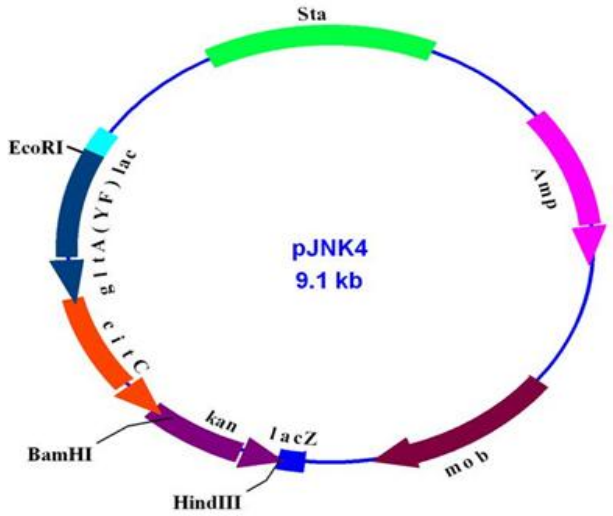


Fig. 6.10: Restriction map of the plasmid used in this chapter (Wagh et al., 2013).

6.3: RESULTS

6.3.1: Heterologous overexpression of *E. coli* NADH insensitive *cs** gene and citrate transporter *citC* gene of *S. typhimorium* in *Rhizobium* spp.

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. 6.11, 6.12)** before studying the effect of overexpression of *E. coli* NADH insensitive *cs** gene and citrate transporter *citC* gene of *S. typhimorium.*

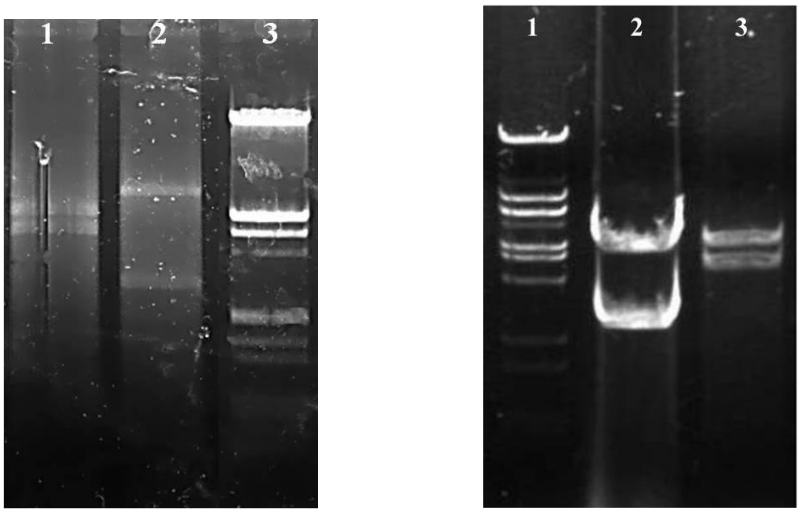


Fig. 6.11: Restriction digestion analysis of (A) *B. japonicum* USDA110 containing pJNK4 plasmid. Lane 1: pJNK4 digested with EcoRI-HindIII (5,298bp, 3,967bp); Lane 2: pJNK4 digested with XbaI (6.6 kbp, 2.6 kbp); Lane 3: Molecular Weight Marker (MWM)-Lambda DNA cut with EcoRV/ Hind III. **(B) *M. loti* MAFF030669 containing pJNK4 plasmid.** Lane 1: Molecular Weight Marker (MWM)-Lambda DNA cut with BstEII; Lane 2: pJNK3 digested with EcoRI-HindIII (5,298bp, 2,967bp); Lane 3: pJNK4 digested with EcoRI-HindIII (5,298bp, 3,967bp).

The CS activity of *B. japonicum* USDA110 and *M. loti* MAFF030669 transformants {*Bj* (pJNK4)} and{*Ml* (pJNK4)} grown on TRP medium with 50 mM glucose, was 3.9 and 4.7 fold higher (72.34 ± 0.80 U and 64.63 ± 1.32 U) in both the strains compared to control which possessed very low 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* (pJNK4) and *Ml* (pJNK4) as compared to the control *Bj* (pAB8) and *Ml* (pAB8). P-solubilizing ability of wild type *B. japonicum USDA110* USDA110 and *M. loti MAFF030669* and its transformants varied in the order of *Bj* (pJNK4) = *Ml* (pJNK4) > *Bj* (pAB8) = *Ml* (pAB8) > *Bj*=*Ml* on PVK medium after 3 days of incubation at 30°C (**Fig. 6.12**).

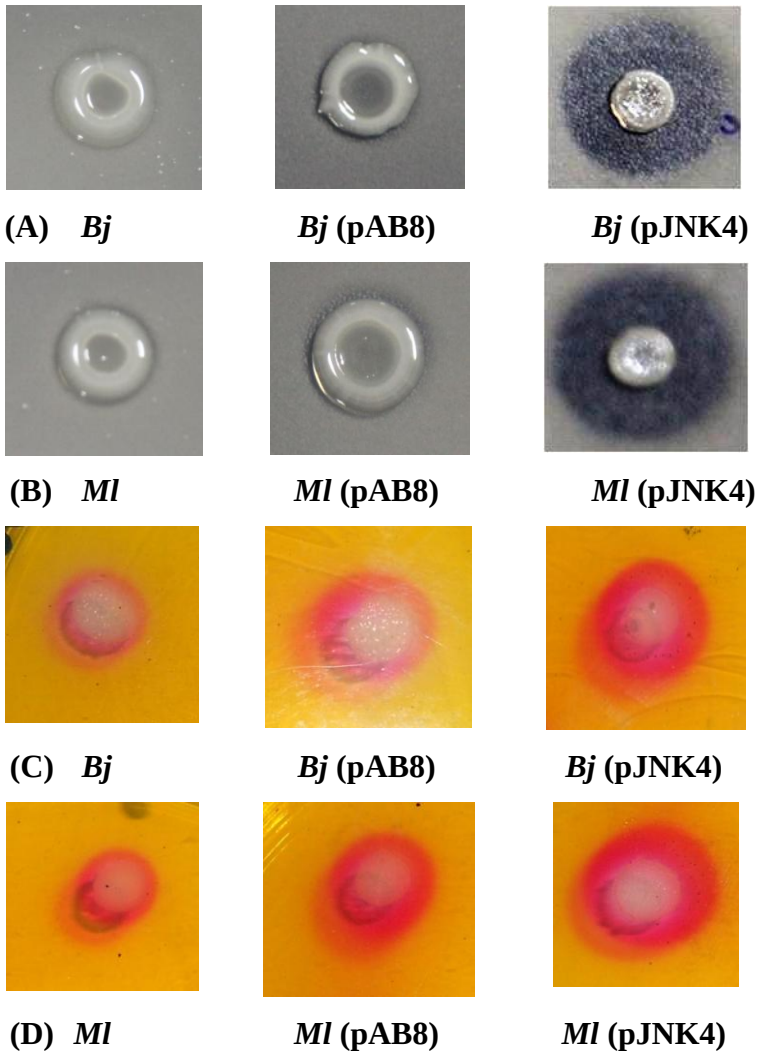


Fig. 6.12: MPS phenotype of *B. japonicum* USDA110 and *M. loti* MAFF030669 strains harboring pJNK4 plasmid. (A) and (B) on Pikovskaya's agar and **(C) and (D)** Tris rock phosphate agar containing 50 mM glucose and 100 mM Tris HCl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 ºC. Media composition and other experimental details are as described in Sections 2.2.4 and 2.7.

Table 6.4 : 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) : *B. japonicum* USDA110 with vector control and *Bj* (pJNK4) **:** *B. japonicum* USDA110 with *cs** and citrate transporter *citC* gene of *S. typhimorium* gene. The results were noted after an incubation of 3 days at 30 ºC and are given as mean ± S.D. of three independent observations as compared to native *Bj and Ml* strains. *** P<0.001.

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* (pJNK4)**	18.50 ± 0.50	6.17 ± 0.29	3.0***
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
***Ml* (pAB8)**	12.17 ± 0.29	10.17 ± 0.29	1.22
***Ml* (pJNK4)**	20.17 ± 0.29	7.50 ± 0.50	2.86***

The pJNK4 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 Section 2.5. And it was highest in *Bj* (pJNK4) and *Ml* (pJNK4) (**Table 6.4).**

6.3.6: Effect of *E. coli* NADH insensitive *cs** gene and citrate transporter *citC* gene of *S. typhimorium* gene overexpression on growth pattern and pH profile in presence of 50 mM glucose concentrations.

The growth profiles and organic acid secretion of *Bj* (pAB8) , *Bj* (pJNK4), *Ml* (pJNK4) and *Ml* (pAB8) along with native, on TRP medium with 50 mM glucose demonstrated that maximum O.D. was reached faster within 12 h transformants compared to 20 h of the control *Bj* (pAB8) and *Ml* (pAB8). Acid production was

monitored and it was found that there was slight pH drop within 20 h in the native and control vector while pH drop to 4.3 and 4.2 was seen in *Bj* (pJNK4) and *Ml* (pJNK4). Significant media acidification was seen within 12 h in both the cases. Both *Bj* (pJNK4) and *Ml* (pJNK4) acidified the medium when grown on TRP medium (**Fig. 6.13).**

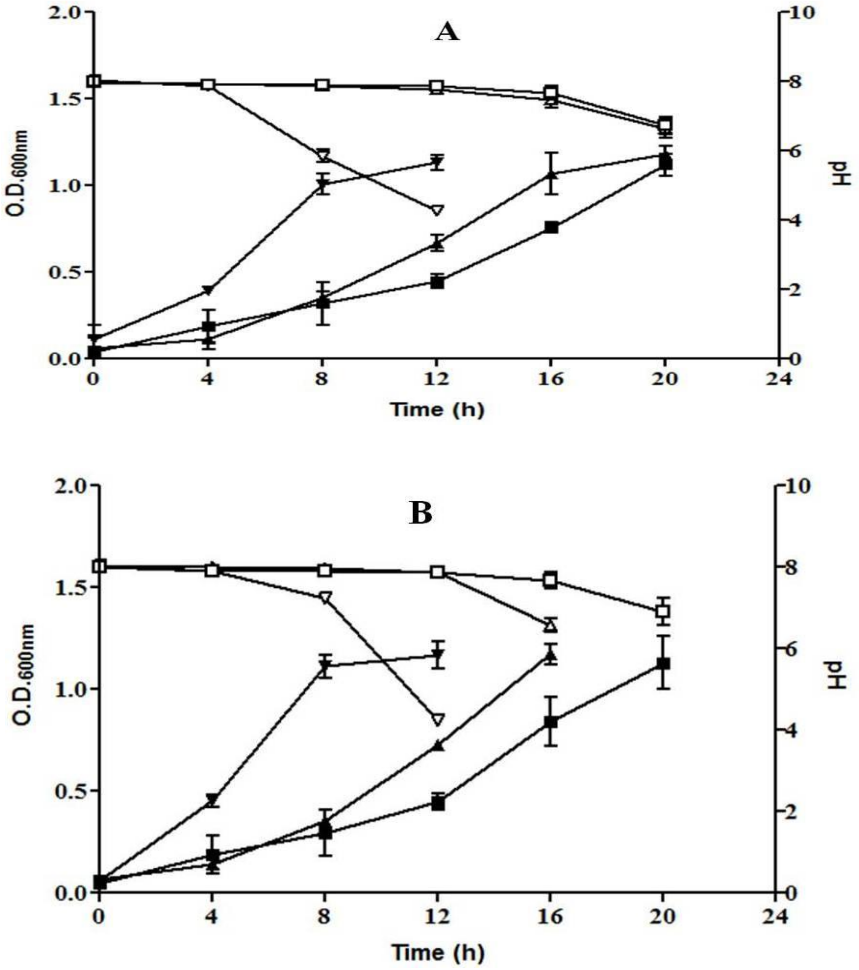


Fig. 6.13 : Effect of *E. coli* NADH insensitive *cs* gene and **citrate transporter *citC* gene of *S. typhimorium* gene overexpression on extracellular pH (□, Δ, ▽,) and growth profile (■, ▲, ▼) of (A) *B. japonicum* USDA110 and (B) *M. loti***, on TRP medium with 50 mM glucose .(□, ■, *Bj, Ml* wild type); {Δ, ▲, *Bj* (pAB8), *Ml* (pAB8)}; {▽, ▼, *Bj* (pJNK4), *Ml* (pJNK4)}. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

6.3.7: Physiological effects of *E. coli* NADH insensitive *cs** gene and citrate transporter *citC* gene of *S. typhimurium* gene overexpression on M9 minimal medium with 50 mM glucose.

In presence of 50 mM glucose, increase in CS activity significantly affected growth profile in transformants of both the strains (**Fig. 6.13**). The total glucose utilization rate remained unaltered and the total amount of glucose consumed showed ~1.9 fold and ~2.0 fold decrease in *Bj* (pJNK4) and *Ml* (pJNK4), respectively at the time of pH drop. The increase in CS activity increased biomass yield by ~1.6 fold and also ~2.2 fold decrease was seen in specific glucose utilization rate in the transformants of both the *Rhizobium* strains (**Table 6.5**).

***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 ±0.2**	**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* (pJNK4)**	**0.337 ±0.02**	**48.34 ±0.21**	**19.64 ±3.09*** **	**2.50 ±0.23*** **	**0.10 ±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* (pJNK4)**	**0.323 ±0.03**	**48.29 ±0.29**	**18.49 ±1.63*** **	**2.35 0.17*** **	**0.11 ±0.01*** **

Table 6.5: Physiological variables and metabolic data from of *B. japonicum* USDA110 and *M. loti* MAFF030669 *ppc* transformants grown on TRP medium 100mM Tris-Cl buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml. The results are expressed as Mean±S.E.M of 6-10 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 2.6 *** P<0.001.

6.3.6: Biofilm, exopolysaccharide and indole acetic acid production by *Bj* (pJNK4) and *Ml* (pJNK4) transformants in TRP medium.

Biofilm and exopolysaccharide production showed significant increase by ~2.6 and ~3.4 fold and ~2.4 and ~1.3 fold in *Bj* (pJNK4) and *Ml* (pJNK4), respectively, compared to control. Indole acetic acid production was increased by ~1.3 fold in both the transformants compared to control vectors. This increase further enhanced P solubilization by the transformants (**Table 6.6**).

Table 6.6: Biofilm, exopolysaccharide and indole acetic acid production by *Bj* (pJNK4) and *Ml* (pJNK4) transformants in TRP medium. The results are expressed as Mean±S.E.M of 6-10 independent observations *** 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* (pJNK4)**	5.31 ±0.20***	32.48 ±0.51***	33.38 ±1.44
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* (pJNK4)**	5.29 ±0.36***	20.03 ±1.07	32.92 ±0.55

6.3.7: P Solubilization and organic acid secretion by *Bj* (pJNK4) and *Ml* (pJNK4) transformants in TRP medium.

There was significant increase in release of P by ~14.7 and ~17 fold in *Bj* (pJNK4) and *Ml* (pJNK4) respectively, compared to control vectors when grown in TRP medium containing 50 mM glucose (**Fig. 6.14)**.

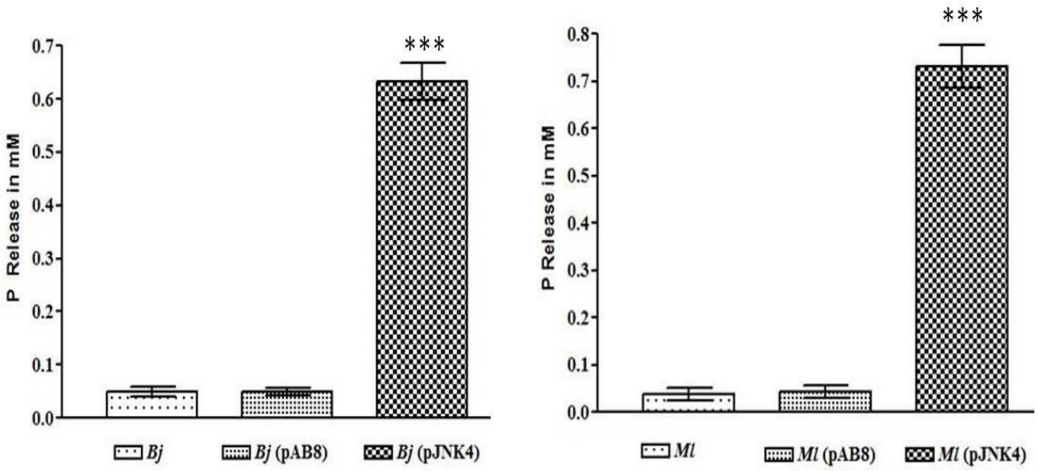


Fig. 6.14: P release by (A) *B. japonicum* USDA110 and (B) *M. loti* MAFF030669 transformants on TRP medium. The values are depicted as Mean ± S.E.M of 7-10 independent observations. ******* P<0.001.

