Chapter 6

Effect of overexpression of *E. coli* NADH insensitive Y145F *cs* and Na^+ dependant citrate transporter in *B. japonicum* USDA110 *and M. loti* MAFF030669

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).

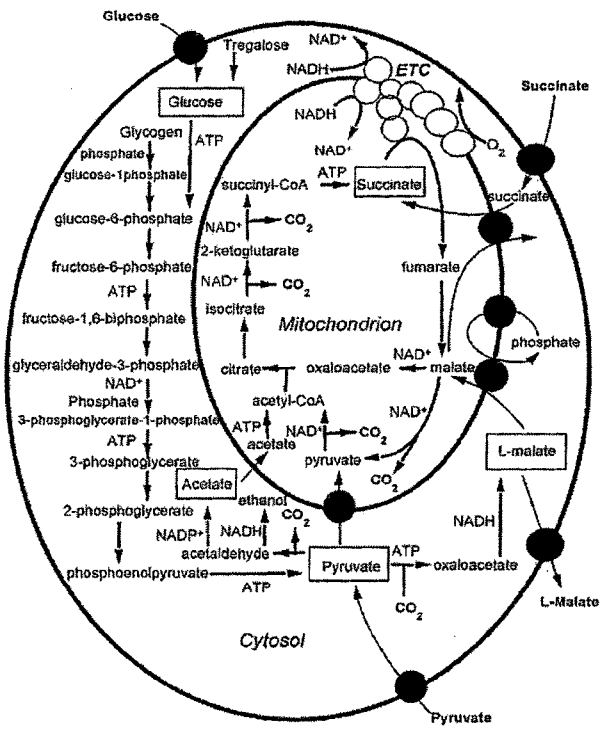


Fig. 6.1: Basic metabolism and the electron transport chain (ETC) in *S. cerevisiae* cells (Aliverdieva et al., 2006)

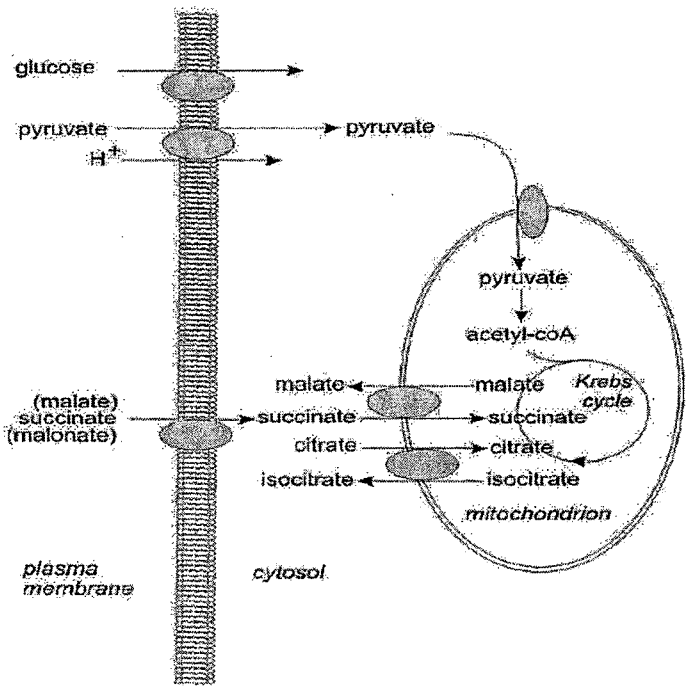


Fig. 6.2 Oxidation of substrates of plasma membrane dicarboxylate transporter in *S. cerevisiae* (Aliverdieva et al., 2008)

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.

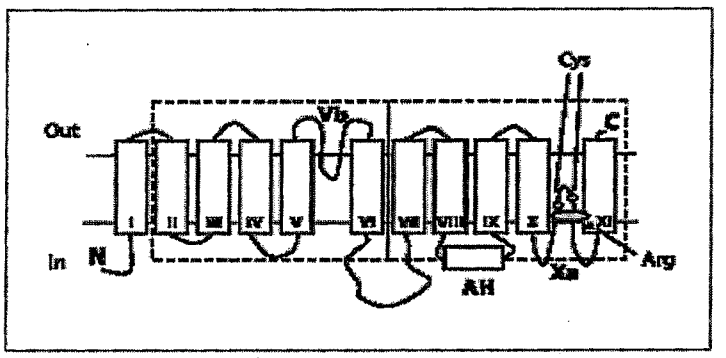


Fig. 6.3: Structural model for 2HCT family transporters (Lolkema, 2006).

