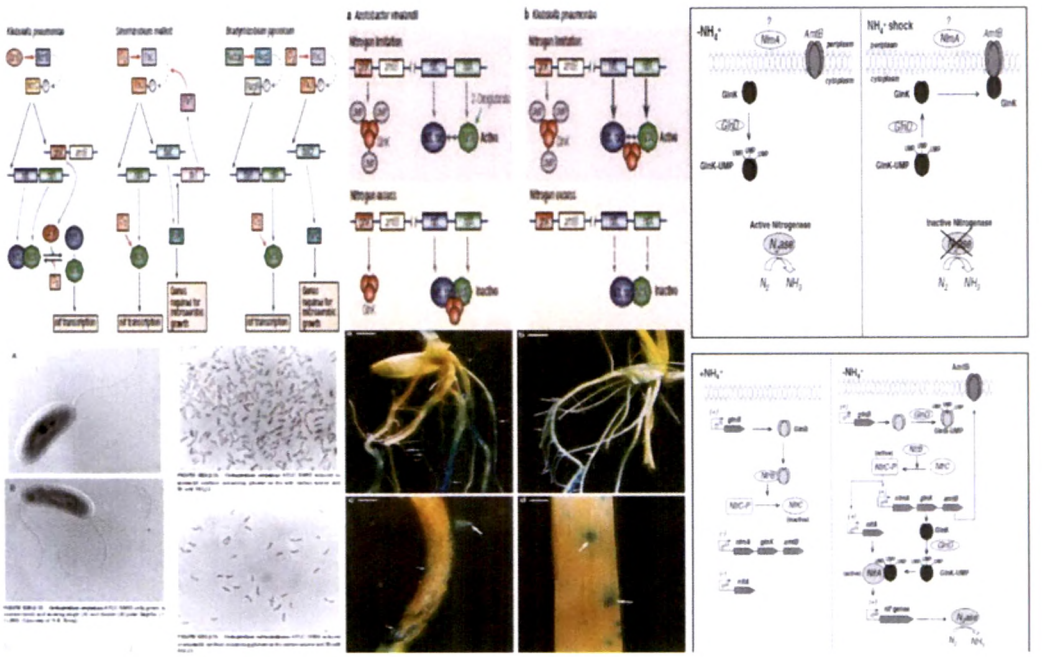
Chapter 3



Monitoring the MPS ability of *H. seropidecae* Z67 containing *E. coli* NADH insensitive citrate synthase (Y145F) - *Salmonella typhimurium* Na^+ dependent citrate transporter (*citC*) operon.

3.1: 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).

Rhizobacteria mainly secret organic acids such as citric, oxalic, gluconic, 2-ketogluconic, lactic, malic, succinic, tartaric and acetic for mineral phosphate solubilizing (MPS) ability (Archana et al., 2012). Solubilization of mineral P from soils is depends on the nature and amount of the acid which varies from 10 to 100 mM from strong to weak organic acid (Gyaneshwar et al., 1998). *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium, Rahnella, Enterobacter, Citrobacter, Azospirillum* and *Erwinia* are known to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate (TCP), dicalcium phosphate (DCP), hydroxyapatite (HAP) and rock phosphate (RP) (Khan et al., 2006). Among all organic acids, citric acid is one of the most efficient organic acids in mineral P solubilization due to its good chelating and strong acid properties (Kpomblekou et al., 1994; Liu et al., 2012).

Rice (*Oryza sativa*) is the staple food for one third of world population. Nitrogen and Phosphate are key nutrients which limit agricultural production and supplementation of P and N chemical fertilizers significantly enhanced plant growth and development (Gyaneshwar et al., 2002; Tilman et al. 2002; Ladha et al. 2003). Alternative to application of chemical fertilizers is the use of nitrogen fixing and plant growth promoting rhizobacteria (PGPR). 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).

Herbaspirillum seropedicae is a diazotrophic endophyte which colonises the external and internal tissues of economically important plants gramineous (*Poaceous*) plants, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*) rice, as well as some non-*Poaceaous* plants (Baldani et al. 1986;

Olivares et al. 1996; Weber et al. 1999). Under field conditions, *H seropedicae* inoculation stimulates root and shoot growth and rice seed germination (Pereira et al. 1988). However, these effects are highly variable which depend on the rice variety and environmental conditions (Malarvizhi and Ladha1999; Gyaneshwar et al. 2002; James et al. 2002).

Citric acid production in *A. niger* was increased by 45% through increasing the influx of glucose (Guebel and Torres, 2001). Overexpression of phospho fructokinase (*pfk*), pyruvate kinase (*pk*) and citrate synthase (*cs*) genes in *A. niger* improved citrate production (Ruijter et al. 1997; 2000). Isocitrate lyase (*icl*) gene over expression 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 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 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). Since these mutants show glutamate auxotrophy, they are not suitable for field conditions.

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* over expressing *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, phosphoenol pyruvate carboxylase (*ppc*) gene of *Synechococcus elongatus* was over-expressed in fluorescent pseudomonads. Overexpression of *ppc* enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increased gluconic, pyruvic and acetic acids but citric acid was not detected (Buch et al., 2010). When *ppc* and *gltA* (gene for citrate synthase) were over expressed, the biomass yield was increased but citric acid secretion was not observed (Buch et al., 2009).

E. coli has Type II CS which is a hexamer of identical subunits, is strongly and specifically inhibited by NADH (Weitzman, 1996). This allosteric regulation of CS may result in tight homeostasis of citrate levels *in vivo* and this might explain why *cs* overexpression did not result in high accumulation of citrate (Buch et al., 2009). Gram positive bacteria, on the other hand, possess Type I CS which is insensitive to NADH inhibition, but it is inhibited by ATP (Stokell et al., 2003). Several specific point mutations in the NADH binding pocket of *E. coli* CS are reported extremely weak inhibition by NADH (Stokell et al., 2003). Citrate transport in *Enterobacteriaceae* family is mainly mediated by cation dependent or ATP dependent transporters (Lolkema, 2006). The *Klebsiella pneumonia* Na^+-dependent citrate carriers CitS and *Salmonella serovars* CitC belong 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 *K. pneumonia* citrate transporter CitH is driven by the proton motive force (Lolkema, 2006). The objective of present study was to investigate the effect of over expressing *E. coli cs** and *S. typhimurium citC* genes in *H. seropedicae* Z67 on citric acid secretion, MPS ability and growth promotion of rice plants.

3.1.1: Rational of the present study

Objective of the present study was to determine the MPS ability of *H. seropedicae* Z67 in field conditions, which is slowed down by low accessibility of carbon sources, high buffering capacity of soils. The improvement in the MPS ability was investigated by targeted genetic manipulation of the central carbon metabolic pathway towards citrate secretion by over expression of NADH insensitive citrate

synthase (*cs**) of *E. coli* and *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene.