On TRP medium in presence of 50 mM glucose, the organic acids identified were mainly gluconic, 2-ketogluconic, acetic and citric acids. As a result of *E. coli* NADH insensitive *cs* gene and citrate transporter *citC* gene of *S. typhimorium* gene overexpression, there was only quantitative change in two organic acids secreted. there was ~14 and ~14.2 fold increase in extracellular citric acid with their corresponding increase in yield ($Y_{C/G}$) by ~7.4 and ~7.5 fold in *B. japonicum* USDA110 and *M. loti* MAFF030669 transformants, respectively. Also extracellular medium of *Bj* (pJNK4) and *Ml* (pJNK4) contained ~4 fold higher amount of gluconic acid, and ~2.3 and ~2.1 fold increase in its yield was seen respectively as compared to *Bj* (pAB8) and *Ml* (pAB8) (**Fig. 6.15)**. There was no change in the intracellular levels of citric acid **(Table 6.7).**

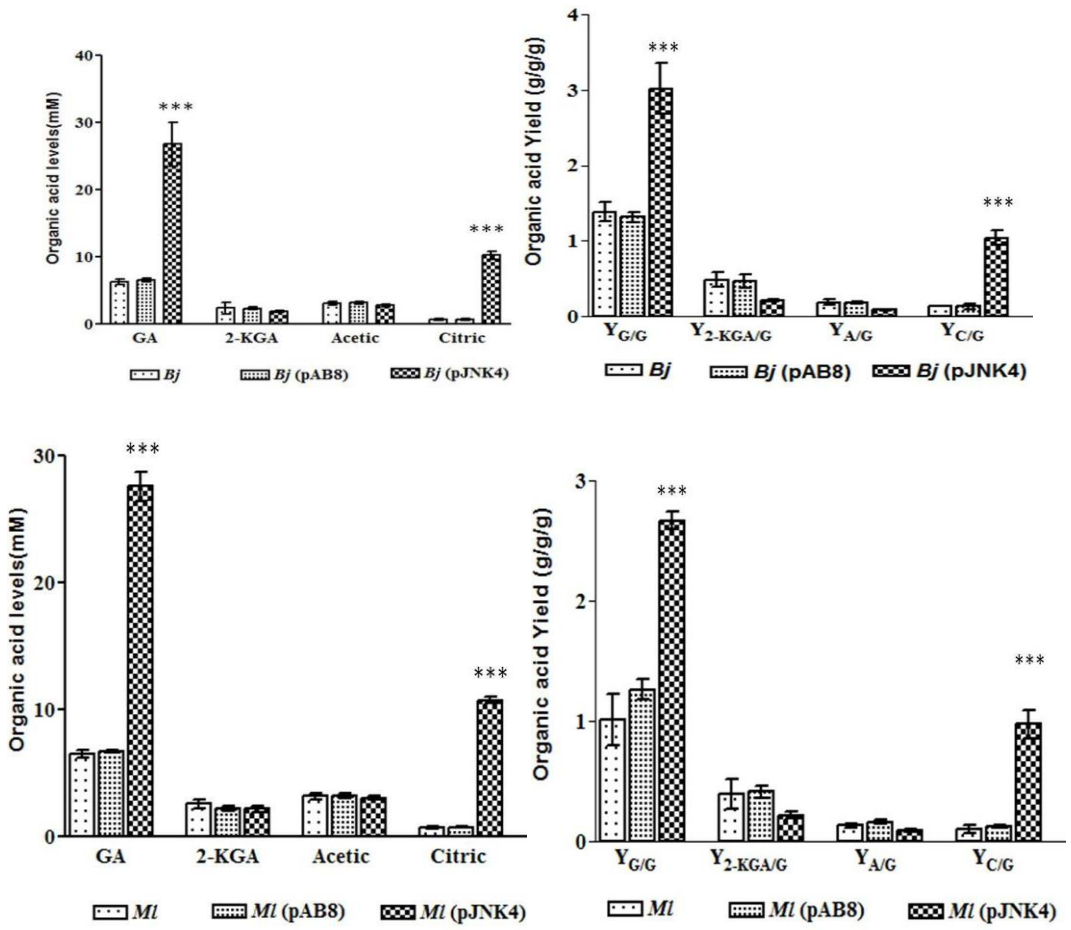


Fig. 6.15: Organic acid production {(a) and (c)} and Yield {(b) and (d)} from *B. japonicum* USDA110 *and M. loti* MAFF030669 *cs* gene transformants, respectively. All organic acids are estimated from stationary phase cultures (at the time of pH drop) grown on TRP medium with 50mM glucose. Results are expressed as Mean ± S.E.M of 4-6 independent observations *** P<0.001.

Table 6.7: Intracellular citric acid production by *Bj* (pJNK4) and *Ml* (pJNK4) 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* (pJNK4)**	0.85 ± 0.05	***Ml* (pJNK4)**	0.82 ± 0.04

(ii) Alterations in enzyme activities in *Bj* (pAB3) and *Ml* (pJNK3)

In order to correlate the alterations in physiological variables and organic acid profile, enzymes involved periplasmic direct oxidation and intracellular phosphorylative were estimated. In response to *cs** gene and citrate transporter *citC* gene of *S. typhimorium* gene overexpression, about ~ 3.9 and ~ 4.7 fold increase is seen in CS activity in *Bj* (pJNK4) and *Ml* (pJNK4), respectively. GDH activity increased by about ~1.4 and ~1.2 fold, respectively, as compared to the control, PYC showed ~2.2 and ~3.3 fold increase, respectively, in the transformants. The activity of G-6-PDH showed ~1.8 and ~1.3 fold increase in both the transformants and also there was ~1.9 and ~1.5 fold increase, respectively, in ICDH activity. The activity of PPC in *Bj* (pJNK4) and *Ml* (pJNK4) increased by ~1.4 and ~1.3 fold, respectively, as compared to the control. Glyoxylate pathway enzyme ICL also increased by ~1.5 and `1.2 fold in *Bj* (pJNK3) and *Ml* (pJNK3), respectively (**Fig. 6.16**).

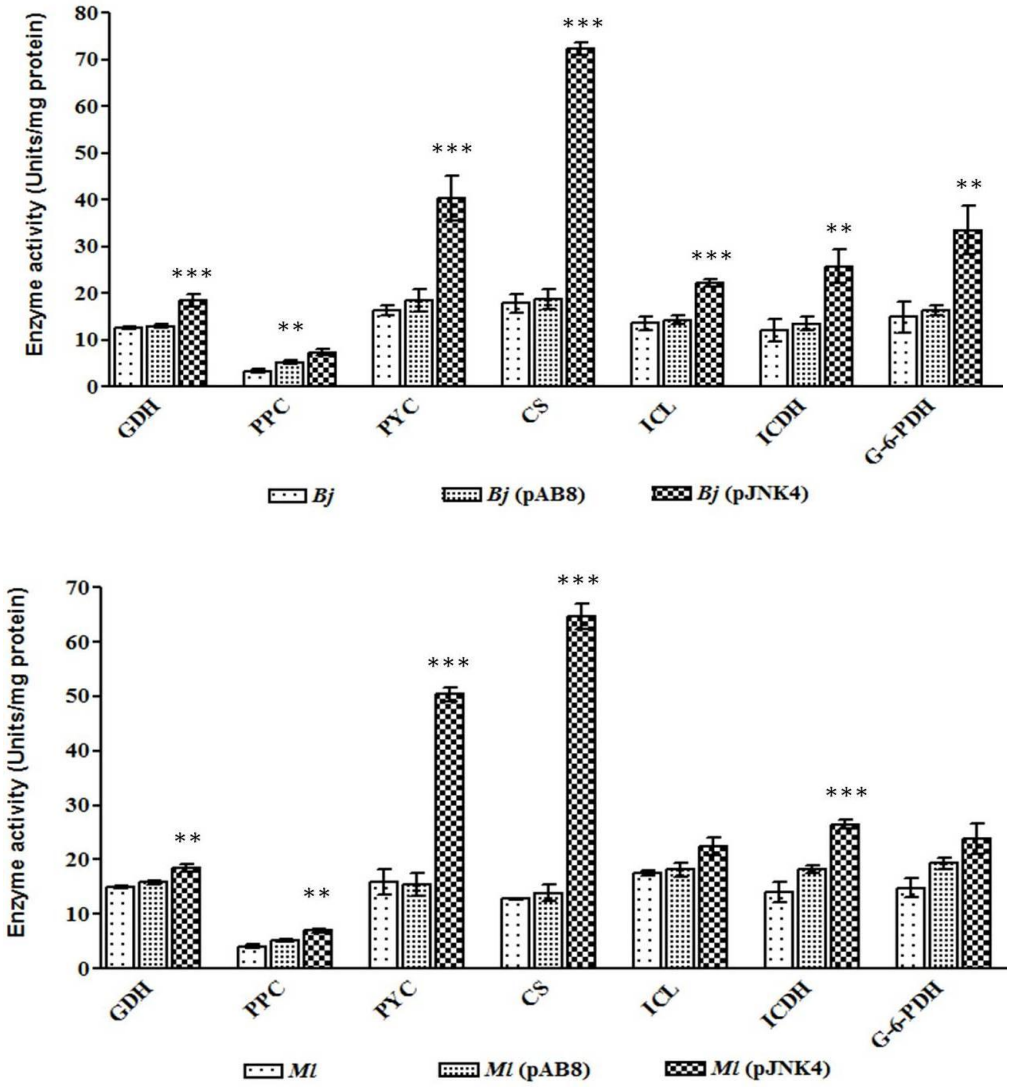


Fig. 6.16: Activities of enzymes PPC, PYC, GDH, G-6-PDH, ICDH and ICL in *B. japonicum* USDA110 and *M. loti* MAFF030669 *ppc* transformant. The activities have been estimated using cultures grown on TRP medium with 50mM glucose. All the enzyme activities were estimated from mid log phase to late log phase cultures except CS, ICDH and ICL which were estimated in stationary phase . 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<0.01and *** P<0.001.

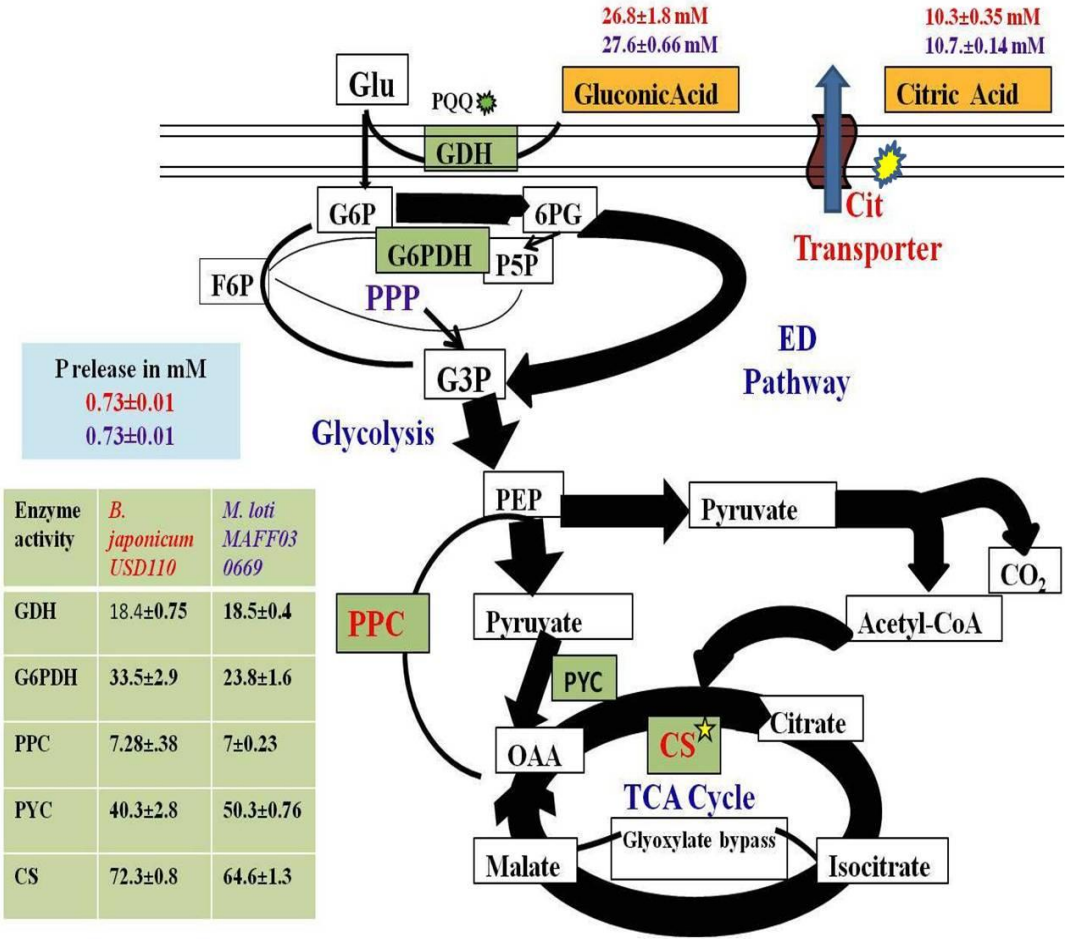
6.4: DISCUSSION

The present study demonstrates heterologous overexpression *E. coli* NADH insensitive *cs* gene along with citrate transporter *citC* gene of *S. typhimorium* in citric acid secretion by *B. japonicum* USDA110 and *M. loti* MAFF030669. Approximately ~14 fold increase in extracellular citrate level (10.3 mM and 10.8 mM) compared to vector control *Bj* (pAB8) and *Ml* (pAB8) and ~1.3 fold increase as compared to *Bj* (pJNK3) and *Ml* (pJNK3) i.e. transformants containing NADH insensitive *cs* and ~1.4 and ~1.8 fold increase compared to wild type *cs*, *Bj* (pAB7) and *Ml* (pAB7), respectively. Increase in extracellular citrate levels are in accordance with the increased levels of extracellular citrate 6 mM, 7 mM and 2.8 mM seen in *P. fluorescens* PfO-1, *Citrobacter* sp. DHRSS and *H. seropedicae* Z67 transformants containing *citC* transporter gene along with NADH insensitive cs gene, respectively (Adhikary, 2012; Yadav, 2013; Wagh, 2013). Increased amount of citric acid is due to ~3.9 and ~4.7 fold increase in CS activity in *Bj* (pJNK4) and *Ml* (pJNK4), respectively. The increase in CS activity is also similar to ~5.6 , ~5 fold and~1.9 folds increase seen in *P. fluorescens* PfO-1, *Citrobacter* sp. DHRSS and *H. seropedicae* Z67 transformants containing *citC* transporter gene along with NADH insensitive cs gene, respectively (Adhikary, 2012; Yadav, 2013; Wagh, 2013).

The higher flux through TCA in Rhizobium strains is supported by ~1.9 and 1.5 fold increase in ICDH activity in *Bj* (pJNK4) and *Ml* (pJNK4), respectively, which is identical to increase seen in transformants containing NADH insensitive *cs* gene. No such change was seen in *P. fluorescens* PfO-1, *Citrobacter* sp. DHRSS and *H. seropedicae* Z67 transformants (Adhikary, 2012; Yadav, 2013; Wagh, 2013).

Enhanced CS activity in *Bj* (pJNK4) and *Ml* (pJNK4) also increased the periplasmic glucose oxidation which is reflected by increase in GDH activity and gluconic acid production**.** Significant increase in GDH and G6PDH activity in *Bj* (pJNK4) and *Ml* (pJNK4) suggests an increase in periplasmic glucose oxidation and phosphorylative pathway. Increased gluconic acid levels could be explained by increased PYC and PPC activity, which could probably divert pyruvate flux towards increased OAA biosynthesis to meet the increased CS activity. Even in *A. niger* the enhancement of

anaplerotic reactions replenishing TCA cycle intermediates predisposes the cells to form high amounts of citric acid (Legisa and Mattey, 2007). Similar increase in flux through glyoxylate shunt was evident from ~1.5 and ~1.2 fold increase in ICL activity detected in *Bj* (pJNK4) and *Ml* (pJNK4), respectively.