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).**

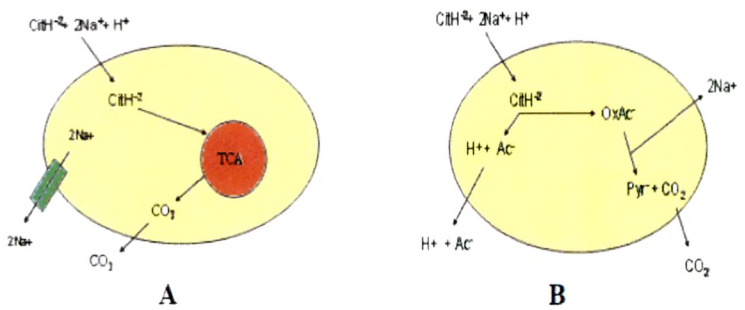


Fig. 6.4: Na^+ efflux mechanisms in bacteria. (A). Aerobic and (B). Anaerobic conditions (Lolkema, 1994).

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.

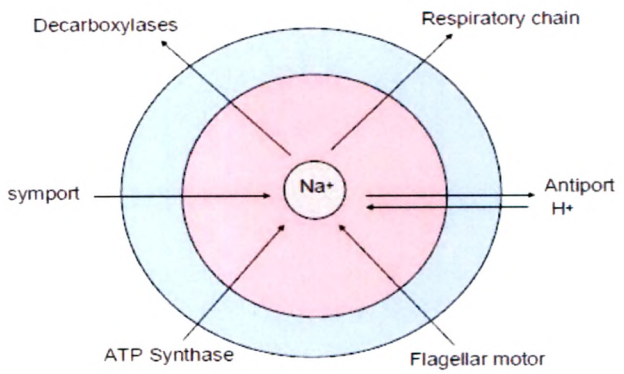


Fig. 6.5: Bacterial stress responses and Na^+ homeostasis (Storz et al., 1996).

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).