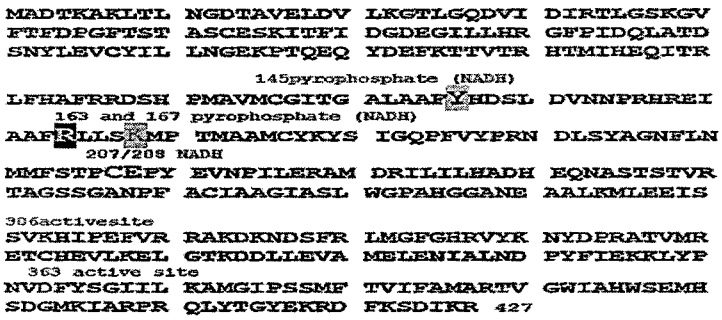


Fig. 3.1: *E. coli cs* protein sequence showing the regulatory variants

Table 3.1: NADH binding and inhibition by variant citrate synthases (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

The present study demonstrates the effect of overexpression of NADH insensitive *cs* Y145F on citric acid accumulation and secretion by *H. seropedicae* Z67.

3.2: The experimental plan of work includes the following-

3.2.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
E. coli DH10B	Used for maintaining plasmids.	(Invitrogen)
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
pAB8	pUCPM18 with Km^rgene; Ampr, Km^r	Buch et al., 2009
pAB7	*gltA* gene of *E. coli* in pAB8, Apr/Km^r	Buch et al., 2009
pY145F	NADH insensitive *cs** gene	Duckworth, H., 2003
citC	Na^+ dependent citrate transporter of *Salmonelatyphimorium*	This study
pJNK3	pUCPM18 with *E. coli* NADH insensitive *cs** gene under *Plac* and km^r gene; Ap^r, Km^r	This study
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	This study
P. putida BBC443 pTOL	TOL*gfp*mut3b plasmid; Km^r	Christensen et al.,1998
Hs Z67	*H. seropedicae* Z 67 with TOL*gfp*mut3b plasmid; Km^r	
Hs Z67	Nitrogen fixer, Natural isolate from gramenae plant; Nal^r	Baldani et al., 1986a

Hs (pAB8)	*H. seropedicae* Z67 pUCPM18 with *nptII* gene, Ap^r/Km^r	This study
Hs Z67 (pAB7)	*H. seropedicae* Z67 Nal^r with pAB7, Km^r	This study
Hs Z67 (pJNK3)	*H. seropedicae* Z67 Nal^r with pJNK3, Km^r	This study
Hs Z67 (pJNK4)	*H. seropedicae* Z67 Nal^r with pJNK4, Km^r	This study

Table 3.2: List of bacterial strains used. Detailed characteristics of these strains are given in **Table 2.1; 2.2.** Parent strains and the transformants of *E. coli* and *H. seropedicae* Z67 were respectively grown at 37°C and 30°C with variations in antibiotic concentrations for rich and minimal media as described in **(Section 2.2).**

3.2.2: Expression of *lac* promoter in *H. seropedicae* Z67

P. putida BBC443 contained a modified pTOL plasmid (~117Kb) conferring several properties to the host, like toluene degradation, kanamycin resistance, and green fluorescence due to green fluorescence protein (GFP) cloned under *lac* promoter (**Table 3.2**; Christensen et al., 1998). Green fluorescence due to GFP expression was monitored in *H. seropedicae* Z67 on Luria Agar as well as BH Agar containing Na-benzoate as sole carbon source **(Section 2.4).** *H. seropedicae* Z67 transformants with pTOL plasmid were selected on kanamycin and the positive *P. putida* KT2440 -TOL plasmid transformants were checked for kanamycin resistant growth and presence GFP mediated fluorescence on BH agar containing Na-benzoate. IPTG was not used to induce *lac* promoter in any of the above experiments.

3.2.3: Construction of *H. seropedicae* Z67 stable plasmid containing *E. coli* NADH insensitive *cs* gene under *lac* promoter

Plasmid pBR322 containing NADH insensitive *E. coli* cs (*gltA*) gene was obtained by restriction digestion of 1281 bp of Y145F.

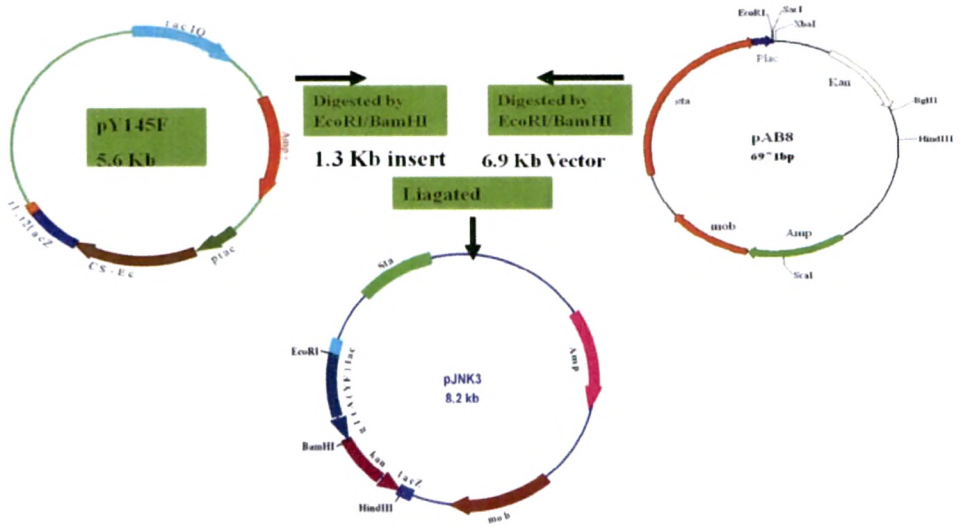


Fig. 3.2: Schematic representation of construction of *H. seropedicae* Z67 stable vectors containing NADH insensitive *E. coli cs* gene under *lac* promoter.

3.2.4: Incorporation of NADH insensitive *E. coli cs* and Na^+ dependent citrate transporter (*citC*) gene of *Salmonella typhimurium* under *lac* promoter in pUCPM18.