Enzyme activity	*B. japonicum USD110*	*M. loti MAFF03 0669*
GDH	18.4±0.75	18.5±0.4
G6PDH	33.5±2.9	23.8±1.6
PPC	7.28±.38	7±0.23
PYC	40.3±2.8	50.3±0.76
CS	72.3±0.8	64.6±1.3

Fig. 6.17: Key metabolic fluctuations in *B. japonicum* USDA110 and *M. loti* MAFF030669 overexpressing NADH insensitive *E. coli cs* gene along with citrate transporter *citC* gene of *S. typhimorium* gene

P solubilization by *Rhizobium* transformants increased by ~14.6 and ~17 fold (0.73 mM and 0.73 mM) compared to vector control *Bj* (pAB8) and *Ml* (pAB8) and ~1.12 and ~1.3 fold increase as compared to *Bj* (pJNK3) and *Ml* (pJNK3) i.e. transformants containing NADH insensitive *cs* and ~2 fold increase compared to wild type *cs*, *Bj* (pAB7) and *Ml* (pAB7), respectively, due to increased amount of gluconic

acid as well as citric acid levels. Similar observation is also reported in *P. fluorescens* PfO-1, *Citrobacter* sp. DHRSS and *H. seropedicae* Z67 transformants containing *citC* transporter gene along with NADH insensitive *cs* gene (Adhikary, 2012; Yadav, 2013; Wagh, 2013).

Both *Rhizobium* transformants had further enhanced growth promoting activities such as biofilm formation, exopolysaccharide, and indole acetic acid production. 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.

Chapter 7

Genomic integration of *E. coli* NADH insensitive *cs* and *Salmonella typhimurium* Na^+ dependent citrate transporter with *vgb, egfp in B. japonicum* USDA110, *M. loti* MAFF303099 and *S. fredii* NGR 234

7.1 Introduction

Inoculation of microbial consortium of P solublizing *Bacillus megaterium* and N fixing free bacteria like *Azotobacter* have been found to be more effective than individual members (Aditya et al., 2009). In field experiments, *Penicillium bilaii* and *Bacillus megatherium* are known to be most effective phosphate solubilizing microorganisms (PSMs) (Asea et al., 1988; Kucey, 1988).

Overexpression of *ppc* gene of *S. elongatus* in *B. japonicum* USDA110 and *M. loti MAFF030669* enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increase in gluconic acid and citric acid secretion, which was due to increased activities of both PYC and PPC. Similar levels of citric acid secretion was seen upon overexpression of wild type *cs* gene but there was a decrease in P solubilization compared to *ppc* gene overexpression which may be attributed to lower levels of gluconic acid secretion in *cs* overexpression. Additionally, overexpression of *E. coli* NADH insensitive *cs* gene in *B. japonicum* USDA110 and *M. loti* MAFF030669 strains also resulted in minor increase in secretion of citric acid but P solubilization showed a significant improved phenotype which is correlated with ~ 2.6 fold increase in gluconic acid secretion. The gluconic acid secretion was more than even *ppc* overexpression. Interestingly, overexpression of citrate transporter along with NADH insensitive *cs* gene increased citric secretion upto ~10 mM citric acid and gluconic acid level remained high which further enhanced MPS phenotype. Earlier studies showed that 10 mM citric and 20 mM gluconic acids released 0.7 mM and 0.5 mM P from alkaline vertisols, respectively (Gyaneshwar et al., 1998). Thus, the *B. japonicum* USDA110 and *M. loti* MAFF030669 transformants containing NADH insensitive *cs* and *citC* genes could be effective PSMs in alkaline vertisols.

Presence of plasmids adversely affected the growth and gluconic acid secretion of phosphate solubilizing *Enterobacter asburiae* PSI3 under phosphorus limited condition (Sharma et al. 2011). Similarly, *Azotobacter vinelandii*, *Azospirillum brasilense*, and *Pseudomonas putida* GR12-2 also showed impaired growth, phosphate solubilization, nitrogen fixation, siderophore production, and indole acetic acid biosynthesis, under various experimental conditions (Glick 1995). The maintenance of plasmid DNA in *E. coli* has been demonstrated to have diverse effects on the physiology and cellular metabolism including alteration in ATP biosynthesis as well as perturbations in host DNA replication, transcription, and translation (Rozkov et al. 2004; Wang et al. 2006; Chou 2007; Ow et al. 2006; 2009). Presence of *colE*1 based plasmid has been demonstrated to significantly alter several metabolic pathways in *E. coli* depending on the growth conditions. Additionally, plasmids are not very stable in natural soil isolates and presence of antibiotic marker also impose problem in field conditions (De Gelder, 2007; Buch et al., 2010). Presence of plasmid affects the host metabolism adversely which results in different metabolic perturbations including alterations of several metabolic pathways in *E. coli* depending on the growth conditions due the presence of colE1 based plasmid (Wang et al. 2006). Variable stability of *P. fluorescens* WCS365 and reduction in competitiveness of *P. putida* GR12-2 were found under rhizospheric conditions (Simons et al. 1996; Schmidt-Eisenlohr et al., 2003).

Previous reports from our laboratory showed that presence of plasmids and overexpression of genes in plasmids exerted greater metabolic alterations on *P. fluorescens* ATCC 13525 metabolism despite of low copy number (Buch et al., 2010). Thus to minimize the plasmid load, instability and antibiotic marker free genetic manipulation an artificial citrate gene cluster consisting NADH insensitive citrate synthase (*cs**), sodium dependent citrate transporter (*citC*), *Vitreoscilla hemoglobin* (*vgb*) and *egfp* were integrated into the genome of *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 by miniTn7 based integration system at *att* site and characterized the biochemical, growth and MPS abilities.

The mini-Tn7 transposon is very effective for single copy tagging of bacteria in a site-specific manner at a unique and neutral site without any deleterious effects. The Tn7 transposon was originally discovered by Barth et al. (1976) on the plasmid R483 (IncIα) as an element carrying the resistance genes trimethoprim (TmR) and streptomycin/ spectinomycin (SmR/ SpR), which could be transposed to other replicons. These genes are flanked by the ends of the transposon, named the left (Tn7L) and the right (Tn7R) end (Lichtenstein and Brenner, 1982; Rogers et al., 1986). The Tn7 transposition process has been studied intensively in *E. coli* in which Tn7 inserts with high efficiency and unique orientation into one specific location named the attTn7 site (Peters et al., 2001)**.** This site of insertion is located just downstream of the coding region, in the transcriptional terminator, of the *glm*S gene and thereby does not disrupt the gene (Gringauz, et al., 1988). The *glm*S gene encodes a glucosamine synthetase, which is required for cell wall synthesis (Vogler et al., 1989). It is conserved among many bacteria and therefore Tn7 is likely to have the same specific insertion site in many different bacteria, some have already been tested. The transposon genes required for specific insertion into the attTn7 site, are *tnsABCD*, and they function in *trans*. Thus, sequences located in the 3‟end of the coding region of *glm*S are recognised by transposase proteins directing the actual insertion into the attTn7 site, down-stream of the *glmS* gene**.** However, if this site is unavailable the transposon can insert into other sites with low frequency.

The mini-Tn7-based gene integration system has been used for gene complementation, gene expression analysis, strain construction, and reporter gene-tagging of *Pseudomonas aeruginosa* and *Yersinia pestis*, particularly in biofilm and animal models. Heterologous genes including *lacZ* (β-galactosidase), *est* (esterase), and *gfp* (green fluorescent protein) under the control of the methanol dehydrogenase promoter have been integrated into the intergenic region between *glmS* and *dha*T via the delivery of mini-Tn7 in *Methylobacterium extorquens*. A gene encoding for different fluorescent protein and luciferase protein along with promoter was integrated into the chromosomes of *Erwinia chrysanthemi, P. fluorescens, Pseudomonas syringae, P. putida* and many gram negative bacteria by the Tn7-based delivery system (Koch et al., 2001, Lambersten

et al., 2004; Choi et al.,2008). This gene delivery system was also applied to other organisms, such as *Burkholderia* spp. and *Proteus mirabilis*, which were determined to have multiple *glmS*-linked attTn7 sites and secondary, non-*glmS*-linked attTn7 site, respectively. Thus mini Tn7 transposition is a powerful technique for the integration or excision of a gene of interest at a single-copy on the chromosomal level, which makes it possible to conduct a variety of experiments, including insertional random mutagenesis, gene expression analysis, protein functional studies, or the gene-tagging of bacteria in living organisms.

MPS ability of the bacterial transformants in field conditions could be affected by various parameters. In addition to the nutrient availability and soil properties, oxygen limitation could be a significant factor. Oxygen is present in limited amounts in the rhizosphere which could limit the colonization and survival of rhizobacteria (Ramírez et al., 1999). Genetically modified *Rhizobium* with *vgb* overexpression has enhanced the growth of *Rhizobium* and also increased nitrogen content in bean plants (Ramirez et al., 1999).

The present study describes the genomic integration of *E. coli* NADH insensitive *cs* Y145F along with *S. typhimurium* sodium citrate transporter *citC* gene, *vhb* gene and *egfp* into *Rhizobium* genome of *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 and compare its effect to plasmid based expression on glucose catabolism and citric acid secretion.

7.2 Experimental design

7.2.1: Bacterial strains used in this study

Table 7.1: Bacterial strains used in this study. The details of the plasmids and the concentration of the antibiotics used are given in the **Table 2.2** and **2.3**.

Plasmid/Strains	**Characteristics**	**Source or Reference**
pGRG36	Intergration vector (Tn7)	Gregory et al., 2006
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
E. coli DH10B	*Str^r F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZ ΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-*	(Invitrogen) USA
pJNK4	pUCPM18 with *E. coli* NADH insensitive *cs** gene citrate transporter *citC* of *Salmonella typhimurium* under *Plac* and km^r gene; Ap^r, Km^r	Wagh, 2013
pJIYC	pGRG36 with with *E. coli* NADH insensitive *cs** gene citrate transporter citC of *Salmonella typhimurium* under *Plac*, *vgb* gene and *egf* gene Ap^r	This study
Bj intYc	Genomic integrant of *B. japonicum USDA110* containing *lac-YF citC, vgb, egfp* Ap^r	This study
Ml intYc	Genomic integrant of *M. loti MAFF030669* containing *lac-YF citC, vgb, egfp* Ap^r	This study
Sf intYc	Genomic integrant of *S. fredii NGR234* containing *lac-YF citC, vgb, egfp* Ap^r	This study

7.2.2: Cloning of artificial citrate operon in integration vector.

Construction of artificial citrate operon containing constitutive *lac* promoter, NADH insensitive citrate synthase (cs*), Na^+ dependent citrate trasporter (*citC*), *vhb* gene and *gfp* was done as per the following strategy. XT-20 polymerase was used for PCR amplification (Bangalore Genei, India) from plasmid pJNK4. Sequence of forward (*lac*) primer 5` TCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACAGGAGG ACGCATGATGGCTGATACAAAAGC 3` and reverse primer of *citC* gene 5' TTACACCATCATGCTGAACACGATGC 3' was used to amplify artificial citrate operon. Plasmid pGRG36-mini-Tn7-Amp-*egfp* was digested with *Sma*I, gel purified and ligated with 2.9 kb amplicon containing artificial citrate operon was ligated with pGRG36 containing *vgb* and *egfp* **(Fig. 7.1).** Clone was confirmed by restriction digestion and PCR amplification. Resultant construct of 17.9 kb, named as pJIYC consisted of artificial citrate operon.

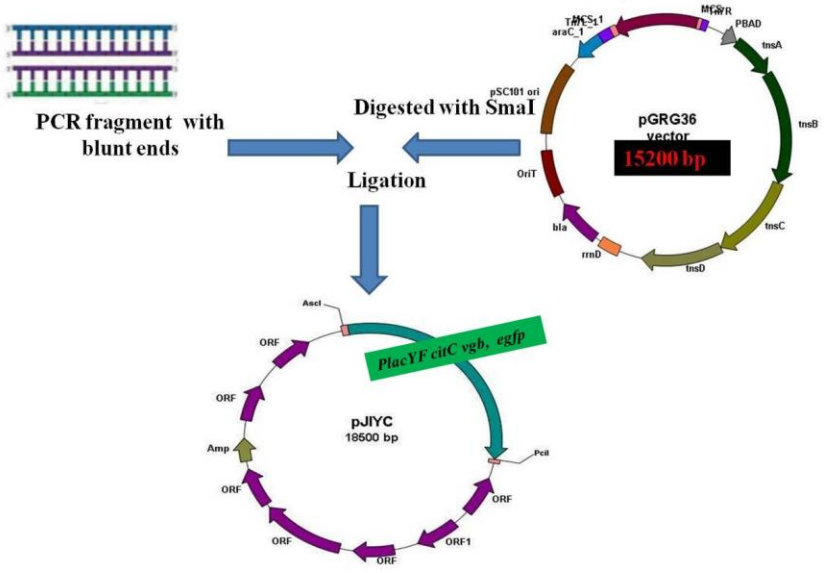


Fig. 7.1: Strategy used for cloning of artificial citrate operon in pGRG36 containing *vhb, egfp* resulted in pJIYC.

7.3: RESULTS

7.3.1: Construction of Genome integrants of *B. japonicum* USDA110 *M. loti* MAFF030669 and *S. fredii* NGR234

PCR amplification of NADH insensitive citrate synthase (*cs**) along with *citC* gene- Na^+ dependent transporter under constitutive *lac* promoter was done from pJNK4 plasmid containing YF*citC* by using specific primers as mentioned above. This PCR amplicon was used for ligation with integration vector (**Fig.7.2).** For further confirmation, the recombinant plasmid pJIYC plasmid containing NADH insensitive Y145F and *S. typhimurium* sodium citrate transporter operon under constitutive *lac* promoter was confirmed by restriction enzyme digestion. pJIYC was digested with PvuII and pGRG36*vhb, egfp* plasmid digested with PvuII **(Fig. 7.3).**

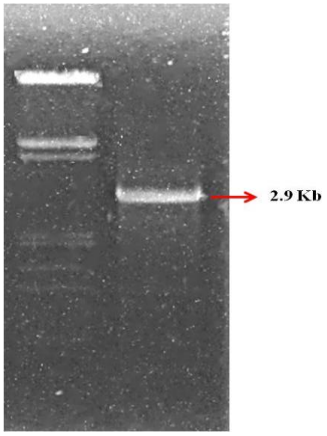


Fig. 7.2: PCR amplification of YF*citC* with constitutive *lac* promoter

Lane1- Marker HindIII/EcoRI, Lane2- PCR amplification with constitutive *lac* promoter

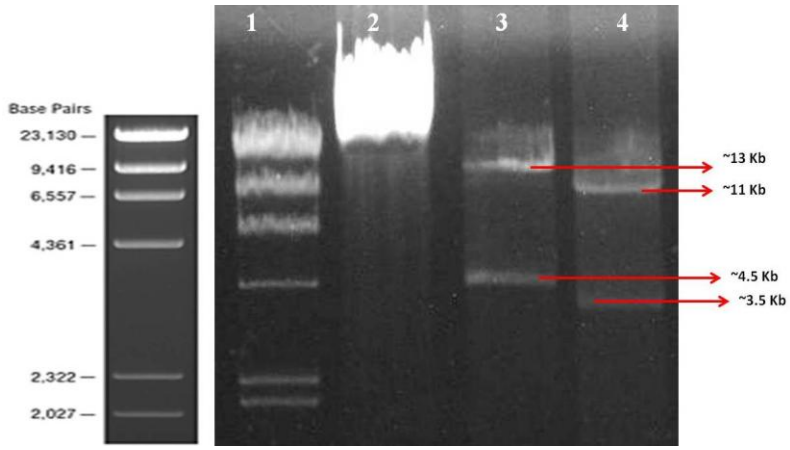


Fig.7.3: Restriction enzyme digestion pattern of pJIYC plasmid containing NADH insensitive Y145F and *S. typhimurium* sodium citrate transporter operon under *lac* promoter. Lane1- Marker HindIII, Lane2- Plasmid undigested, Lane-3 pJIYC digested with PvuII, and Lane-4 pGRG36*vhb*, *egfp* plasmid digested with PvuII.

B. japonicum USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 integrants were confirmed based on PCR amplification of YF-CitC from genomic DNA (**Fig. 7.4).**

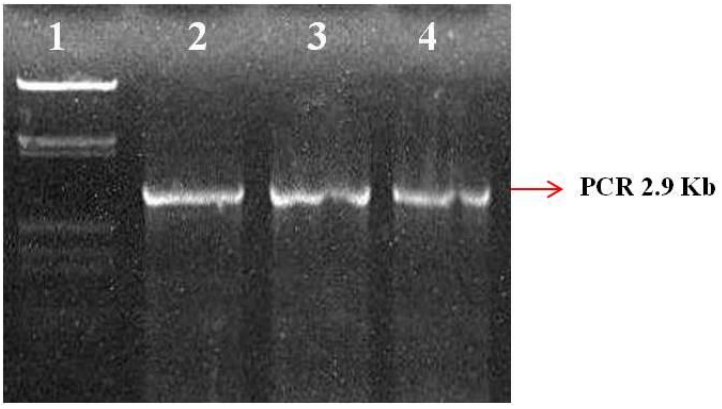


Fig. 7.4: Confirmation of Genome integrants by PCR amplification of YF-citC from *B. japonicum* USDA110 , *M. loti* MAFF030669 and *S. fredii* NGR234 integrants: Lane1- Marker HindIII/EcoRI. Lane- 2,3,4 PCR amplicon of YF-citC from genomic DNA of *B. japonicum* USDA110 , *M. loti* MAFF030669 and *S. fredii* NGR234 integrants.