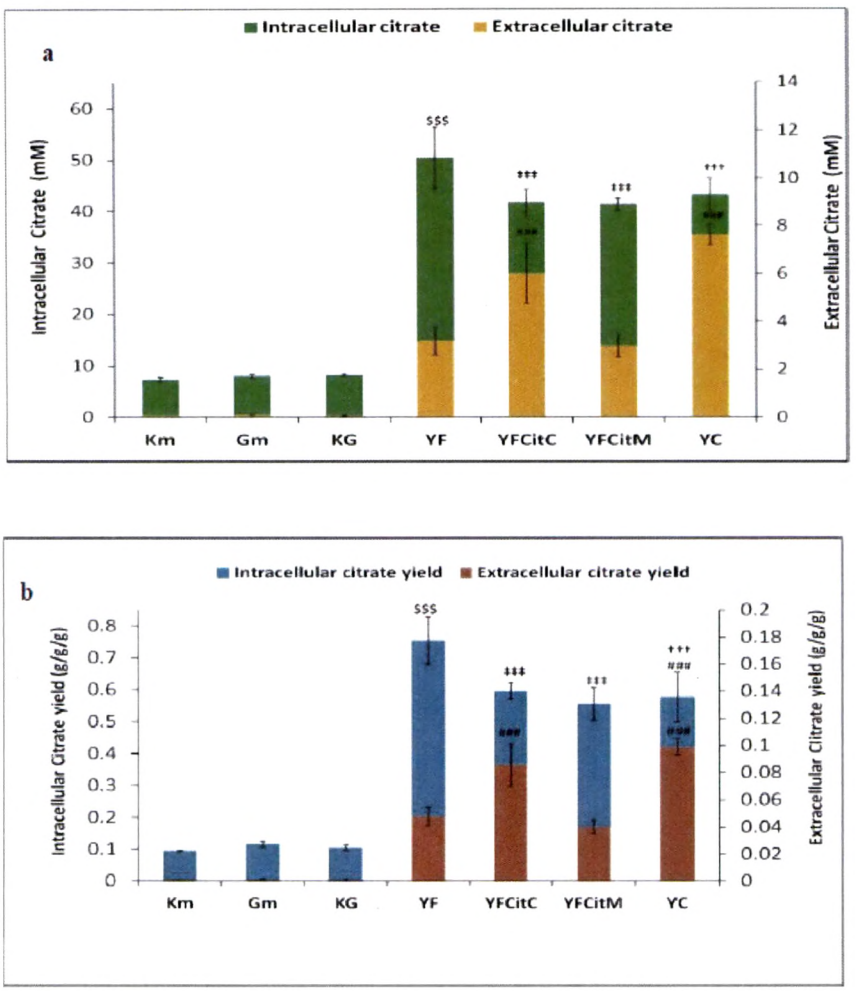


Fig. 6.6: Citric acid levels and yields in *P. fluorescens* PfO-1 overexpressing citrate transporter. Intracellular and extracellular citrate levels (a) are represented in **green** and **orange** bars respectively. Intracellular and extracellular citrate yields are represented in **blue** and **magenta** 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**).

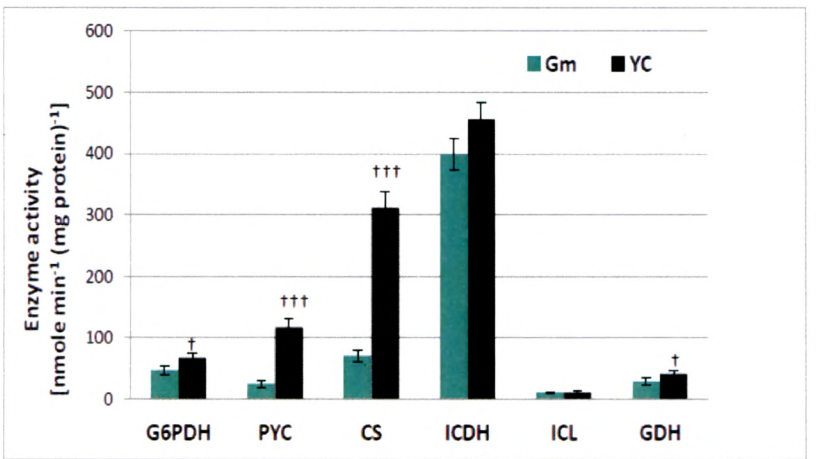


Fig. 6.7: Activities of enzymes G-6-PDH, ICDH, ICL, PYC, and GDH in *P. fluorescens* PfO-1 overexpressing citrate transporter.

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).