PCR amplification of pBR322 plasmid containing NADH insensitive *E. coli cs* (*gltA*) gene are carried out using gene specific primer (**Table 3.5**) to get an amplification of 1281 bp of Y145F

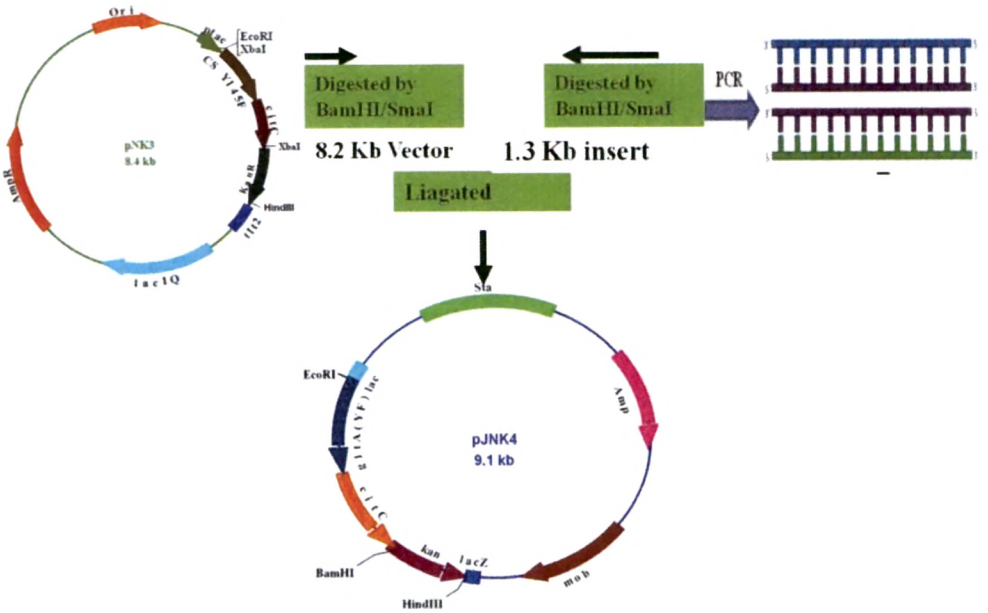


Fig. 3.3: Schematic representation of construction of plasmid containing NADH insensitive *E. coli cs* gene and citrate transporter (*citC*) gene under *lac* promoter.

E. coli NADH insensitive *cs* (pY145F) gene cloned in pBR322 was a generous gift from Dr. H. W. Duckworth, University of Manitoba, Canada. The *E. coli* NADH insensitive *cs* gene was ligated in pUCPM18 Km^r was selected using Kanamycin and further designated as pJNK3. *S. typhimurium citC* gene was obtained by polymerase chain reaction amplification using gene specific primer pair. Underline sequence shows BamHI/ *Sma*I restriction enzyme site used for cloning. PCR amplification was done using *Pfu* polymerase (Thermo scientific, USA) from plasmid pBKS-citC. Amplicon of 1.3 kb size, containing *citC* gene, was digested with *Bam*HI, gel eluted, purified, ligated in *BamHI*/*Sma*I digested and gel purified plasmid pJNK3. The resultant construct containing citrate operon was designated as pJNK4. The construct was confirmed by restriction digestion pattern. Further plasmids pAB8 as vector control, pAB7, pJNK3, pJNK4 were transformed by electroporation in *H. seropedicae* Z67 as described by Unge et al. (1998).

Table 3.3: Specific primer pair for Na^+ dependent citrate transporter (*citC*) gene.

Primer		Tm	GC%
FP-	5'GGATCCCGCACGGAGGAATCAACTTATGAC CAACATGACCCAGGCTTC3'	65	54.2
RP-	5'CCCGGGTTACACCATCATGCTGAACACGAT GC3'	66.5	56.3

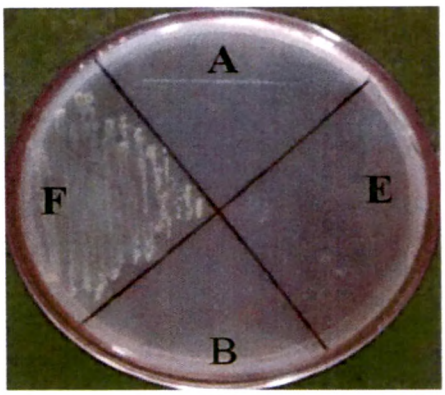
GGATCCCG- BamHI **CCCGGG -SmaI**

3.3: RESULTS.

3.3.1: Use of *lac* promoter for constitutive expression of desired genes in *H. seropedicae* Z67.

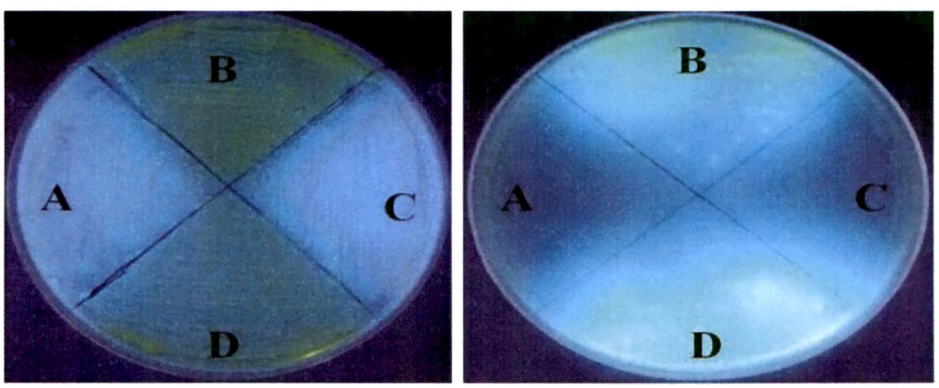
Lactose utilization of the *H. seropedicae* Z67 strain was checked on M9 minimal medium supplemented with lactose as sole carbon source. *H. seropedicae* Z67 and *E. coli* JM101 (used as negative control) could not grow on lactose whereas *Enterobacter asburiae* PSI3 showed good growth (utilizes lactose; used as a positive control) as shown in (**Fig 3.4**). *P. putida* KT2440 containing TOL*gfp*mut3b plasmid could grow and fluoresce due to GFP expression from *lac* promoter on rich media like Luria Agar as well as BH agar with Na-benzoate as sole carbon source in the presence of kanamycin. On the contrary *H. seropedicae* Z67 lacking pTOL plasmid grew on Luria Agar but could not fluoresce while failed to utilize Na-benzoate (**Fig. 3.5**). On the other hand, conjugal transfer of pTOL from *P. putida* BBC443 to *H. seropedicae* Z67 not only imparted the ability to utilize Na-benzoate as the sole carbon source in presence of kanamycin and but also conferred GFP expression as evident from green fluorescence (**Fig. 3.5**). These results confirmed that *lac* promoter was constitutively functional and thus could be used to express foreign genes in *H. seropedicae* Z67

Fig. 3.4: Lactose utilization of *H. seropedicae* Z67 strain on M9 minimal medium.



A - *H. seropedicae* Z67 W.T., **B** - *H. seropedicae* pTOL, **E** – *E. coli* JM1O1 negative control, **F** – *Enterobacter asburiae*

Fig. 3.5: pTOL mediated Na-benzoate utilization and GFP expression in *H. seropedicae* Z67.



Growth and GFP mediated fluorescence of **(a) A** - *H. seropedicae* Z67 W.T on Luria Agar, **(b) B** - *H. seropedicae* (+TOL*gfp*mut3b plasmid) on Luria agar, **C**- *P.putida* KT2440 and **D** - *P. putida* KT2440 (+TOL*gfp*mut3b plasmid), on BH Agar containing Na-benzoate as sole carbon source and 7.5µg/ml kanamycin.