7.3.2: CS activity and MPS ability of *B. japonicum* USDA110 , *M. loti* MAFF030669 and *S. fredii* NGR234 integrants on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

The CS activity of *B. japonicum* USDA110 *M. loti* MAFF030669 and *S. fredii* NGR234 integrants grown on 50 mM Tris-Cl Buffer pH 8 and 50 mM glucose, was ~2.6, ~4.3 and ~2.2 fold higher (47.13 ± 1.04 U, 54.8 ± 1.23 U and 34.5 ± 1.02 U) in the three strains, respectively, compared to control which possessed low levels of CS activity (17.85 ± 1.14U), (12.86 ± 0.07) and (16.02 ± 0.12 U), respectively. To check MPS ability a zone of clearance and acidification was observed on PVK and TRP plates. Maximum zone of clearance and acidification was shown by *Bj*. intYc, *Ml.* intYc and *Sf.* intYc as compared to the wild type *B. japonicum* USDA110 , *M. loti* MAFF030669 and *S. fredii* NGR234 . P-solubilizing ability of wild type *B. japonicum, M. loti MAFF030669* and *S. fredii* NGR234 and its transformants varied in the order of *Sf intYc > Bj intYc > Ml intYc> Sf >Bj = Ml* on PVK medium after 3 days of incubation at 30°C (**Fig. 7.5**). The integrants of *S. fredii* NGR234, *B. japonicum* USDA110 and *M. loti* MAFF030669 showed enhanced zone of clearance as compared to the wild type. Also among the three integrants showed maximum PSI (**Table 7.2).**

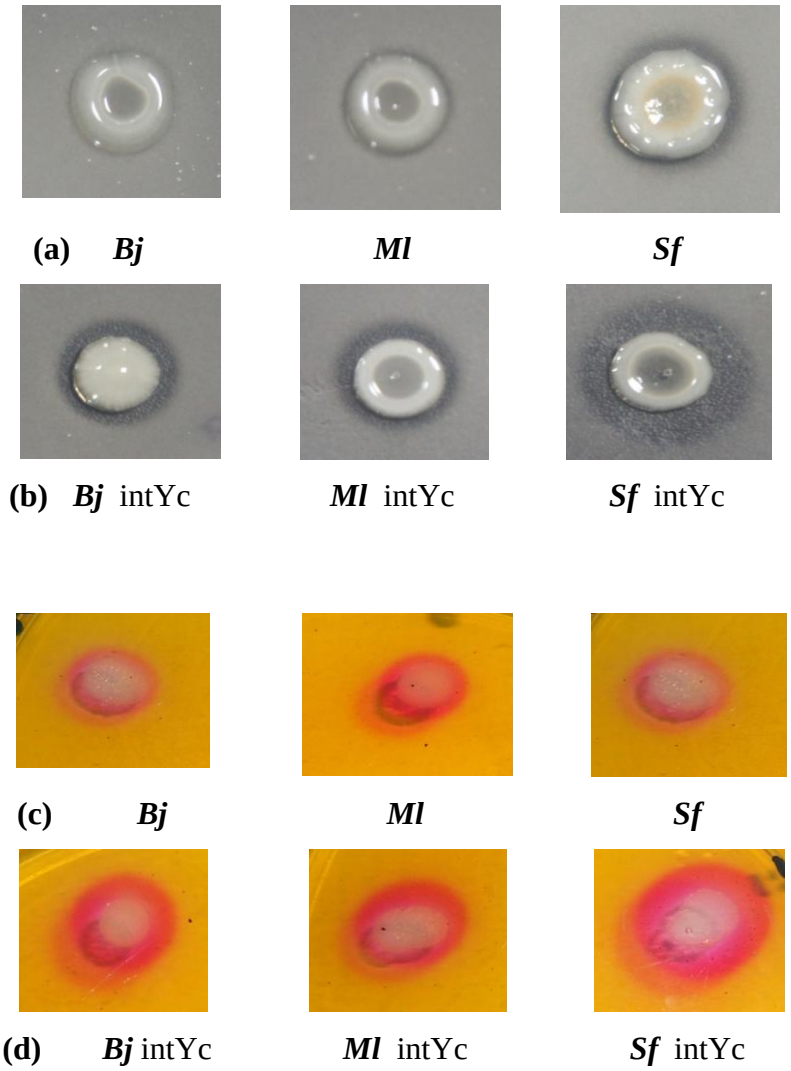


Fig. 7.5: MPS phenotype of *B. japonicum* USDA110 *M. loti* MAFF030669 and *S. fredii* NGR234 integrants (a), (b) Zone of clearance formed by *Rhizobium* integrants on Pikovskaya's agar and (c), (d) zone of acidification Tris rock phosphate agar containing 50 mM glucose and 50 mM Tris Cl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 ºC. Media composition and other experimental details are as described in Sections 2.2.4 and 2.7.

Table 7.2: P solubilization index on Pikovskyas agar of *B. japonicum* USDA110 , *M. loti* MAFF030669 and *S. fredii* NGR234 integrants during 3 days of growth on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate. *Bj, Ml* and *Sf* : wild type strain; and *Bj intYc*, *Ml intYc*and *Sf intYc* : *B. japonicum* USDA110 , *M. loti* MAFF030669 and *S. fredii* NGR234 integrants. The results were noted after an incubation of 3 days at 30 ºC and are given as mean ± S.D. of three independent observations as compared to native *Bj, Ml* and *Sf*.

***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.. intYc	13.17± 0.29	9.50 ± 0.50	1.44
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
Ml.. intYc	14.50 ± 0.50	10.17 ± 0.29	1.40
Sf	12.17 ± 0.29	10.50 ± 0.50	1.20
Sf.. intYc	16.50 ± 0.50	9.17 ± 0.29	1.78

7.3.4: Growth pattern and pH profile of *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 integrants on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

The growth of *Bj intYc*, *Ml intYc*and *Sf intYc* on 50 mM Tris-Cl buffer pH 8 and 50mM glucose reached to a maximum of 1.8 O.D. within 12 h in integrants compared to 20 h of the native *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 strains. pH of the medium dropped to 6.8, 6.8 and 5.7 within 20 h in the native *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234,while pH drop to 3.84, 4.22 and 4.31 was seen within 12 h in *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 integrants, respectively (**Fig. 7.6; 7.7; 7.8**).

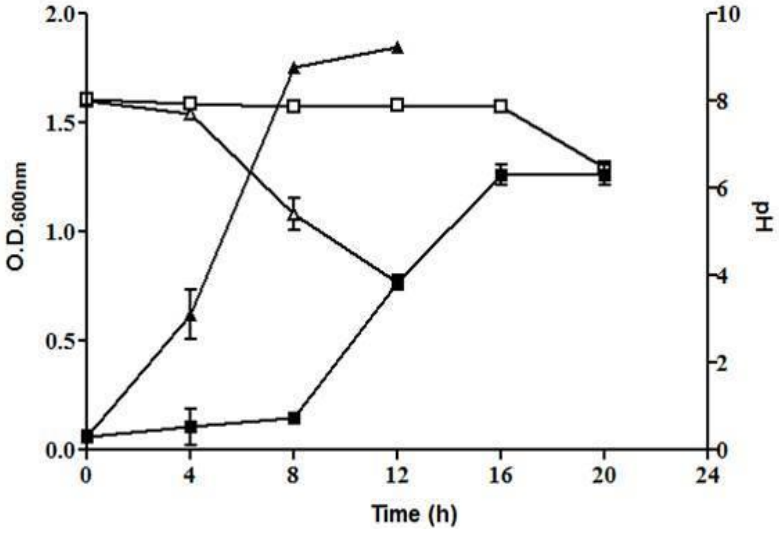


Fig. 7.6: Extracellular pH (□, ∆,) and growth profile on glucose 50 mM, Tris-Cl 50 mM rock phosphate medium (■,▲,) of *B. japonicum* USDA110 integrant containing *YFcitC, vhb, egfp*. □, ■, *Bj* (wild type); ∆, ▲, *Bj* intYc. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

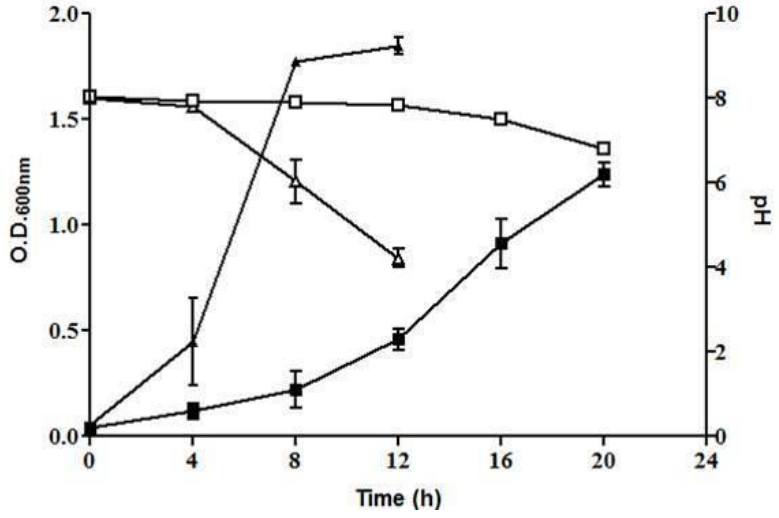


Fig. 7.7: Extracellular pH (□, ∆,) and growth profile on glucose 50 mM , Tris-Cl 50 mM rock phosphate medium (■,▲,) of *M. loti* MAFF030669 integrant containing *YFcitC, vgb* and *egfp*. □, ■, *Ml* (wild type); ∆, ▲, *Ml* intYc. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

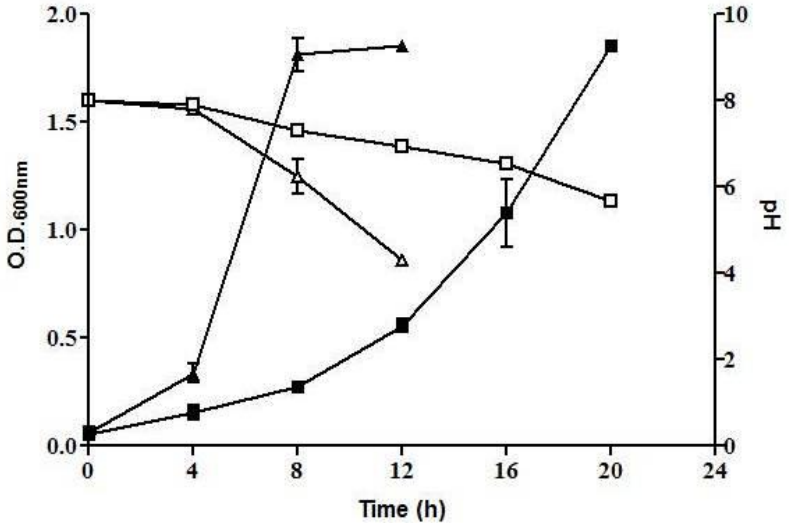


Fig.7.8: Extracellular pH (□, Δ,) and growth profile on glucose 50 mM , Tris-Cl 50 mM rock phosphate medium (■,▲,) of *S. fredii* NGR234 integrant containing *YFcitC, vgb, egfp*. □, ■, *Sf* (wild type); Δ, ▲, *Sf* intYc. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

7.3.5: Physiological effect of genomic integration on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

In presence of 50 mM glucose, genomic integration showed ~1.73,~1.72 and ~2.2 fold increase in growth profile by *Bj intYc*, *Ml intYc* and *Sf intYc*, respectively. The total glucose utilization rate at the time of pH drop remained unaffected and the total amount of glucose consumed at the time of pH drop showed ~1.15, ~1.10 and ~1.4 fold decrease in *Bj intYc*, *Ml intYc*and *Sf intYc*, respectively, and Specific Glucose utilization Rate Q_{Glc} (g.g $dcw^{-1}.h^{-1}$) decreased ~1.6, ~2.1 and ~1.9 fold. Increase in enzyme activity improved the biomass yield by ~1.5, ~2.1 and ~2.1 fold in the integrants of *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234, respectively, compared to native *B. japonicum*, *M. loti* MAFF030669 and *S. fredii* NGR234 (**Table 7.3**).

Table 7.3: Physiological variables and metabolic data of *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 integrants grown on TRP The results are expressed as Mean ± S.E.M of 6-10 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 ±0.2	38.23 ±1.33	1.78 ±0.14	0.14 ±0.01
Bj intYc	0.321 ±0.01***	48.28 ±0.12	33.15 ±0.92	2.66 ±0.15**	0.09 ±0.01
Ml	0.221 ±0.03	45.91 ±0.64	37.07 ±0.55	1.36 ±0.26	0.19 ±0.04
Ml intYc	0.380 ±0.01***	48.29 ±0.15	33.56 ±0.40	2.80 ±0.28***	0.09 ±0.01
Sf	0.260 ±0.02	46.10 ±0.42	37.17 ±0.55	1.85 ±0.1	0.15 ±0.02
Sf intYc	0.569 ±0.01***	48.26 ±0.1	26.82 ±1.07	3.95 ±0.17***	0.08 ±0.01

7.3.6: P solubilization and organic acid by *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 integrants in 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

There was significant increase in release of P by ~11.2, ~8.6 and ~11.24 fold by *Bj intYc*, *Ml intYc*and *Sf intYc*, respectively, compared to wild type when grown in 50 mM Tris-Cl Buffer pH 8 containing 50 mM glucose containing Rock Phosphate 1mg/ml (**Fig. 7.9**).

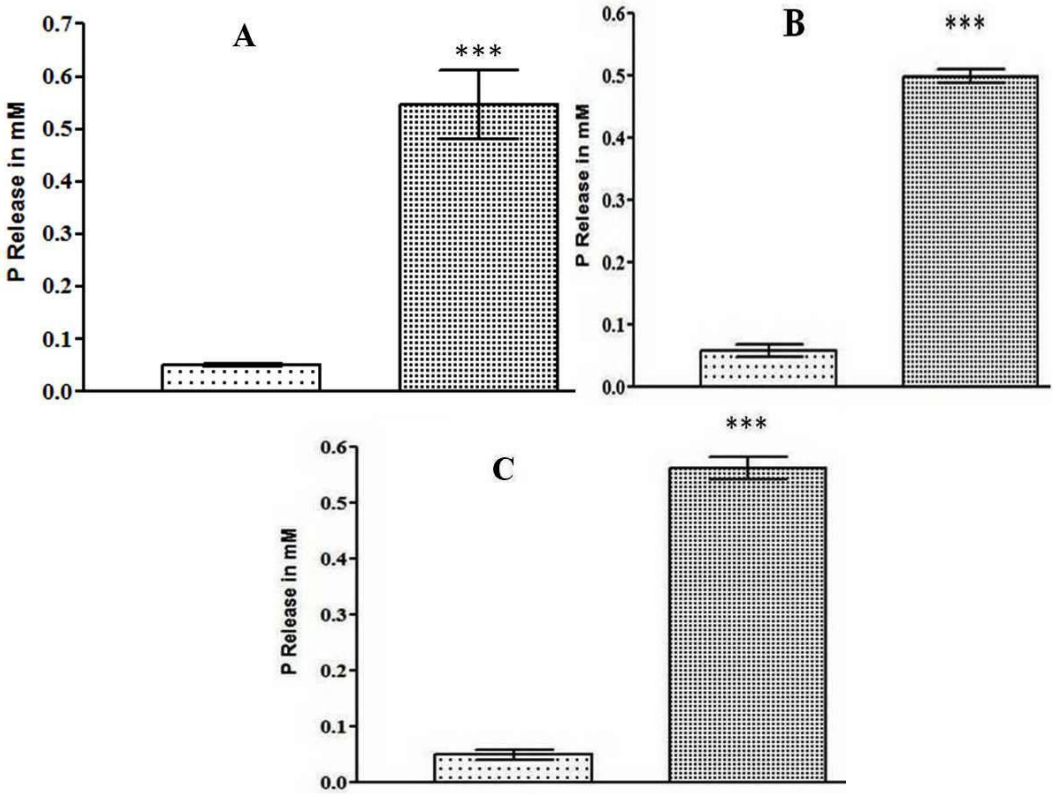


Fig. 7.9: P Solubilization by different integrants. (A)- ▭, *Bj*, ▭, *Bj* intYc,) (**B**)- ▭, *Ml*, ▭, *Ml* intYc and (**C**)▭, *Sf*, ▭, *Sf* intYc; on **50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.** Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