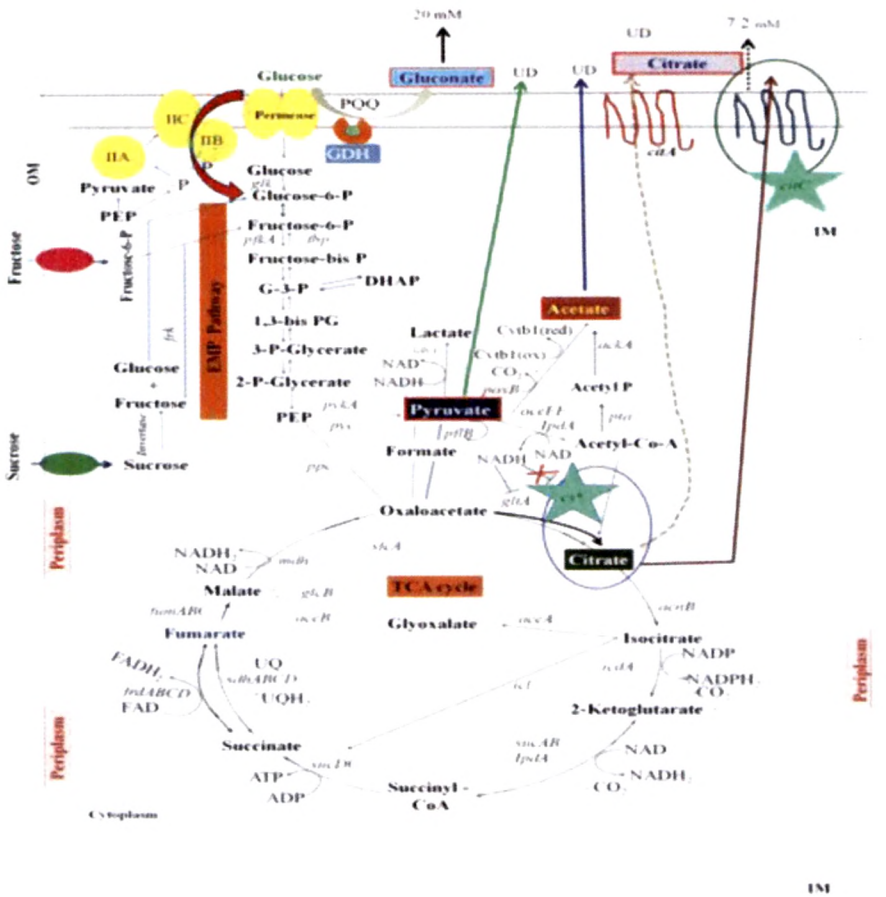


Fig. 6.8: Schematic representation of citric acid secretion in *Citrobacter* sp. DHRSS containing artificial citrate operon (Yadav,2013).

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).**

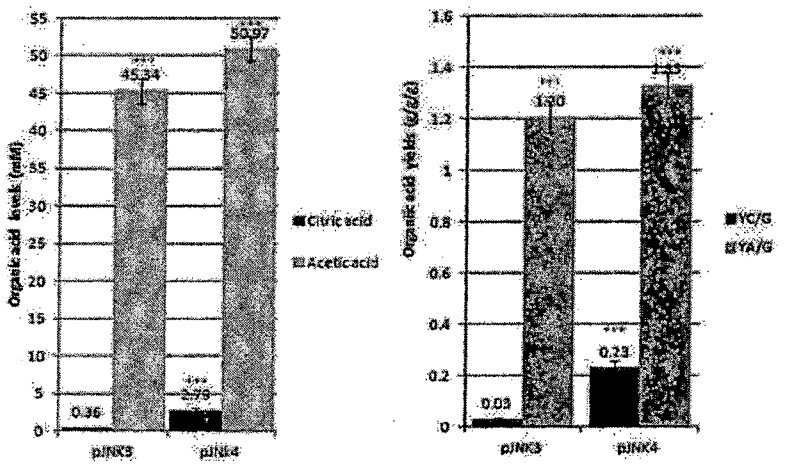


Fig. 6.9: Organic acid production from *H. seropedicae* Z67 transformants (Wagh, 2013).

6.1.3 Rationale of the present work

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.

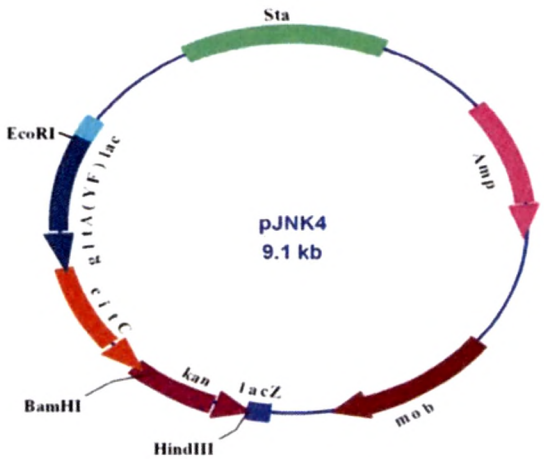


Fig. 6.10: Restriction map of the plasmid used in this chapter (Wagh et al., 2013).