3.3.2: Construction and functionality of pJNK3 and pJNK4 plasmids containing $P_{lac}cs^*$ gene and $P_{lac}cs^*citC$ operon.

*E. coli cs**gene was obtained as 1.3Kb DNA fragment by digesting pY145F plasmid with EcoRI and BamHI enzymes. The *cs**fragment was gel eluted, purified and ligated to purified EcoRI-BamHI digested pUCPM18 (Km^r) plasmid (6.9Kb) resulted in pJNK3 plasmid (8.2Kb) containing *cs** gene under *lac* promoter. Plasmid pJNK3 was digested with *BamHI*/*Sma*I and ligated to the PCR product of *citC* to form pJNK4 (9.2Kb). All plasmids were confirmed by restriction digestion analysis. Functionality of *cs** gene in these plasmids was determined by complementation of *E. coli* W620 glutamate auxotrophy while CitC function was monitored by the growth on citrate as sole carbon source.

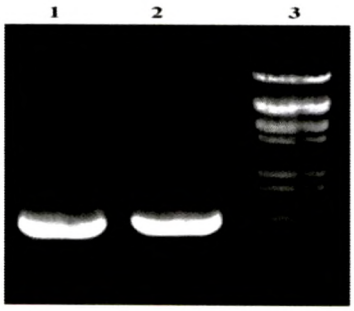


Fig. 3.6: PCR amplification of *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene.

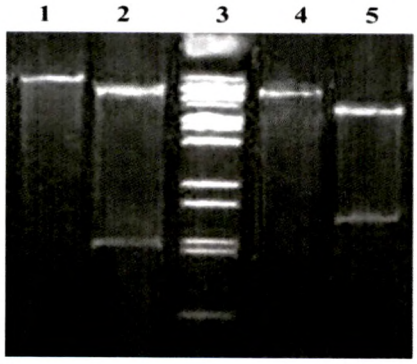


Fig. 3.7: Restriction digestion pattern for pAB8 and pAB7. Lane 1: pAB7 linearized with BamHI (8,265bp); Lane 2: pAB7 digested with XbaI (1,294bp, 6,971bp); Lane 3: Molecular

Weight Marker (MWM)-Lambda DNA cut with BstEII; Lane 4: pAB8 linearised with XbaI (6,971bp); Lane 5: pAB8 digested with EcoRI-HindIII (5,298bp, 1,673bp)

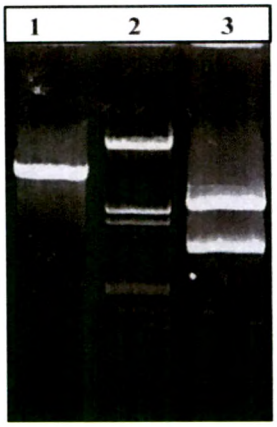


Fig. 3.8: Restriction digestion pattern for pUCPM18 Km^r containing NADH insensitive *cs* gene Lane 1: pJNK3 linearized with BamHI 8,265bp); Lane 2: Molecular Weight Marker (MWM)-Lambda DNA cut withEcoRI/HindIII Lane 3: pJNK3 digested with EcoRI-HindIII (5.310 bp, 2.900 bp)

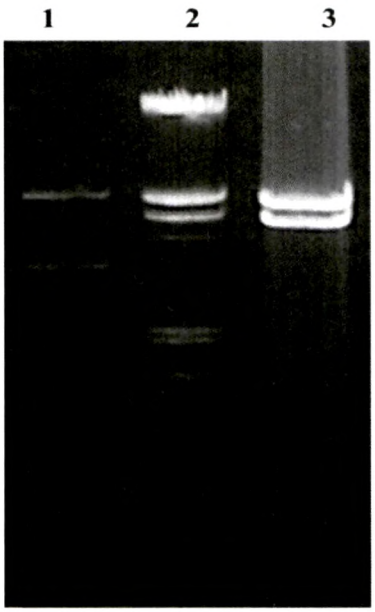


Fig. 3.9: Restriction digestion pattern for pUCPM18 Km*r* containing NADH insensitive *cs* gene and of Na^+ dependent citrate transporter (citC) of *Salmonella typhimurium* Lane 1: pJNK4 digested by XbaI (6.5 Kb, 2.6 Kb); Lane 2: Molecular Weight Marker (MWM)-Lambda DNA cut with EcoRI/HindIII ; Lane 3: pJNK4 digested with EcoRI-HindIII(5.210 bp, 3.900 bp)

3.3.3: *E. coli cs* mutant complementation

E. coli cs mutant strain harboring the pAB7, pAB8, pJNK3 plasmids were subjected to growth on M9 minimal medium containing glucose as carbon source in presence and absence of glutamate. *E. coli cs* mutant strain harboring pAB7 and pJNK3 could grow on glucose when induced with 0.1mM IPTG without glutamate supplementation unlike the controls (pAB8) (**Fig. 3.10**).

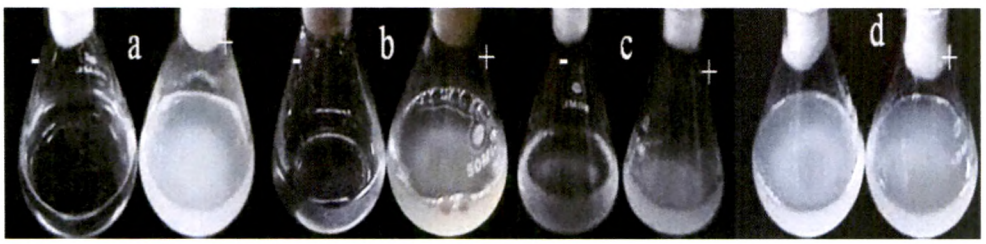


Figure 3.10: Complementation of *E. coli* W620 mutant phenotype by wild type and NADH insensitive *cs* plasmids. **a**: *E. coli* W620-deletion mutant of *cs* gene **b**: E. coli W620 with pAB8 plasmid **c**: *E. coli* W620 with pAB7 plasmid **d**: *E. coli* W620 with pJNK3 plasmid. All plasmid bearing strains are induced with 0.1mM IPTG. Growth is monitored on M9 medium with 0.2% glucose (Section) +/– at the corners of each image indicates absence and presence of 340µg/ml glutamate in the medium, respectively.

3.3.4 Growth and MPS ability of *H. seropedicae*Z67 transformants of pJNK3 and pJNK4 plasmids.

Hs (pAB8), *Hs* (pAB7), *Hs* (pJNK3), and *Hs* (pJNK4) transformants were obtained by electroporation (Eppendorf electroporator). *Hs* (pJNK3) and *Hs* (pJNK4) showed good P solubilization on Pikovaskya's agar and on 50 mM HEPES buffer methyl red plates with very good acidification on 50 mM glucose (**Fig. 3.11**).