On 50 mM Tris-Cl pH 8 and in presence of 50 mM glucose, the organic acids identified were mainly gluconic, 2-ketogluconic, acetic and citric acids. Extracellular medium of *Bj* intYc, *Ml* intYc and *Sf* intYc showed ~9.4, ~9.6 and ~7.9-fold increase in citric acid with specific citric acid yield $Y_{C/G}$ ~2.7, ~3.5 and ~1.5 fold, respectively, which is ~2.1, ~1.9 and ~2.8- fold higher amount of gluconic acid as compared to native with specific gluconic acid yield, $Y_{G/G}$, increasing by ~1.7,~1.5 and ~1.8 fold, respectively (**Fig. 7.10**).There was no change in the intracellular citric acid levels.(Table

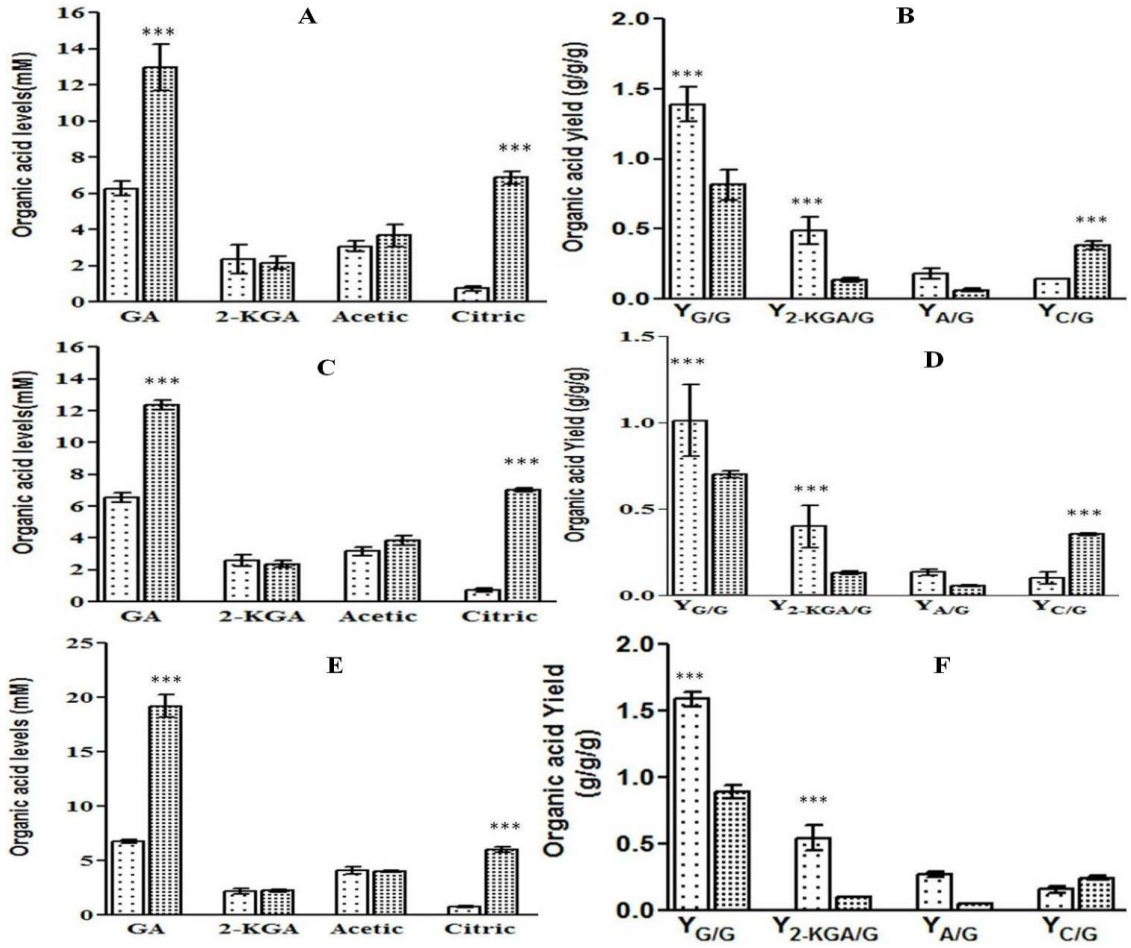


Fig.7.10: Organic acid production by three integrants. (A, B)-▭, *Bj* , ▭ , *Bj*. intYc,) (**C, D**)- ▭, *Ml*, ▭, *Ml.* intYc and (**E, F**)▭, *Sf*, ▭, *Sf.* intYc; **(A), (C), (E) Organic acids in mM (B), (D), (E) Organic acid yields, grown on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.** All organic acids are estimated from stationary phase cultures (at the time of pH drop). Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Table 7.4: Intracellular citric acid levels of *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 integrants grown on TRP.

***Rhizobium* Strains**	**Intracellular Citric acid in mM**
***B.japonicum* USDA110**	0.83 ± 0.06
***Bj* intYc**	0.85 ± 0.04
***M. loti* MAFF030669**	0.85 ± 0.04
***Ml* intYc**	1.07 ± 0.09
***S. fredii* NGR234**	1.48 ± 0.08
***Sf* intYc**	1.32 ± 0.31

7.3.7: Alterations in enzyme activities in *B. japonicum* USDA110 *M. loti* MAFF030669 and *S. fredii* NGR234 integrants .

Genome integrants were further analyzed for alterations in physiological variables and organic acid profile, enzymes involved periplasmic direct oxidation and intracellular phosphorylative pathways. There was an overall increase in all the enzyme activities. **GDH** activity increased by about ~1.95, ~1.3 and ~2.1- fold; **PPC** activity increased by ~1.7, ~1.3-fold and no change; **PYC** increased by ~2.6, ~2.5 and ~2.0-fold; **CS** increased by ~2.6, ~4.3 and ~2.2-fold; ICL increased by -~1.5, ~1.3-fold and no change, ICDH increased by ~1.5, ~1.8 and~1.6-fold and G-6-PDH increased by ~1.6,~1.4 and ~1.5-fold in *Bj*. intYc, *Ml.* intYc and *Sf.* intYc or *B. japonicum USDA110 M. loti MAFF030669* and *S. fredii NGR234* integrants respectively compared to the native strains (**Fig. 7.11**,**7.12 and 7.13).**

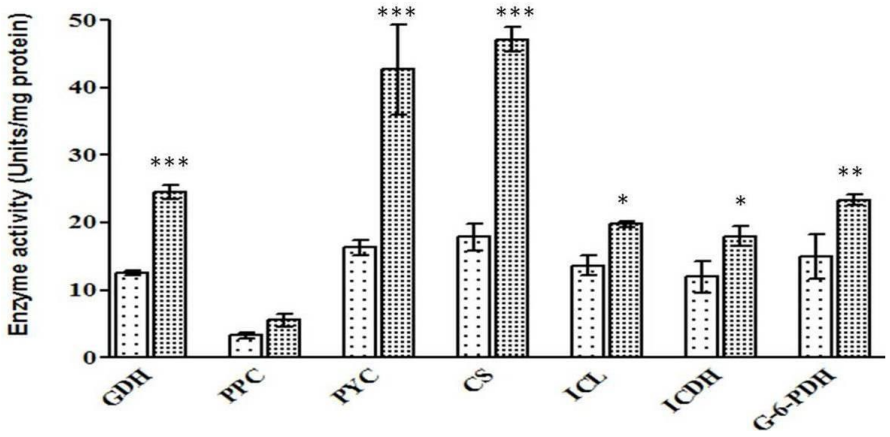


Fig.7.11: Alterations in enzyme activities in *B. japonicum* USDA110 integrants (▭, *Bj*;▭ , *Bj*. intYc). The activities have been estimated using cultures grown on M9 minimal medium with 50 mM glucose. All the enzyme activities are represented in the units of nmoles/min/mg total protein. Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

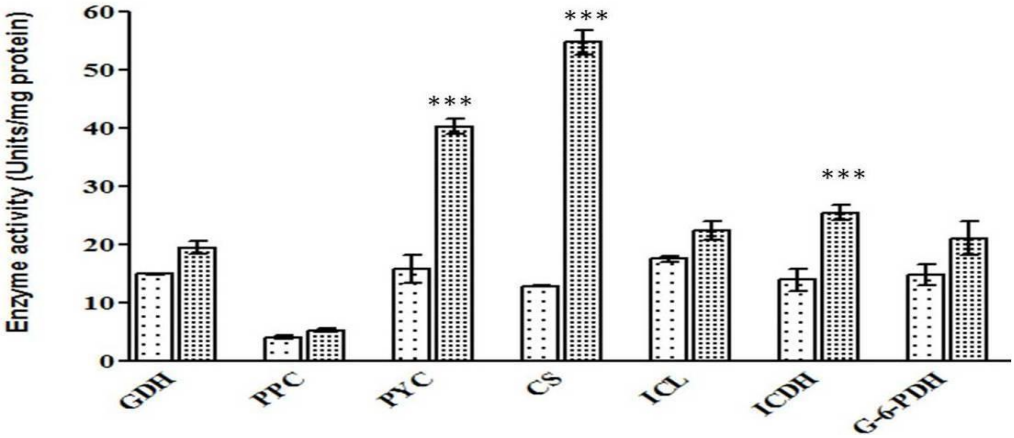


Fig. 7.12: Alterations in enzyme activities in *M. loti* MAFF030669 integrants (▭, *Ml*, ▭, *Ml.* intYc). The activities have been estimated using cultures grown on M9 minimal medium with 50 mM glucose. All the enzyme activities are represented in the units of nmoles/min/mg total protein. Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

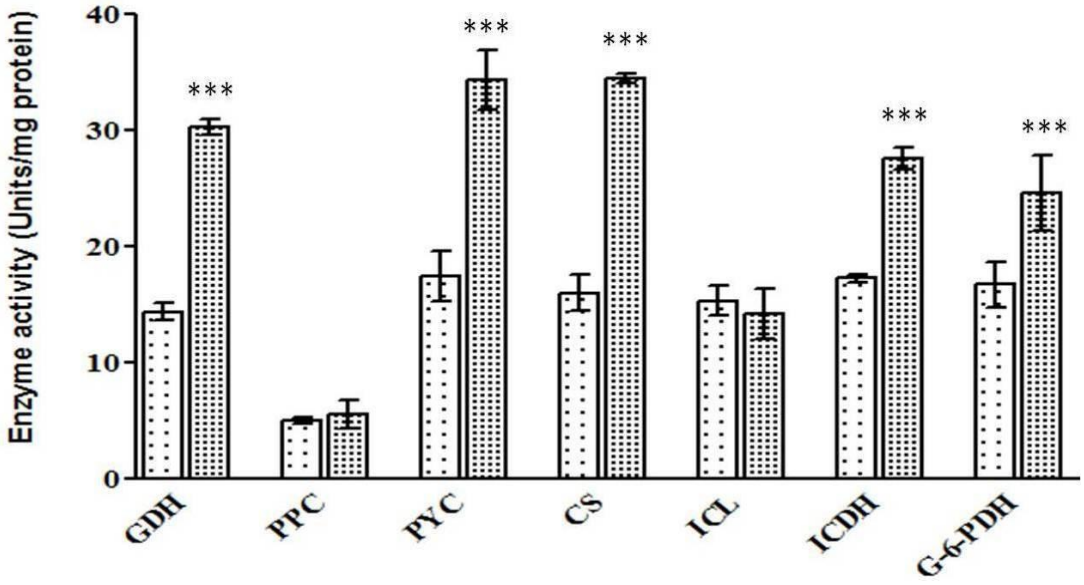


Fig. 7.13: Alterations in enzyme activities in *S. fredii* NGR234 integrants (▭, *Sf;* ▭, *Sf.* intYc). The activities have been estimated using cultures grown on M9 minimal medium with 50 mM glucose. All the enzyme activities are represented in the units of nmoles/min/mg total protein. Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

7.4: DISCUSSION

Genetic engineering of microorganisms involves the use of extra-chromosomal plasmid for heterologous expression of desired genes. Plasmid DNA is known to cause metabolic burden on the cell and alter its metabolism depending on the host organism, plasmid nature, and environmental conditions (Buch et al., 2010b; Sharma et al., 2011). Hence, genetic manipulations need to be directed towards chromosomal integration as it would lead not only to increased stability but also decrease the metabolic load caused by the presence of the plasmids to nullify the pleotropic effects on the host metabolism. The present study demonstrates the effect of genomic integration of *E. coli* NADH insensitive *cs* Y145F gene and *S. typhimurium citC* gene on *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234.

In our study, genomic integrants of *B. japonicum, M. loti* MAFF030669 and *S. fredii* NGR234 showed MPS phenotype in 50 mM Tris pH 8.0 while corresponding plasmid transformants showed phenotype even on 100 mM Tris pH 8.0 medium. Hence, the strength acidification by the genomic integrants had decreased. This was contrary to the phenotype expected on the basis of metabolic load between plasmid transformants and genomic integrants. This, it appears that the higher level overexpression of genes in plasmid transformants, due to high copy number of the genes, may be responsible for their better MPS phenotype.

The metabolic differences among the various transformants and the integrants may be due to the differences in the copy numbers, the former having 10-16 plasmid copy number. Plasmid nature and copy number are known to exert load on cellular physiology leading to significant alterations in the metabolism (Schweder et al, 2002; Wang et al., 2006; De Gelder, 2007). The copy number of the plasmids used in this study (pAB4, pAB3, pAB8, pAB7) in *P. fluorescens* 13525 was found to be 10-16 and was independent of the nature of antibiotic resistance.(Buch et al., 2010) The increased phenotype in plasmid transformants is due to high copy number of plasmids, so more

overexpression, and more enzyme activity resulted in increased amount of acid. While in the integrants, single copy number produced less amount of acid, and thus showed decreased phenotype compared to transformants. Here metabolic load is not so high in the laboratory conditions compared to copy number effect.

There was significant increase in activities of GDH, G-6-PDH, PPC, PYC, CS, ICL and ICDH in integrants of all the three strains, suggesting increase in oxidative and phosphorylative pathway and higher flux of glucose through TCA in *Rhizobium* leading to increase in gluconic acid and citric acid (Fuhrer, 2005). In addition to the metabolic alterations in the central carbon pathway, this work also deals with the overexpression of *vgb* gene in *Rhizobium* spp. along with artificial citrate operon to improve the recombinant protein production and better survival of host under microaerobic conditions (Frey and Kallio, 2003). A loss of MPS ability under low aeration conditions was seen in case of *Citrobacter* sp. DHRSS containing citrate operon with a concomitant loss of citric and gluconic acid secretion. Presence of *vgb* gene restored the citric and gluconic acid secretion along with MPS ability under microaerobic conditions (Yadav, 2013). In this study *vgb* effect was seen under aerobic condition and effect in microaerobic condition is yet to be investigated.

The genetically modified strains are expected to be an efficient phosphate solubilizing bacterium (PSB) in rhizosphere soil. This paves the way for genetic modification of other plant growth promoting rhizobacteria to enable them to secrete higher amount citric acid and converting into efficient PSB.

Chapter 8

Effect of *Sinorhizobium fredii* NGR 234 genomic integrant containing *E. coli* NADH insensitive *cs* and *S. typhimurium citC, vgb* and *egfp* gene cluster on growth promotion of Mung bean plants

8.1 Introduction

Legumes are second only to cereals as a source of human food and animal feed. Their importance as food lies primarily in their high protein content. Legumes' grain protein is the natural supplement to cereal grain protein. They also provide fat and carbohydrates. Moreover, legumes are high in bone building minerals and vitamins essential for good health (Porres *et al*., 2003). Many efforts were directed to improve yield and protein content of legumes through breeding, fertilization and genetic engineering. Biofertilizers are inputs containing microorganisms which are capable of mobilizing nutritive elements from non-usable form to usable form through biological processes; they include mainly the nitrogen fixing, phosphate solubilizing and plant growth–promoting microorganisms (Goel *et al*., 1999). To maintain the sustainability and soil fertility research efforts are concentrated to make use of less expensive, eco friendly sources of P nutrients such as rock phosphate (Whitelaw, 2000; Arcand 2006). Rock phosphate originates from igneous, sedimentary, metamorphic, and biogenic sources, with sedimentary being the most widespread (van Straaten, 2002). Microorganisms are an integral part of the soil P cycle and in particular, are effective in releasing P from rock phosphate (Richardson 2011).