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.*

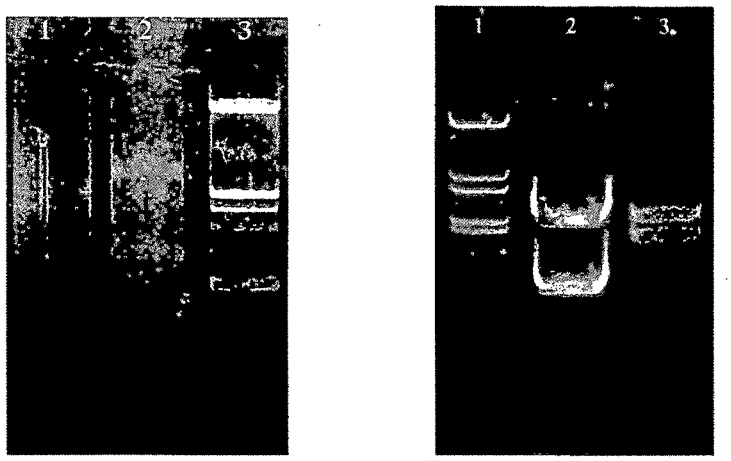


Fig. 6.11: Restriction digestion analysis of (A) *B. japonicum* USDA110 containing pJNK4 plasmid. Lane 1: pJNK4 digested with EcoRI-HindIII (5,298bp, 3,967bp); Lane 2: pJNK4 digested with XbaI (6.6 kbp, 2.6 kbp); Lane 3: Molecular Weight Marker (MWM)-Lambda DNA cut with EcoRV/ Hind III. **(B) *M. loti*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

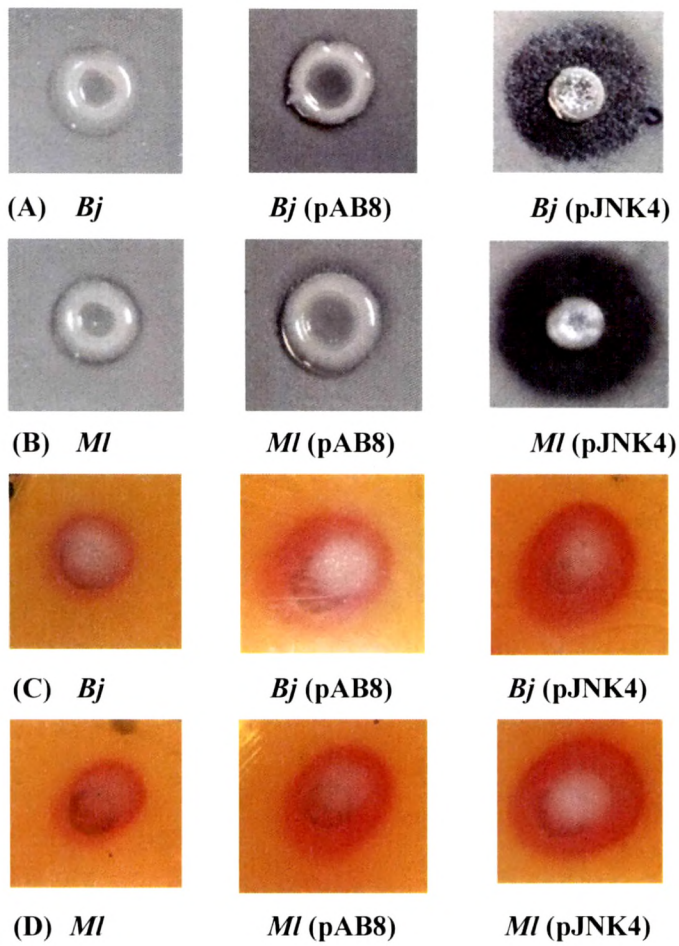


Fig. 6.12: MPS phenotype of *B. japonicum* USDA110 and *M. loti* MAFF030669 strains harboring pJNK4 plasmid. (A) and (B) on Pikovskaya's agar and **(C) and (D)** Tris rock phosphate agar containing 50 mM glucose and 100 mM Tris HCl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 °C. 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 °C 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).**