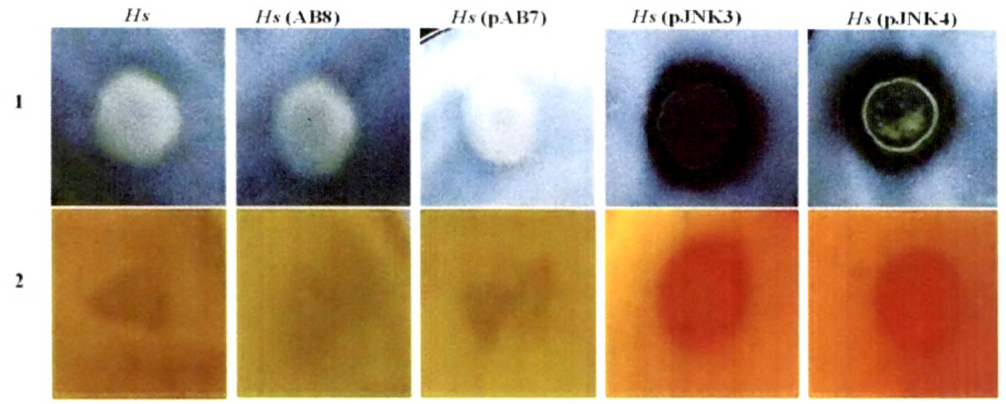


Fig. 3.11: MPS phenotype. 1-Pikovskaya's medium 2 - HRP medium containing 50 mM glucose and 50 mM HEPES rock phosphate (1 mg/ml)

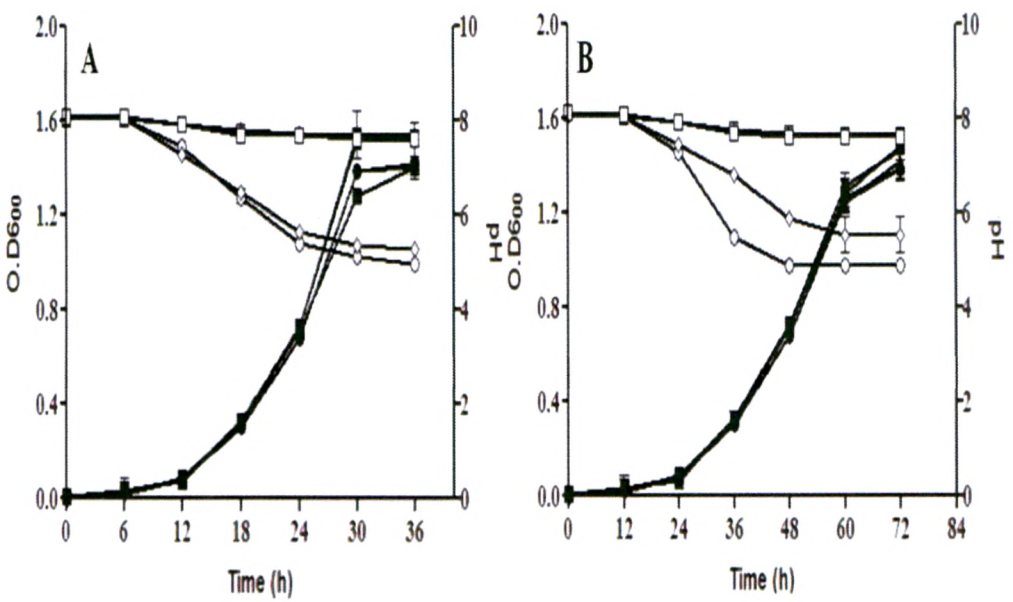


Fig. 3.12: Growth and pH profile. Extracellular pH (□, Δ, ▽, ◊, ○) and growth profile (■,▲,▼,◆,●) of *H. seropedicae* Z67 transformants on glucose 50 mM and HEPES 50mM rock phosphate(1 mg/ml) in medium. □, ■, *Hs* (wild type); Δ, ▲, *Hs* (pAB8); ▽,▼, *Hs* (pAB7) ; ◊, ◆, *Hs* (pJNK3), & ● ,○, *Hs* (pJNK4). A –Aerobic condition and B - Nitrogen free medium OD_{600} and pH values at each time point are represented as the mean ± SD of 3 independent observations

3.3.5 Effect of NADH insensitive (*cs**) and Na^+ dependent citrate transporter on growth, biomass and glucose utilization of *H. seropedicae* Z67 transformants grown on HRP minimal medium with 50mM glucose as C source.

Growth rates of *H. seropedicae* Z6 and the transformants *Hs* (pAB7), *Hs* (pJNK3) and *Hs* (pJNK4) were similar on HRP medium (**Table 3.4**). Total glucose depletion of *Hs* (pJNK3) and *Hs* (pJNK4) was ~3.38 and ~ 3.4 folds higher as against native strain and *Hs* (pAB7), respectively, on HRP medium. On the other hand, the specific glucose utilization rate of *Hs* (pJNK3) and *Hs* (pJNK4) was ~2.35 and ~ 2.68 folds higher than wild type and *Hs* (pAB7), respectively, on HRP medium. However, glucose consumption was almost similar in all *H. seropedicae* Z67 transformants when compared with wild type. In contrast, biomass yield of *Hs* (pJNK3) and *Hs* (pJNK4) was decreased by ~ 2.3 and ~ 2.6 folds on HRP medium when compared with wild type. *Hs* (pAB7) showed ~1.5 fold increase in biomass yield as compared with native strain. When inoculated in HRP minimal medium, pH change was not found in *Hs* (pAB7) but *Hs* (pJNK3) and *Hs* (pJNK4) showed good growth and a drop in pH up to 5.0 within 36 h in the presence of glucose (Fig. 3.12). *Hs* (pJNK3) and *Hs* (pJNK4) released P up to ~80.16 μM, ~110.24 μM, on 50 mM glucose (**Table 3.4**).

Table 3.4: Physiological attributes and metabolic record of *H. seropedicae* Z 67 transformants grown on HRP minimal medium with 50mM glucose as C source under aerobic condition.

Bacterial Strain	Growth rate μ (h^{-1})[a]	Total glucose depleted (mM)[b]	Glucose consumed (mM)[b]	Biomass yield $Y_{dcw/Glc}$[a] (g /g)	Sp. glucose utilization rate Q_{Glc}[a] (g.g $dcw^{-1}.h^{-1}$)	CS activity (U)		Phosphate released. P (μM)[b]
						M9 minimal medium	HRP medium	
Hs	0.25 ±0.00	13.60 ± 0.55	13.60 ± 0.55	0.051 ± 0.007	0.54 ± 0.07	12.90± 1.51	17.43± 0.96	UD
Hs (pAB8)	0.34 ±0.01	12.93 ± 1.68	12.93 ± 1.68	0.066 ± 0.016	0.43 ± 0.08	12.10 ± 1.31	17.13± 2.05	UD
Hs (pAB7)	0.30 ± 0.05	12.33±1.22^{ns}	12.33 ± 1.22^{ns}	0.075 ± 0.020	0.38 ± 0.10^{ns}	18.50 ± 0.60	26.16 ± 1.20	UD
Hs (pJNK3)	0.32 ± 0.01	46.66±0.66***	46.66 ±0.66***	0.022 ± 0.002**	1.27± 0.13 ***	34.50 ± 3.95***	51.70 ± 3.17***	80.16 ± 3.58
Hs (pJNK4)	0.32 ± 0.00	47.00±0.95***	47.00 ± 0.95***	0.019 ±0.0005**	1.45 ± 0.042***	36.03 ± 3.20***	53.89 ± 2.86***	110.24± 2.76

The results are expressed as Mean ± SEM of readings from 3 independent observations. *** P<0.001, ns=non-significant (as compared to wild type culture controls) ; [a]Determined from mid log phase of each experiment. [b]Determined at the time of pH drop (36 h).