The inoculation of seeds with *Rhizobium* increased nodulation, nitrogen uptake, seed protein (Solaiman, 1999**;** Rudresh, 2005). *Rhizobium* inoculant significantly increased number of pods, nodule dry weight compared to uninoculated control in chickpea (Solaiman ,1999; Daza et al., 2003; Rabbani et al., 2005; Togay et al., 2008). The combined inoculation of *Rhizobium* and phosphate solubilizing bacteria has increased nodulation, plant height, seed protein and yield parameters in chickpea [Khurana and Sharma, 2000; Namvar et al., 2011). Co-inoculation with nodule-forming and rhizospheric soil bacteria improved growth and nodulation through multiple mechanisms (Contesto et al. 2008; Zadeh et al. 2008). Several PGPR produced ACC-deaminase (e.g. Dimkpa et al. 2009; Shaharoona et al. 2012) and thus modulated C_2H_4 levels in plant through the cleavage of ACC (Glick et al. 1997).

The conducive effect of dual inoculation of seeds with *Rhizobium* and microelements application on nodulation and N_2 fixation in legumes has been established (Zehirov and Georgiev, 2001). Inoculation of seed with *Rhizobium* in combination with microelement fertilizer significantly affected the yield and nodules formation of chickpea (Bejandi et al., 2012). Concentration of chlorophyll dyes is a reliable index of physiological plant condition (Swedrzynska and Sawicka, 2000). However, little work has been done on the combined effects of *Rhizobium* inoculation, microelements application and plant density on nodulation, seedling emergence, chlorophyll content, seed protein and grain yield.

After nitrogen, phosphorus is generally the most limiting nutrient affecting plant growth and productivity. In legumes, phosphorus availability stimulates the nodulation and N_2 fixation since nodule initiation and nodule growth require P (Gentili and Huss-Danell 2003). Phosphorus deficiency generally results in impaired symbiotic N fixation by limiting plant growth, survival of rhizobia, nodule formation, and nodule functioning (Tang et al. 2001; Bargaz et al. 2011). Low organic matter content coupled with native low soil P pool is a major factor limiting agricultural productivity in the Indian subcontinent (Manna et al. 2001). Soil organic amendments increased soil biological activity and improved physical and chemical properties (Gaind et al. 2006). Addition of organic amendment to soil stimulated microbial activity that in turn increased organic matter decomposition rate and nutrient dynamics (Abbott and Murphy 2007; Chakraborty et al. 2011). Microbial biomass has vital role in regulating nutrient source sink. Soil enzymatic activities are key determinants of soil available nutrient pools including N, P, and K (Sinsabaugh et al. 2009; Nannipieri et al. 2012).

PGPR benefit the plant growth directly by solubilization of insoluble phosphorous, nitrogen fixation, sequestering of iron by production of siderophores, producing metabolites such as auxins, cytokinins, gibberellins, lowering of ethylene concentration. The indirect growth promotion occurs via antibiotic production, synthesis

of antifungal metabolites and cell wall lysing enzymes, competing for sites of root colonization, induced systemic resistance (Ahmad and Khan, 2011). The beneficial effects of PGPR seen under greenhouse conditions are often not repeatable under field conditions and the results in terms of crop growth and yields are highly variable (Gyaneshwar et al. 2002; Viveros –Martinez et al., 2010). Understanding the influence of environmental factors is widely recognized as a key to improve the level and reliability of PGPR (Dutta et al., 2010). The dissemination of bacteria in the field has remained marginal, with the exception of rhizobia and agrobacteria. Inoculation of plants with P-solubilizing microorganisms in controlled experiments improved the growth and P nutrition, especially under glasshouse conditions and in fewer cases in the field (Whitelaw, 2000; Gyaneshwar et al., 2002; Jakobsen et al., 2005; Rodríguez and Fraga, 2006; Khan et al., 2007; Harvey et al., 2009; Khan et al., 2010). But, the effectiveness of PSMs in the laboratory or controlled conditions is not operable in soils (Richardson 2011). Plant growth promotion abilities of biofertilizers are strongly influenced by climate changes and are restrictive to certain cultivars, climate, and soil conditions (Figueiredo et al., 2010; Kern et al., 2011). The phosphate solubilization is accompanied by a number of activities such as production of plant stimulants, enzyme production, biocontrol activity and organic acid production (Vassilev et al. 2006; 2007a,b; 2008; 2009b).

Efficacy of PGPR in field conditions is determined mainly by their survival in harsh environmental conditions including high concentrations of environmental contaminants, salts, extremes of pH and temperature, and competition with other organisms. Isolation of stress tolerant PSM is gaining importance to enhance the efficacy of PSM (Thakuria et al. 2004; Chaiharn et al., 2008; Vassilev 2012). Inoculation of *Sinorhizobium cicero* and *Pseudomonas sp*. with 18/20 kg NP ha^{-1} as urea and di-ammonium phosphate increased nodule number, nodule dry weight, nodule volume and dry matter compared to uninoculated control at mid flowering stage in chickpea plant (Messele and Pant, 2012).

A large number of PGPR representing diverse genera have been isolated over past 50 years. Despite of their natural means of plant growth promotion ability, strains are genetically modified because of their inconsistent performance and requirement of uneconomically high dose application (Carmen, 2011). Additionally, several plant growth promoting traits are combined in a single organism for long-term cell survival under a variety of environmental conditions (Defez, 2006). Inoculation of *Medicago tranculata* plant with indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti* improved both the shoot and root fresh weigh, nitrogen fixation ability, P mobilization, oxidative damage and salt tolerance (Bianco and Defez, 2009; 2010a,b; Imperlini et al., 2009). Hence, genetic modification of native strains may help to survive, adapt and function better in the rhizosphere and improve plant nutrition.

Plant growth promoting rhizobacteria (PGPR) stimulated or inhibited nodule formation in a given symbiotic association, depending upon the type, nature, and concentration of secondary metabolites produced by the rhizobacteria. PGPR carrying ACC-deaminase enzyme intrinsically improved symbiotic efficiency of legumes by lowering the plant ethylene level that inhibited nodulation process (Nascimento et al. 2012).

Over-expression of genes involved in soil inorganic phosphate solubilization in natural PGPR improved the capacity of microorganisms when used as inoculants (Bashan et al., 2000). Studies carried out so far have shown that following appropriate regulations, genetically modified microorganisms can be applied safely in agriculture (Armarger, 2002; Morrissey et al., 2002). Chromosomal insertion of the genes is one of the tools to minimize the risks of using genetically modified microbes in agricultural filed.

MPS ability of the bacterial transformants in field conditions could be affected by various parameters. In addition to the nutrient availability and soil properties, oxygen limitation could be a significant factor. Oxygen is present in limited amounts in the rhizosphere which limits the colonization and survival of rhizobacteria (Ramírez et al.,

1999). The obligate aerobic bacterium, *Vitreoscilla*, synthesizes elevated quantities of homodimeric hemoglobin (VHb) under hypoxic growth conditions which improved growth under microaerobic conditions when dissolved oxygen is less than 2% of air saturation (Khosla and Bailey, 1988a; b). Expression of *vgb* gene encoding VHb protein in heterologous hosts often enhanced growth and metabolism by facilitating oxygen transfer to the respiratory membranes (Stark et al., 2011). Genetically modified *Rhizobium* with *vgb* overexpression has enhanced the ATP, ADP, NADH and NADPH generation, plasmid bearing *vgb* gene generated 0.624 nmoles of ATP, which significantly enhanced the nitrogenase activity up to ~170 µmol at low concentration of oxygen, while nitrogen content was increased up to ~56 mg/plant in bean plants (Ramirez et al., 1999).

In addition to the metabolic alterations in the central carbon pathway, overexpression of *vgb* gene in *Rhizobium* spp. along with artificial citrate operon improved the recombinant protein production and better survival of host under microaerobic conditions (Frey and Kallio, 2003). The MPS ability was lost when grown without an air in case of *Enterobacter intermedium* which secreted 2-ketogluconic acid (Hwangbo et al., 2003). A similar loss of MPS ability under low aeration conditions was seen in case of *Citrobacter* sp. DHRSS containing citrate operon with a concomitant loss of citric and gluconic acid secretion. Presence of *vgb* gene restored the citric and gluconic acid secretion along with MPS ability under microaerobic conditions (Yadav, 2013).

Plant growth promoting rhizobacteria (PGPR) are soil bacteria that benefited plant growth by different mechanisms (Glick, 1995; Archana et al., 2012), and P-solubilization ability of the microorganisms is one of the most important traits. The effect of genomic integration of *yc* operon containing *E. coli* NADH insensitive *cs* and *S. typhimurium citC, vgb* and *egfp* gene cluster in fluorescent pseudomonad strains showed enhancement of all plant growth parameters (leaf number, plant height and dry weight, nodule number, and dry weight) and remarkably at par with the SSP control **(Fig. 8.1; 8.2; 8.3)** (Adhikary et al., 2012).

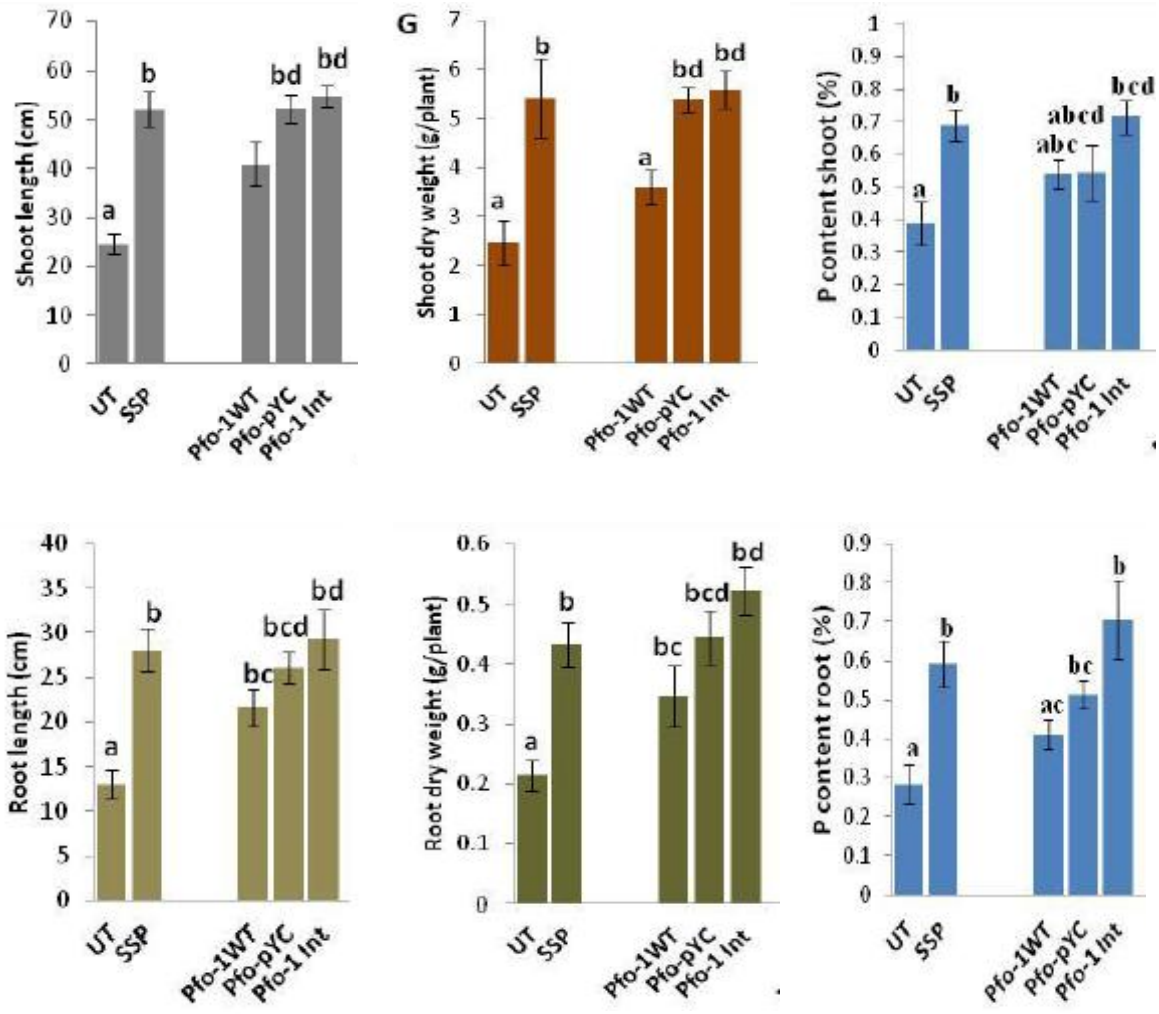


Fig. 8.1: Effect of *P. fluorescens* genomic integrants on shoot length, weight and P content and root length, weight and P content of mung bean (*Vigna radiata*) at 45 Days after sowing.

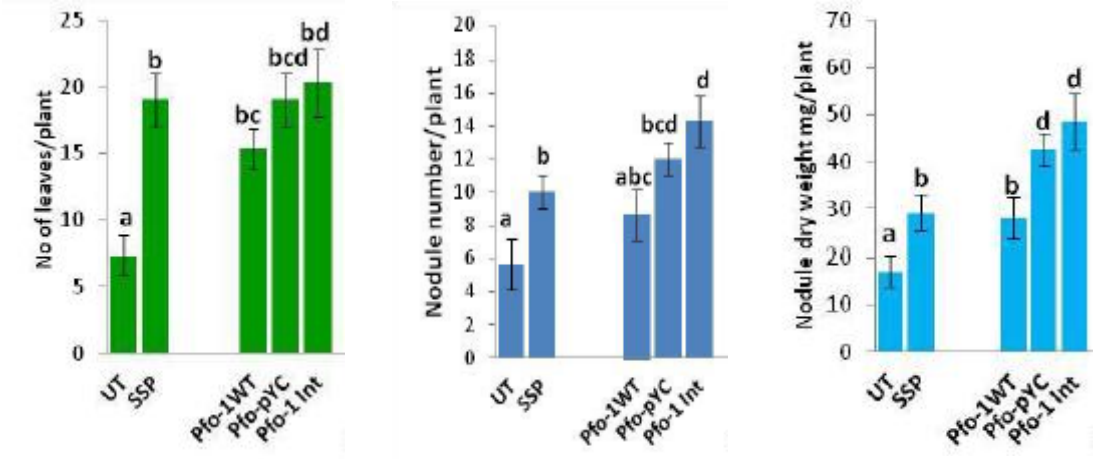


Fig. 8.2: Effect of *P. fluorescens* genomic integrants on number of leaves, nodule number and weight of mung bean (*Vigna radiata*) at 45 Days after sowing.

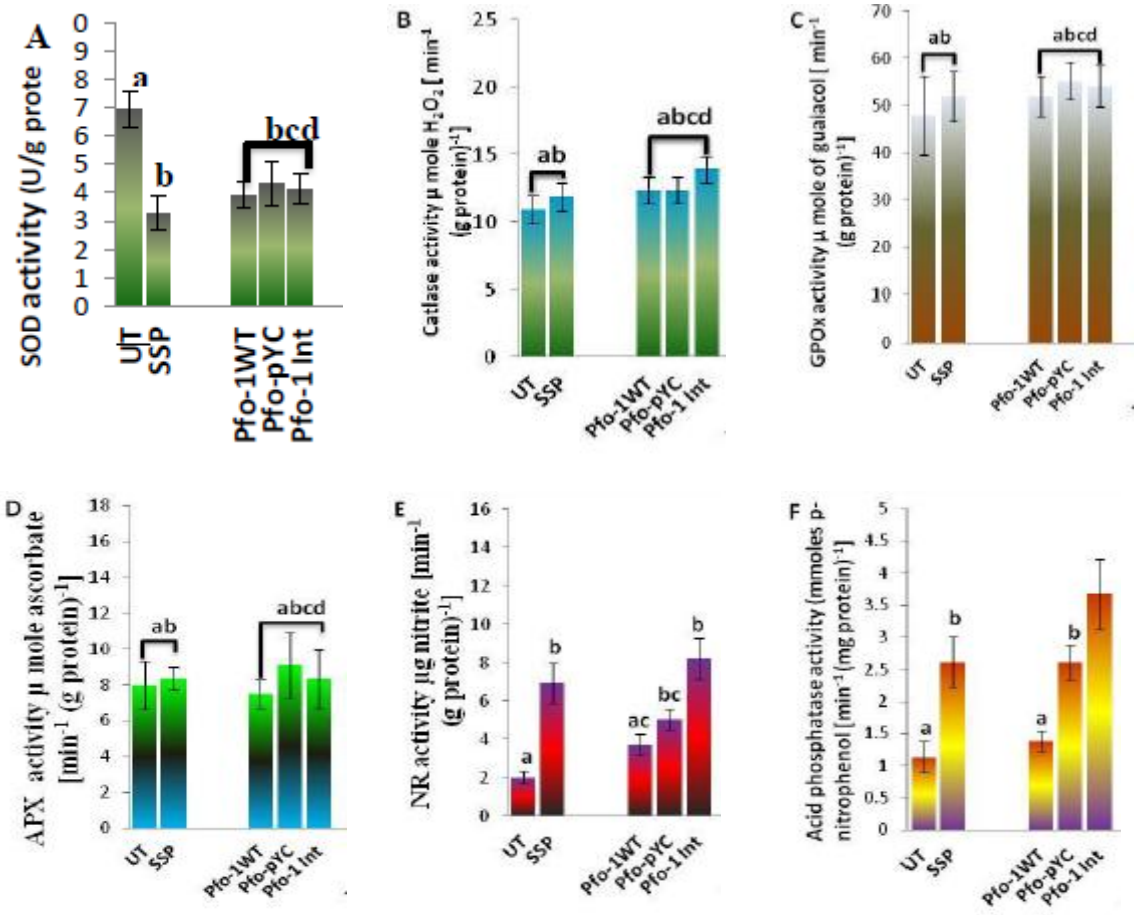


Fig. 8.3: Effect of *P. fluorescens* genomic integrants on enzyme activities of mung bean (*Vigna radiata*) at 45 Days after sowing.