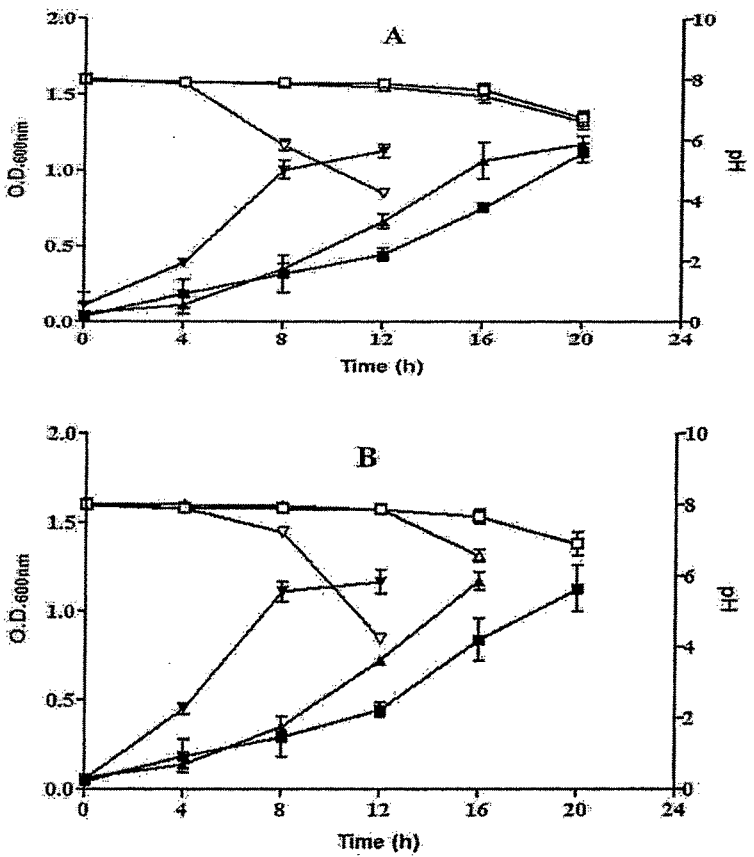


Fig. 6.13 : Effect of *E. coli* NADH insensitive *cs* gene and citrate transporter *citC* gene of *S. typhimorium* gene overexpression on extracellular pH (□, Δ, ▽,) and growth profile (■, ▲, ▼) of (A) *B. japonicum* USDA110 and (B) *M. loti*, on TRP medium with 50 mM glucose .(□, ■, *Bj, Ml* wild type); {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**).

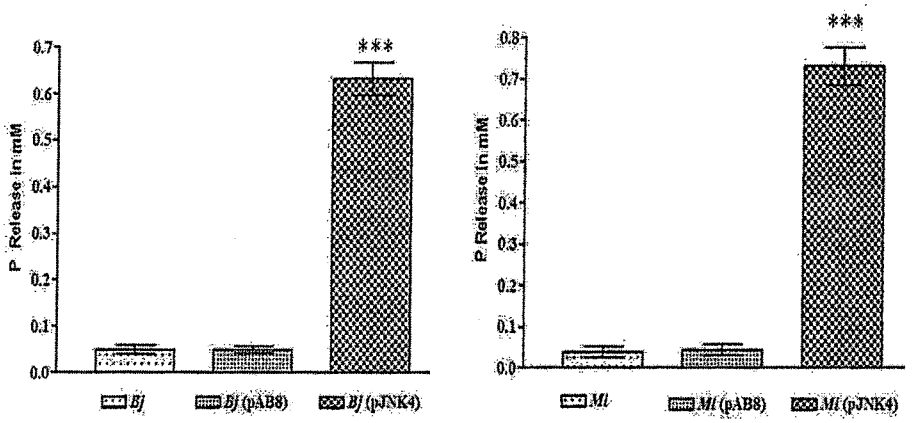


Fig. 6.14: P release by (A) *B. japonicum* USDA110 and (B) *M. loti* MAFF030669 transformants on TRP medium. The values are depicted as Mean ± S.E.M of 7-10 independent observations. *** P<0.001.