Table 3.5: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown under micoaerobic condition on HRP minimal medium with 50mM glucose as C source.

Bacterial Strain	Growth rate μ (h^{-1})[a]	Biomass yield $Y_{dcw/Glc}$[a] (g /g)	Sp. glucose utilization rate Q_{Glc}[a] (g.g $dcw^{-1}.h^{-1}$)	Phosphate released P (μM)[b]
Hs	0.11 ± 0.01	0.025 ± 0.00	0.27 ± 0.03	
Hs (pAB8)	0.11 ± 0.00	0.021 ±0.00	0.33 ± 0.07	
Hs (pAB7)	0.12 ± 0.00	0.027 ±0.00	0.27 ± 0.09	
Hs (pJNK3)	0.12 ± 0.00	0.0088 ±0.00***	0.78 ± 0.03***	77.60 ± 1.37
Hs (pJNK4)	0.13 ± 0.00	0.0078 ±0.00***	0.88 ± 0.04***	116 ± 3.46

The results are expressed as Mean ± SEM of readings from 3 independent observations. *** P<0.001, ns=non-significant (as compared to wild type culture controls) ; [a]Determined from mid log phase of each experiment. [b]Determined at the time of pH drop (72 h).

Under N free HRP medium, growth rates of *Hs* (pAB7), *Hs* (pJNK3) and *Hs* (pJNK4) were similar as compared with wild type culture (**Table 3.5**). On the other hand, the specific glucose utilization rate of *Hs* (pJNK3) and *Hs* (pJNK4) was ~2.5 and ~ 3 folds higher than wild type and *Hs* (pAB7), respectively, on N free HRP medium. In contrast, biomass yield of *Hs* (pJNK3) and *Hs* (pJNK4) was decreased by ~ 2 folds on N free HRP medium when compared with wild type. *Hs* (pAB7) showed similar pattern of biomass yield as compared with native strain. Additionally, no change in pH was found in *Hs* (pAB7) but *Hs* (pJNK3) and *Hs* (pJNK4) showed good growth and decreased the pH up to 5.0 within 72 h in the presence of glucose and solubilized RP (**Fig. 3.12**). Native culture and *Hs* (pAB7) neither showed acidification nor solubilized RP whereas *Hs* (pJNK3) and *Hs* (pJNK4) released P up to ~77.60 µM and ~116 µM, respectively, on 50 mM glucose (**Table 3.5**). Extent of P release in microaerobic condition is similar to that of aerobic conditions.

3.3.6 CS activity and organic acid secretion in *H. seropedicae* Z67 transformants.

H. seropedicae Z67 and *Hs* (pAB7) had12.9 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 (**Table 3.4**). 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 mMcitric acid secretion was observed with *Hs* (pJNK3) and *Hs* (pJNK4) (**Fig. 3.13**). Acetic acid was also secreted by all *Hs* transformants. Acetic acid level in the native strain was (mM), while *Hs* (pAB7) were more and highest levels (~ up to 50 mM) in *Hs* (pJNK3) and *Hs* (pJNK4). Similar pattern of yields of citric acid and acetic acids were found in *H. seropedicae* Z67 transformants.

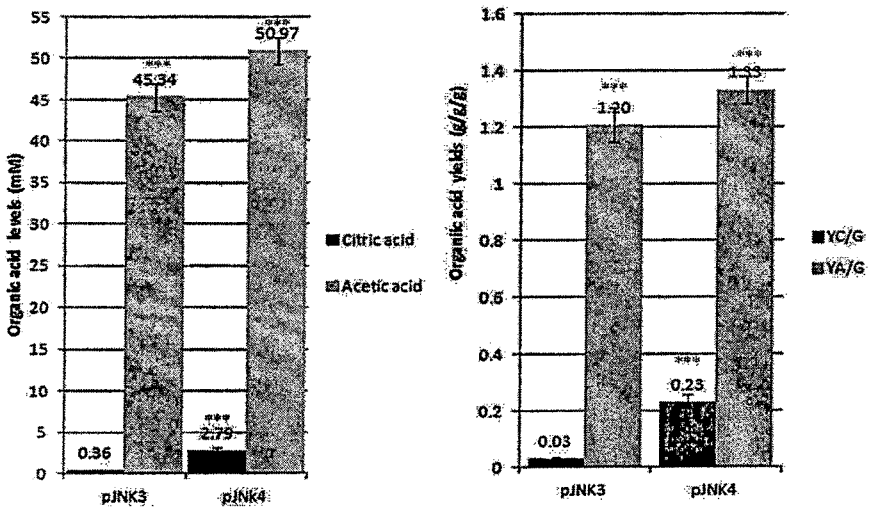


Fig. 3.13: Organic acid production from *H. seropedicae*Z67 transformants.

3.3.7 Influence of phosphorous levels on IAA, EPS and biofilm production.

Hs native culture and *Hs* transformants did not show significant change in EPS production under aerobic and under microaerobic condition (**Fig. 3.14 A, B**). However, biofilm formation was increased by ~4.5 fold in *Hs* (pJNK3) *Hs* (pJNK4) under both N-sufficient aerobic and N-free microaerobic conditions when compared with wild type

culture (**Fig. 3.14 C & D**). IAA production was also enhanced in *Hs* (pJNK3) and *Hs* (pJNK4) to ~1.4 and ~1.7 fold, respectively, under aerobic conditions than that of native culture, *Hs* (pAB8) and *Hs* (pAB7) (**Fig. 3.14 E**). All *Hs* transformants showed almost 40% decrease in IAA under N free microearobic conditions as compared to aerobic conditions (**Fig. 3.14 F**).