8.1.2: Rationale of the Study

Metabolic engineering strategies developed for a particular organism may not necessarily work for other organism or even organism of the same species. Therefore, it is necessary to investigate the effects of genetic modification in multiple host organisms. In this study, *Sinorhizobium fredii* NGR 234 was subjected to similar genetic modification for citric acid secretion leading to P solubilization and plant growth promotion. *S. fredii* NGR234 having wide host range for nodulation and its integrant gave better results compared to the integrants of *Bradyrhizobium japonicum* USDA110 and *Mesorhizobium loti* MAFF030669. Thus, *S. fredii* NGR234 has been selected to monitor its efficacy in providing P and promoting the growth of mung bean (*Vigna radiata*) plants. The objectives of this study were to determinate the effects of *S. fredii* integrant on rhizospheric soil, plant height and weight, leaf number and area, chlorophyll content, number and size of nodules, number and size of pods and grain yield, and determine the nitrogenase and antioxidant enzyme activities of mung bean.

8.2 Experimental design

8.2.1: Bacterial strains used in this study

Table 8.1: Bacterial strains used in this study.

Plasmid/Strains	**Characteristics**	**Source or Reference**
S. fredii NGR 234	NC_012587.1	
Sf intYc	Genomic integrant of *S. fredii NGR 234* containing *lac-YF citC, vgb, egfp* Ap^r	This study Chapter 7

8.2.2 Plant Inoculation Experiments

Pure bacterial cultures were grown in nutrient broth at 30^oC, centrifuged, and diluted to a final concentration of 10^8 CFU/ml in sterile distilled water. Seeds of mung

bean obtained from local market and were washed repeatedly with autoclaved distilled water and soaked in distilled water for 10 minutes. Later seed directly soaked into respective cultures. For uniform treatment of the seed with culture, flasks were kept in an orbital shaker at 500 rpm for 2 h. Seeds were treated with *Rhizobium* strains each containing native strain (N), and *yc* operon genomic integrant (Int). In the experiment, one control was used, where no inoculum was added and designated as control (C).

8.2.3 Greenhouse experiment

Bacteria coated Mung bean seeds were sown in pots containing unsterile field soil and reared in a green house (25-30 °C). The pots were irrigated time to time to maintain the moisture level in green house. The growth parameters were recorded out at 20 days and 45 days after sowing and biochemical characterization was carried out at 45 days after emergence. Each treatment had 5 replications (6 seeds per replicate).

8.2.4 Growth parameter assessment

All the plant growth parameters were estimated at 20 days and 45 days after sowing (DAS).

8.2.5 Biochemical characterization

Superoxide dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX), Guaicol Peroxidase (POX) and nitrogenase enzyme activities were estimated at 45 days after sowing (DAS).

8.2.6 PQQ determination

PQQ production was estimated using the method of Rajpurohit et al. (2008). Fresh leaves and nodules were crushed using liquid nitrogen. Acetonitrile 50% was added

to powder and kept for digestion at 65°C for 2 h. The mixture was centrifuged at 15,000 *g* for 10 min; the clear supernatant was collected and dried with a concentrator under a vacuum. The residue was dissolved in 50% *n*-butanol at 1 mg/ml, and PQQ was extracted at 50°C overnight. The clear supernatant was dried under a vacuum and dissolved in 100% methanol. The identity of the PQQ was ascertained by comparing with standard PQQ on spectrofluorometer. Fluorescence was monitored at *ex* 360 and *em* 480 nm.

8.2.7 Isolation of bacteria from rhizospheric soil and nodules.

Bacteria isolated from rhizospheric soil and nodules from mung bean plants of 45 days and were isolated on agar plate with appropriate dilutions. Further, total bacterial count was mentioned in CFU.

8.2.8 Statistical analysis

The experiments were carried out in a completely randomized design (CRD) for mung bean. The experimental data was analyzed statistically using Prism 3.

8.3 RESULTS

8.3.1 Effect of genomic integrant on bacteria of rhizospheric soil and bacteroids of nodules.

Fluorescent colonies were not seen in the control and native plate but were seen in experimental plate under UV light in the samples isolated from rhizospheric soil. Mucoid colonies of *Rhizobium* were seen in the control and native plates and fluorescent colonies of *S. fredii* NGR 234 with the integrant were seen in the plate containing isolate from nodules of nodules from the inoculated mung bean plant (**Fig. 8.4**). There was increase by 24 fold and 150 fold in CFU count from the rhizospheric soil and bacteroids from nodules of mung bean plant, respectively, inoculated with the integrant (**Table 8.2**).

Weight of nodules increased by 3.3 fold of the integrant treated plant as compared to the control plant.

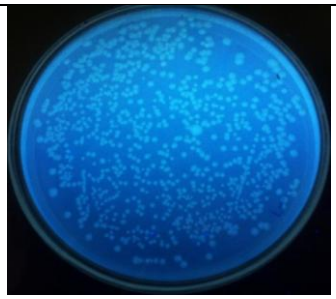
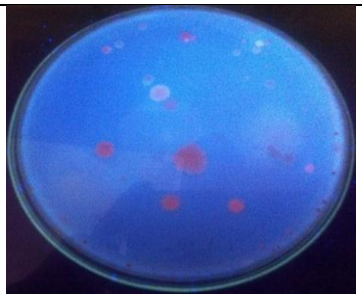
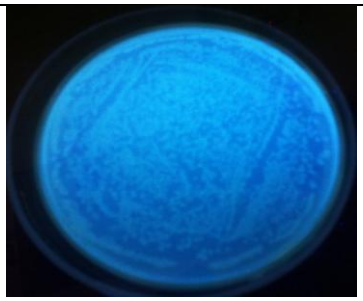
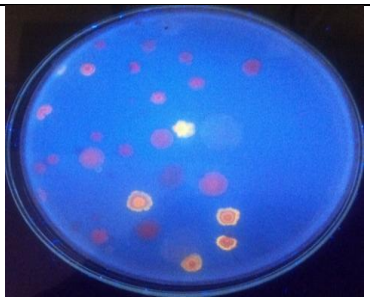
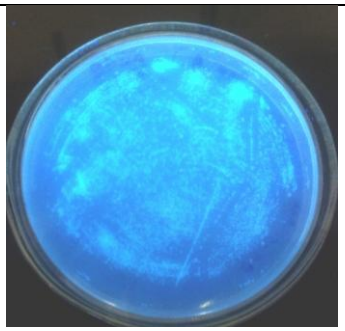
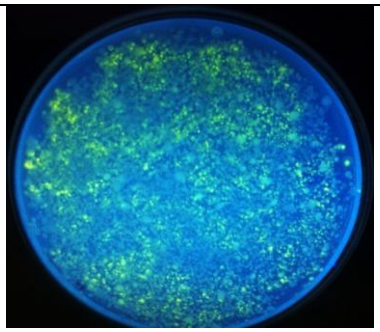
Bacteria isolated from nodules of mung bean plant	**Bacteria isolated from experimental soil**
	
Control	**Control**
	
Native	**Native**
	
Integrant	**Integrant**

Fig. 8.4: Effect of genomic integrant on bacteria of rhizospheric soil and bacteroids of nodules.

Table 8.2: Effect of genomic integrant on number of *S. fredii* NGR 234 integrants in the rhizospheric soil and in nodules from 45 days old mung bean plants.

	Total bacterial count. CFU /mg of soil	Total bacterial count. CFU / nodule	Wt. of 10 nodules (mg)
Control	6×10^3	1.1×10^4	13.9
Native	1.6×10^4	1.7×10^4	14.2
Integrant	3.8×10^5	2.6×10^6	47.3

8.3.2 Effect *S. fredii* NGR 234 genomic integrant on nitrogenase activity, available Soil P and N, K content.

S. fredii NGR 234 genomic integrant increased ~5.5 fold and ~2.8 fold increase in nitrogenase activity of integrant and native, respectively, as compared to control (**Fig. 8.5**).

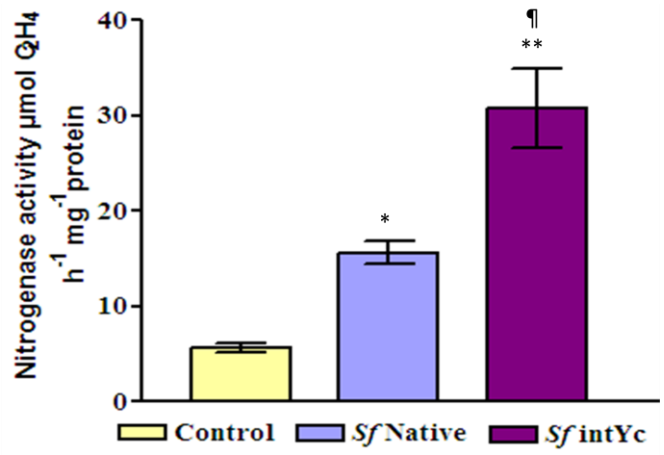


Fig. 8.5: Effect of *S. fredii NGR 234* genomic integrant on nitrogenase activity of mung bean at 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ** P<0.01 and *, ¶ P<0.05.

Inoculation with integrant (***Sf* intYc)** increased ~7.4 fold soluble P as compared to the uninoculated control and ~2.4 fold increase compared to native **(Table 8.3)**. The native strain resulted in ~3.1 fold increase in soil P while N and K levels remained unaltered compared to control. Also there was ~1.25 fold and ~2.32 fold increase in N and K content of the soil from plant inoculated with integrant compared to control**.**

Table 8.3: Effect of genomic integrant on the N, P and K content of rhizospheric soil from mung bean plants of 45 days old.

Soil	**N Kg/hac**	**P_2O_5 kg/hac**	**K_2O kg/hac**
Control	158.7	12.9	338.7
Native	161.6	40.0	364.9
***Sf* intYc**	198.9	95.7	788.9

8.3.3 Effect of genomic integrant on N P K content in plant and pods

Significant increase in N, P, K and protein content was observed in plants inoculated with the native (*S. fredii* NGR 234) and the integrant (***Sf* intYc).** N, K and protein showed ~1.5 fold increase in plants inoculated with the integrant compared to control and ~ 1.2 fold increase compared to native while P content increase was ~2.7 fold and ~1.64 fold, respectively, compared to control. Integrant also showed~1.64 fold increase in P and ~1.2 fold increase in N, K and protein content compared to native (**Table 8.4**).

Significant increase in N, P, K and protein content was observed in pods from plants inoculated with the integrant (***Sf* intYc).** All parameters showed ~1.3 and ~1.1 fold increase in pods from plants inoculated with the integrant compared to control and native, respectively. Integrant also showed ~1.2 fold increase in N, P, K and Protein content compared to native strain (**Table 8.5**).

Table 8.4: Effect of genomic integrant on total plant N, P, K and protein content of 45 days old mung bean plants.

Bacterial inoculation	**N %**	**P %**	**K %**	**Protein %**
-	1.39	0.34	1.02	8.71
Native *Sf*	1.65	0.57	1.25	10.30
***Sf* intYc**	2.07	0.93	1.57	12.96

Table 8.5: Effect of genomic integrant on N, P, K and protein content of pods from 45 days old mung bean plants.

Pods from plants with bacterial inoculation	**N %**	**P %**	**K %**	**Protein %**
-	2.61	1.40	1.05	16.29
Native *Sf*	3.05	1.51	1.24	19.08
***Sf* intYc**	3.41	1.74	1.39	21.29

8.3.4 Growth parameters

8.3.4.1. Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean plant.

S. fredii NGR 234 genomic integrant containing *E. coli* NADH *i*nsensitive *cs* and *S. typhimurium citC, vgb and egfp* gene cluster upon inoculation to mung bean resulted in significant increase in all growth parameters. In 20 day plants, ~1.3 fold increase in shoot length and ~2.8 fold increase in root length, ~2.4 fold increase in plant weight, ~2.9 and ~1.4 fold increase in leaf number and leaf area were found in comparison to control. Increase was also seen in all the above parameters in plants inoculated with genomic integrant inoculated with native *S. fredii* NGR 234 compared to control plants **(Fig. 8.5).**

In 45 day plants, ~1.45 fold increase in shoot length and ~1.35 fold increase in root length, ~2.5 fold increase in plant weight, ~2.3 and ~1.3 fold increase in leaf number and leaf area was seen (**Fig. 8.6**). In addition, there was ~1.7 fold and ~1.3 fold increase in chlorophyll content in the leaves of plants inoculated with integrant and native, respectively, compared to control **(Fig. 8.7).**

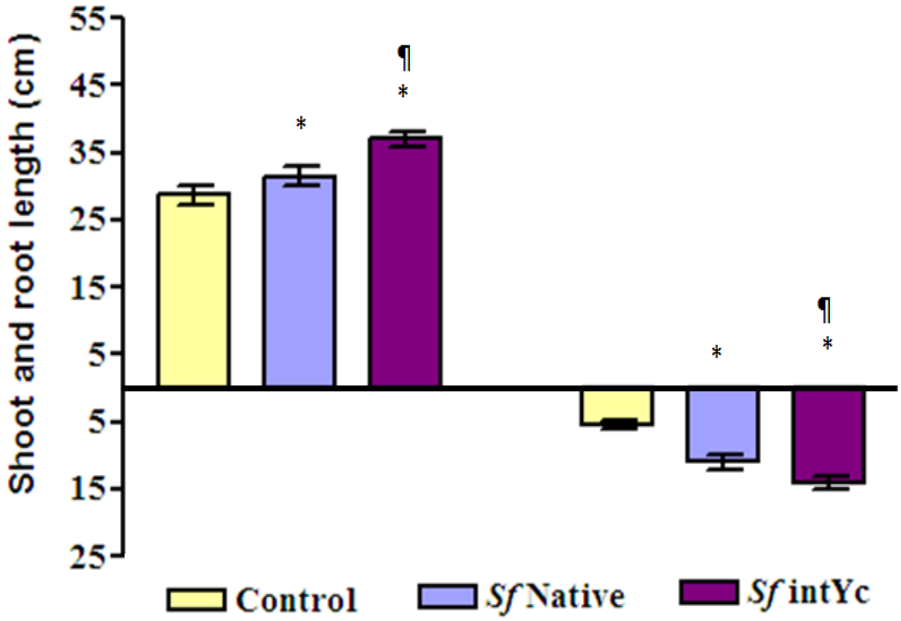


Fig. 8.5: Effect of *S. fredii* NGR 234 genomic integrant on shoot length and root length of mung bean at 20 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. *,¶ $P<0.05$.