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).**

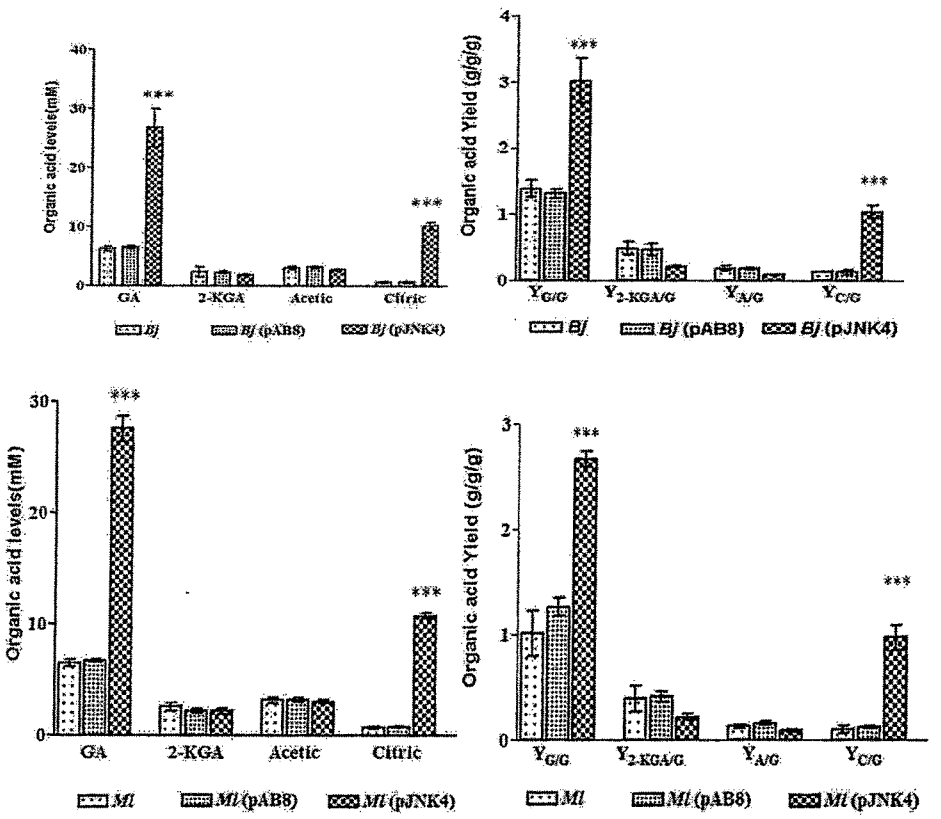


Fig. 6.15: Organic acid production {(a) and (c)} and Yield {(b) and (d)} from *B. japonicum* USDA110 *and M. loti* MAFF030669 *cs* gene transformants, respectively. All organic acids are estimated from stationary phase cultures (at the time of pH drop) grown on TRP medium with 50mM glucose. Results are 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**).