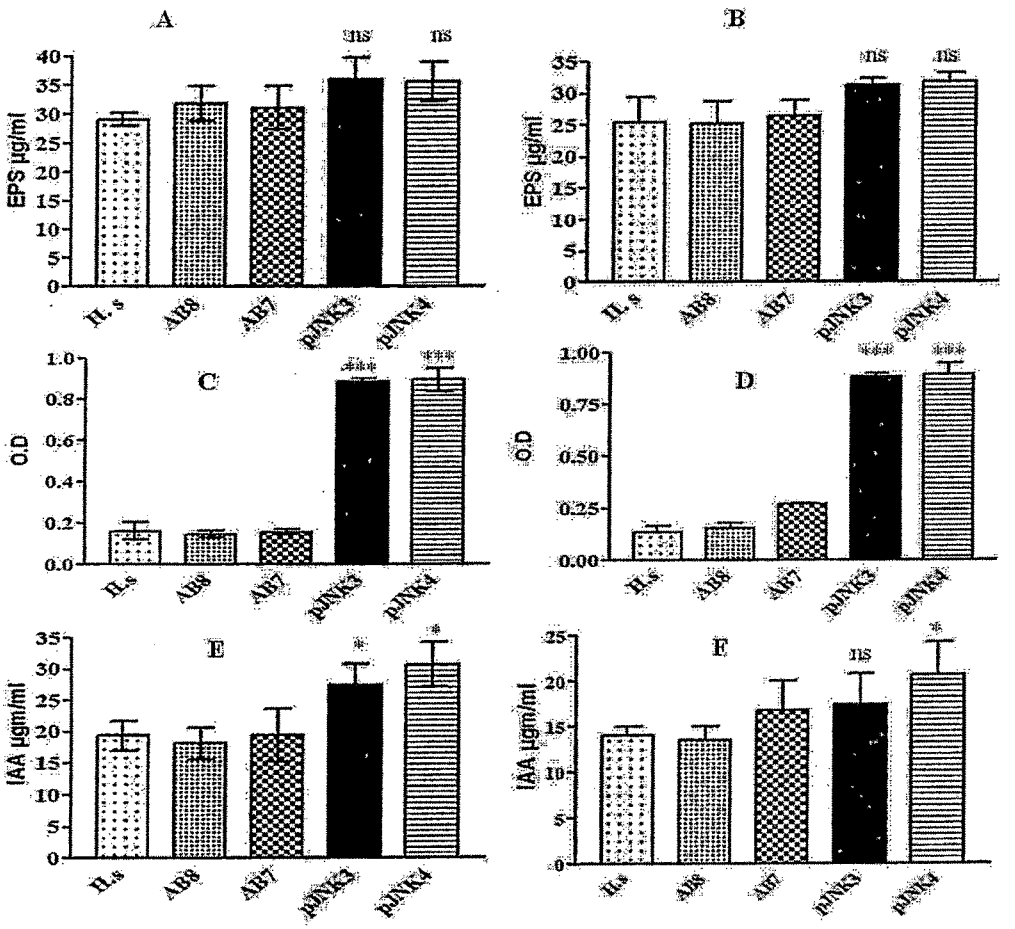


Fig. 3.14: Plant growth promoting factors. The results are expressed as Mean ± SEM of readings from 3 independent observations, ***P < 0.001, ** P < 0.01, * P < 0.05 and ns=non-significant (as compared with wild type culture controls); **A, C** and **E** under aerobic , **B, D** and **F** under microaerobic condition

3.3.8 Effect of *Hs* (pJNK3) and *Hs* (pJNK4) on the nutrient status of Rice plants

Rice plant shoot length, root length, fresh weight, dry weight, N, P and K content, in rice plants were monitored after 30 days after inoculation (**Table 3.7 and Table 3.8**). The shoot length of *Hs* native strain and *Hs* (AB7) inoculated rice plants showed ~1.3 fold increase when compared with control plants without any culture, while *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.2 and ~1.5 folds increase when compared with native strain. Root length was increased in both *Hs* and *Hs* (AB7) by ~1.8 folds when compared with control plants, while *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.6 fold increase in root length as compared with *Hs* plants. Fresh weight of plants inoculated with *Hs* and *Hs* (AB7) increased by ~1.9 folds when compared with control set, while *Hs* (pJNK3) and *Hs* (pJNK4) showed significant increase in fresh weight by ~1.3 and ~1.4 folds when compared with *Hs* treated plants. Total chlorophyll content was found similar in control, *Hs* and *Hs* (AB7), while both *Hs* (pJNK3) and *Hs* (pJNK4) increased by ~1.5 folds when compared with control and native strain. Nitrogen content in plants leaves was significantly increased with *Hs* and *Hs* (pAB7) by ~1.6 folds as compared with control plants while N levels in *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.4 and ~1.7 folds increase, respectively, when compared *Hs* plants. N levels in roots in *Hs* and *Hs* (pAB7) showed ~1.4 folds increase against control, while *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.4 folds and ~1.7 folds increase when compared with *Hs*. Phosphorus content in leaves and roots in both *Hs* (pJNK3) and *Hs* (pJNK4) by ~3.87 folds and ~ 4.35 folds, respectively, when compared against *Hs* plants. K content of leaves and roots was increased in *Hs* (pJNK3) and *Hs* (pJNK4) by almost ~2 folds when compared against *Hs*.

Table 3.6: Physical and chemical properties of experimental soil used for *H. seropedicae* Z67 transformants

E.C	pH	Total phosphorus (%)	Total nitrogen (%)	Total potassium (%)
0.63	7.86	19	16	65

Table 3.7: Effects of *H. seropedicae* transformants on plant fresh weight, dry weight, plant height leaf chlorophyll content at 30 DAI.

H. seropedicae **transformants**	**Shoot length (cm)**	**Shoot/Root ratio**	**Root length/Plant (cm)**	**Root/Shoot ratio**	**Total plant fresh weight /Plant (mg)**	**Total plant Dry weight /Plant (mg)**	**Total Leaf chlorophyll, Content (mg/g fr wt.)**
Control	21.66 ± 2.51	1.91 ± 0.48	11.66 ± 2.08	0.54 ± 0.16	105.33 ± 5.18	10.03 ± 0.96	2.86 ± 0.17
Hs	29.66 ± 1.52	1.38 ± 0.39	22.33 ± 4.72	0.75 ±0.19	201.11 ±18.90	16.11 ± 0.48	3.310 ± 0.27
Hs (pAB8)	27.33 ± 4.16	1.36 ± 0.08	20.00 ± 2.00	0.73 ±0.04	191.22 ±16.98	14.44 ± 1.16	3.050 ± 0.05
Hs (pAB7)	28.66±1.52	1.59 ± 0.14	18.00 ± 1.00	0.62 ±0.05	212.22± 10.88	19.88 ± 0.45	3.07 ± 0.31
Hs (pJNK3)	35.33 ± 3.05***	0.98 ± 0.14	36.33 ± 3.78***	1.03 ±0.14	265.88 ± 3.25***	31.55 ± 1.49***	4.51 ± 1.01***
Hs (pJNK4)	42.33 ± 2.51***	1.13± 0.09	37.33 ± 3.05***	0.88 ±0.07	294.88 ± 0.28***	37.22 ± 2.27***	4.72 ± 0.86***

The results are expressed as Mea n± SEM of readings from 6 plants of treatments. *** P<0.001, ns=non-significant (as compared with wild type culture inoculant and controls plants).

Table 3.8: N, P, K levels in rice plants after 30 DAI with *H. seropedicaeZ67* transformants.