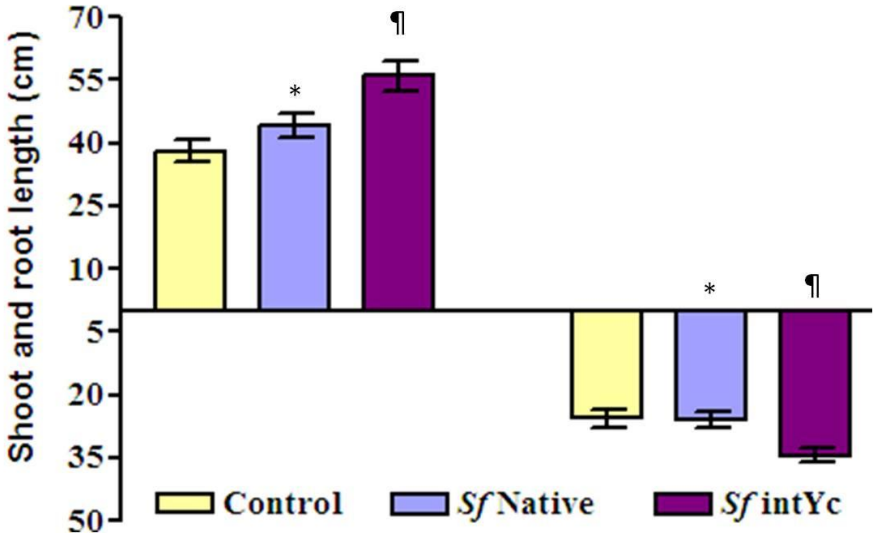


Fig. 8.6: Effect of *S. fredii* NGR 234 genomic integrant on shoot length and root length of mung bean 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. *,¶ P<0.05.

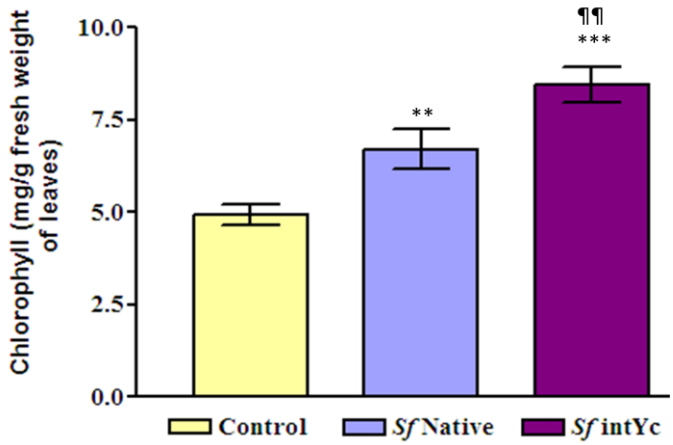


Fig. 8.7: Effect of *S. fredii NGR 234* genomic integrant on chlorophyll content of mung bean at 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ***, P<0.001, **, ¶ ¶ P<0.01.

There was ~2.4 fold, ~1.45 fold and ~1.6 fold increase in number, length and number of grains per pod, respectively, from plants treated with the integrant as compared to control. Fresh weight and dry weight of pods also showed ~2.2 and ~3.1 fold increase, respectively in integrant, compared to control.

Sf intYc genomic integrant in 20 days old plants showed, ~3.1 increase in number of nodules and ~4.3 fold increase in weight of 10 nodules compared to the control. Native Plants treated with native culture showed nodules having double weight compared to untreated plants and nodule number was increased by ~1.35 fold (**Table 8.6**). 45 days old plants treated with integrant showed less increase compared to increase seen in 20 days old plants (**Table 8.7**). ~2.8 increase in number of nodules and ~3.4 fold increase in weight of 10 nodules were found as compared to the control. Nodules from 45 days old integrant plants were ~3.3 times heavier than those of native plant.

Table 8.6: Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean at 20 Days after sowing

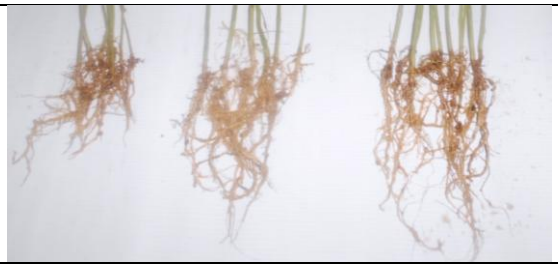
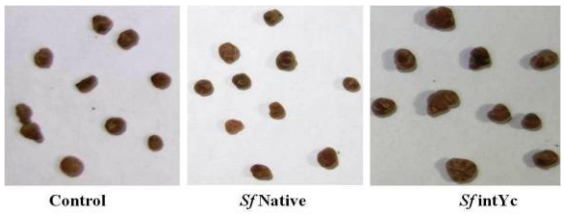
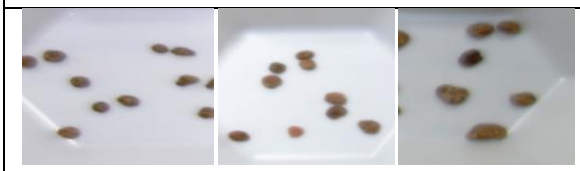
Details of 20 Days old Plants	Parameter	Control	Native	Integrant
	**Plant fresh weight in g**	2.20 ± 0.26	3.72 ± 0.10	5.33 ± 0.50
	No of leaves	6.33 ± 1.52	10.00 ±1.73	18.33 ±1.52
	Leaf area cm^2	17.33 ±2.08	21.66 ± 2.08	24.66 ± 2.51
	**No of nodules /plant**	23.33 ±3.51	31.66 ± 3.08	72.66 ± 2.51
	**Weight of 10 nodules in mg**	21.66 ± 2.08	46.00 ± 3.0	93.00 ± 4.58

Table 8.7: Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean at 45 Days after sowing

Details of 45 Days old Plants	Parameter	Control	Native	Integrant
	**Fresh weight in g**	29.08 ± 5.22	44.93 ± 1.67	72.07 ± 13.33
	Dry weight in g	0.25 ± 0.13	0.33 ± 0.04	1.35 ± 0.06
	**No of leaves**	18.00 ± 3.00	23.00 ± 4.35	42.00 ± 5.29
	Leaf area in cm^2	26.33 ± 2.08	27.00 ± 1.00	34.66 ± 1.52

Contd…

Table 8.7: Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean at 45 Days after sowing (contd.)

Details of 45 Days old Plants	Parameter	Control	Native	Integrant
	**No of pods/ plant**	10.33 ± 1.52	15.00 ± 3.00	24.66 ± 1.52
	Pod length in cm	6.26 ± 0.25	6.60 ± 0.26	9.13 ± 0.15
	**No of grains per/pod**	8.66 ± 1.5	10.00 ± 1.00	13.66 ± 1.52
	Pod fresh weight	1.176 ± 0.10	1.33 ± 0.15	2.58 ± 0.50
	Pods dry weight/mg	0.14 ± 0.04	0.25 ± 0.04	0.43 ± 0.11
	**No of nodules / plant**	12.33 ± 2.51	13.66 ± 2.05	34.33 ± 2.51
	**Weight of 10 nodules**	13.86 ± 1.95	14.20 ± 1.11	47.33 ± 3.05

The genomic integrant treatment had also decreased oxidative stress of mung bean plants as found in the specific activities of antioxidant enzymes (**Fig. 8.8**),

Superoxide Dismutase (SOD), **Catalase**, **Guaiacol Peroxidase** (POX) and **Ascorbate peroxidase** (APX), at 45 days after sowing. There was ~2.2 fold, ~1.4 fold, ~2 fold and ~ 1.5 fold decrease in POX, SOD, CAT and APX, respectively, in the plants inoculated with integrant compared to control. Compared to native, the integrants showed ~1.2 to ~1.4 fold decrease in the enzyme activities. PQQ levels increased by ~1.5 fold in the nodules and leaves of native as well as genome integrant inoculants as compared to control plant while (**Table 8.8**).

Table 8.8: PQQ levels in leaves and nodules of mung bean plants

Bacterial inoculation	**PQQ ng/g fresh wt of nodule**	**PQQ ng/g fresh wt of leaves**
-	**3.26± 0.40**	**1.73± 0.25**
***S. fredii* NGR 234**	**5.23± 0.51**	**2.43± 0.32**
***Sf* intYc**	**6.10± 0.20**	**2.73± 0.32**

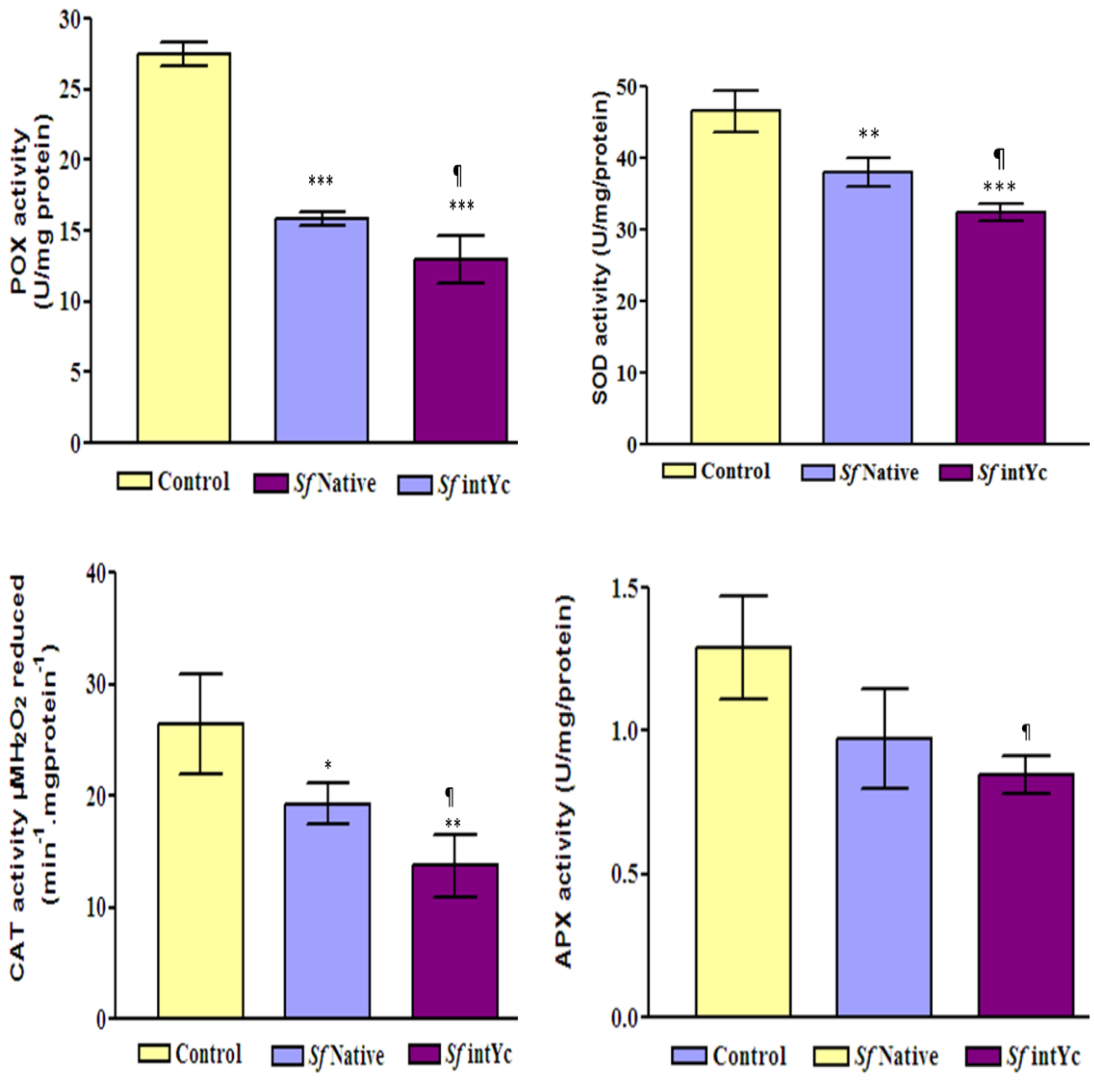


Fig. 8.8: Effect of *S. fredii NGR 234* genomic integrant on enzyme activities of mung bean at 45 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ***, P<0.001, ** P<0.01 and *,¶ P<0.05 .

8.4 Discussion

Availability of phosphate in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes. Productivity of legumes is severely affected by P limitation, as both the plants and their symbiotic bacteria require P for nodule formation. Development of integrant of *S. fredii* NGR234 from TRP medium released 0.56 mM P as compared to 0.05 mM P of native strain. *S. fredii* NGR 234 integrant was efficient in increasing the N and P levels in soil.

Fluorescent colonies of *S. fredii* NGR234 containing integrant were present in rhizospheric soil and nodules isolated from plant (**Fig. 8.4).** Increase in cfu count of integrant was accompanied by increase in P, N and K content, respectively, as compared to native strain. Similar results of increase in soil P was found with co-inoculation of *Rhizobium* strain and PSM in chick pea and soybean (Argaw, 2011; Singh and Sharma, 2011). P levels play important role in nodule formation and nitrogen fixation as nitrogen fixation which is a high energy consuming process (Sulieman and Tran, 2012). Increase in free P content in soil helped plant growth and increased the number and weight of nodules compared to native strain.

Cfu count of *S. fredii* NGR 234 integrant in nodules also increased with concomitant increase in nitrogenase activity. Improvement in nodulation due to inoculation of P solubilizers was seen in chick pea and other leguminous plant (Tang et al., 2001). Enhanced nodulation after inoculation of the rhizobium strain increased available P for the plant as leguminous plant require high amount of P for nodule formation and maintenance of high rate of bacterial activity inside the nodule (Leidi et al., 2000; Zaman et al., 2007; Singh et al., 2011).

Increased nitrogenase activity led to increase in N, P, K and Protein content of whole plant in the integrant compared to control. Similar results were found with co

inoculation of legumes and crops with *Rhizobium* and different PSMs (Yazdani et al., 2011; Sharma et al., 2012; Tahir and Sarwar, 2013). Additionally, increase in NPK content of plant enhanced chlorophyll content and all growth parameters like fresh weight, dry weight, length of root, shoot and pod growth and yield parameters up to 100% compared to native. Simliar studies with barley and chick pea when grown in soil treated with insoluble phosphate and PSM *Mesorhizobium mediterraneum* PECA21 showed 100 and 125%, respectively, increase in the P content as compared to control (Peix et al., 2001).

P deficiency is one of the critical limiting factors, adversely affecting nodulation and N_2 fixation, and thus legume growth and productivity, worldwide (Tesfaye et al., 2007). *M. truncatula* plants inoculated with either the *S. meliloti* 102F51 or 2011 strain but due to P deficiency severely inhibited plant growth and development of nodules as well as N and P assimilation (Sulieman and Schulze, 2010*a*). Similar results were seen with co-inoculation studies with *Rhizobium*, PSMs, supplementation with fertilizers on *Zea mays*, cow pea, walnut, mung bean and chick pea (Gulati et al., 2010; Deepa et al., 2010; Yu et al., 2011; Jha et al., 2012; Verma et al., 2013).

S. fredii NGR 234 encodes *pqq* genes and incorporation of *yc* operon in the genome increased PQQ secretion probably due increased available P. PQQ is a strong antioxidant compared to ascorbate and other antioxidants. Addition of PQQ showed increased growth and scavenging of ROS and hydrogen peroxide (Choi et al., 2008; Ahmed and Shahab, 2010; Misra et al., 2012). Significant decrease in SOD, POX, CAT and APX activities after inoculation with the integrant of *S. fredii* NGR 234 indicates decreased oxidative stress in mung bean plants. Inoculation of PGPR reported to reduce oxidative stress in plants. Abiotic stress conditions cause an increase in ROS formation such as superoxide radical (O_2^-), hydrogen peroxide, and hydroxyl radicals (OH) at the cellular level (Sgherri et al., 2000; Hemavathi et al., 2010). Al toxicity and P deficiency both increased SOD and POD activities in maize and rice plants (Tewari et al, 2004; Sharma and Dubey, 2007). Induction of antioxidant enzymes (catalase, SOD, APX, GR

and POX) is involved in the alleviation of salinity stress in lettuce plants inoculated with PGPR strains (Bianco and Defez, 2009; Kohler et al., 2010). In contrast, PGPR inoculated plants showed significantly lower activity of antioxidant enzymes as compared to uninoculated plants (Omar et al., 2009; Sandhya et al., 2010). Significant increase of catalase and peroxidase activities is found in salt-stressed leaves of two barley cultivars differing in salinity tolerance after inoculation with *Azospirillum brasilense* (Omar et al., 2009). In contrast, the mRNA expression of SOD, CAT, DHAR, GR and APX in bacteria-inoculated considerably increased in plants grown under stress conditions when compared with that of uninoculated stressed plants (Gururani et al 2012). Mung bean plant showed significant increase in all parameters related to growth and yield, decreased oxidative stress with *S. fredii* integrant compared to native. This clearly shows that *S. fredii* integrant is a strong nitrogen and phosphate solubilizer showing enhanced plant growth promoting ability. *In vitro* study of *vgb* on P solubilization, effect of inoculation of plasmid transformants on plant growth promotion and efficacy of integrant with respect to mung bean plant supplemented with super phosphate and urea will determine the potential of the genome integrants applicability in field conditions.

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)

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