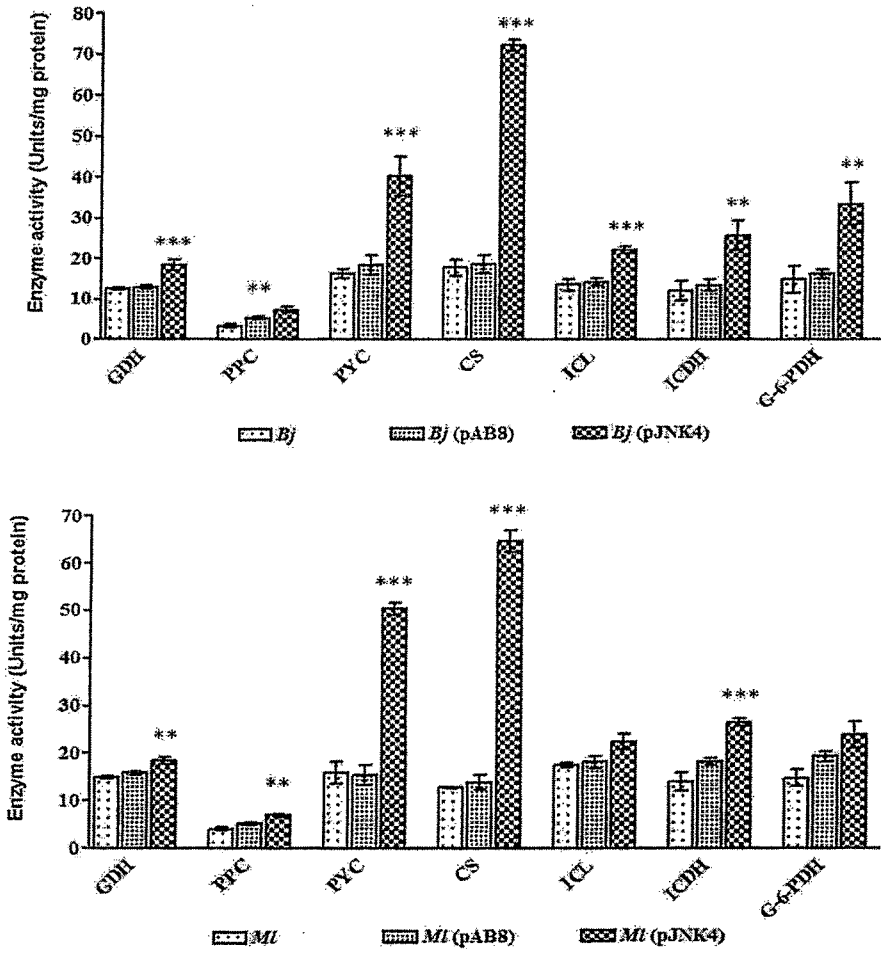


Fig. 6.16: Activities of enzymes PPC, PYC, GDH, G-6-PDH, ICDH and ICL in *B. japonicum* USDA110 and *M. loti* MAFF030669 *ppc* transformant. The activities have been estimated using cultures grown on TRP medium with 50mM glucose. All the enzyme activities were estimated from mid log phase to late log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

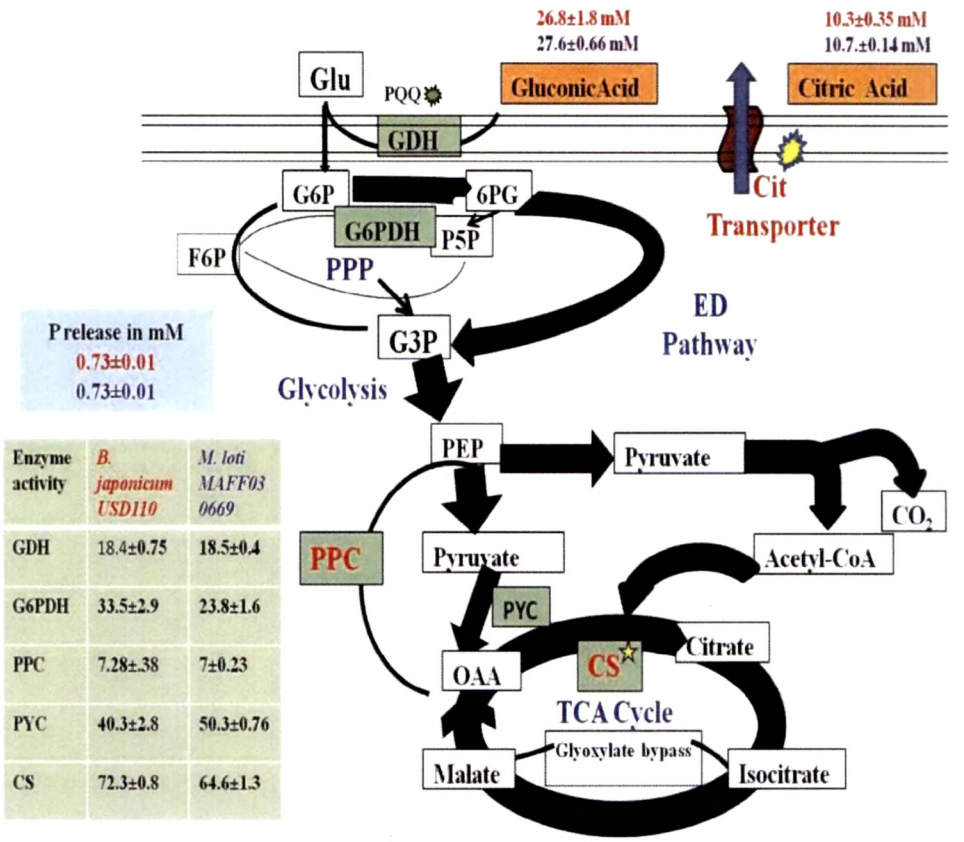
6.4: DISCUSSION

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 MAFF030669*
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.