Hs transformants	N content in leaves (%)	N content in roots (%)	P content in leaves (%)	P content in roots (%)	K content in leaves (%)	K content in roots (%)
Control	0.95±0.02	0.44±0.03	0.035±0.002	0.023±0.002	0.60±0.05	0.37±0.01
Hs	1.34±0.02***	0.92±0.03***	0.062±0.003***	0.045±0.003***	1.09±0.09***	0.85±0.08***
Hs (pAB8)	1.36±0.04	0.83±0.03	0.047±0.004	0.035±0.003	1.14±0.05	0.81±0.07
Hs (pAB7)	1.62±0.03	0.91±0.02	0.074±0.003	0.054±0.003	1.17±0.16	0.84±0.09
Hs (pJNK3)	1.91±0.6***	1.47±0.04***	0.24±0.004***	0.094±0.003***	2.03±0.01***	1.21±0.07***
Hs (pJNK4)	2.32±0.11***	1.55±0.04***	0.27±0.008***	0.097±0.002***	2.06±0.06***	1.35±0.05***

The results are expressed as Mea n± SEM of readings from 6 plants of treatments. *** P<0.001, ns=non-significant (as compared with wild type culture inoculant and controls plants).

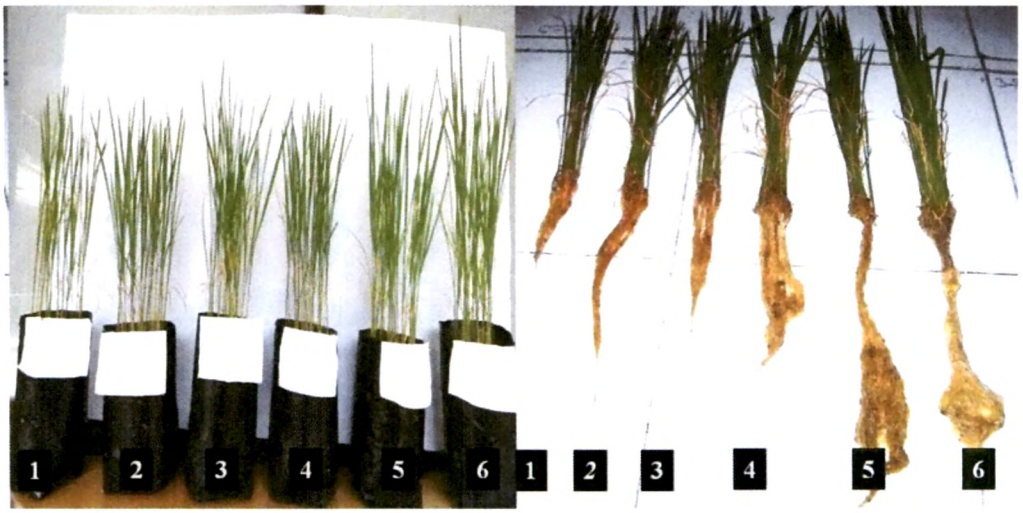


Fig. 3.15: Effect of Hs transformants in rice plants. 1- Control, 2- *Hs* native, 3-*Hs* (pAB8), 4-*Hs* (pAB7), 5-*Hs* (pJNK3), 6-Hs (pJNK4)

3.4 Discussion

Organic acids such as acetate, citrate, oxalate, gluconate and 2 ketogluconate are responsible for mineral phosphate solubilisation (Hazen et al., 1991; Srivastava et al., 2006; Archana et al, 2012). Secretion of gluconic (GA) and 2-ketogluconic acids (2KG) is known to be mediated by direct oxidative pathway in the periplasm and governed by nutritional and environmental factors. Strategy of increasing the activities of GDH and GAD enzymes has been successful in enhancing the secretion of these acids (Zaidi et al., 2009; Kumar et al., 2013). On the other hand, citric acid secretion was detected bacteria with decrease in the enzymes of TCA cycle pathway. Manipulation of TCA cycle for citric acid is complex in nature due to the central role in catabolic and anabolic pathway. Oxaloacetate, which is a substrate for CS enzyme, along with pyruvate and phosphoenolpyruvate forms the central components of the metabolic network known as *anaplerotic node* (Sauer and Eikmanns, 2005). Previously, genetic modifications related to the *anaplerotic node* was carried out in fluorescent pseudomonads resulting in secretion of citric acid up to 1.2 mM (Buch, 2008; 2009). These modifications have also diverted the glucose catabolism from phosphorylative pathway towards direct oxidative pathway leading to gluconic acid secretion.

H. seropedicae Z67 primary metabolism is similar to that of fluorescent pseudomonads but differs by the absence of PQQ biosynthesis genes (Pedrosa et al., 2011). Thus, glucose catabolism is only occurs by phosphorylative pathway. Overexpression of *cs* in *H. seropedicae* Z67 has increased CS activity by ~ 1.9 which is similar to that found in *P. fluorescens* 13525 but citric acid secretion was not detected while 1.2 mM level was secreted in *P. fluorescens* 13525 (Buch et al., 2009). This could be attributed to the lesser CS activity in *H. seropedicae* Z67 (pAB7) (26 U) as against 110 U in *P. fluorescens* 13525 transformant. Acetic acid was found in both the transformants with *P. fluorescens* 13525 was slightly higher which is in agreement with efficient metabolism of fluorescent pseudomonads (Rojo, 2010).

Over-expression of NADH insensitive (*cs**) in *Hs* (pJNK3) resulted in enhanced CS activity ~51 U, while citric acid secretion was too less 0.36 mM. CS

activity of *Hs* (pJNK3) was less than ~2 folds to that of *Pf* (pAB7) (Buch et al., 2009). Both these bacteria had similar effects as amount of citric acid secreted ids directly related to citric acid secretion while *Pf* (pAB7) also secreted high amount of gluconic acid. On the other hand, *Hs* (pAB7) secreted high acetic acid (~45 mM) while *Pf* (pAB7) had secreted only 15 mM. Thus, in *Hs* (pAB7) increase in CS activity diverted pyruvate towards acetic acid secretion. High citric acid levels inside *P. fluorescens* 13525 and *H. seropedicae* Z67 appear to have similar consequences on the metabolism. Catabolism of citric acid appears to be limited either by isocitrate dehydrogenase or by 2-ketoglutarate dehydrogenase enzymes. Diversion of glucose in *P. fluorescens* 13525 towards direct oxidative pathway and acetic acid in *H. seropedicae* Z67 could help in generation of ATP. In *P. fluorescens* 13525, during the conversion of glucose to gluconic acid pyrroloquinoline quinone (PQQ) gets reduced in the periplasm and is oxidized by supplying electrons to cytochrome C resulting in 1 ATP per molecule (Dijkstra et al., 1989). Since *Hs* does not possess PQQ as cofactor for GDH, acetic acid formation can also lead to ATP generation during the conversion of acetly CoA to acetate *via* acetylphosphate, involving acetate kinase which catalyzes the second reaction. Additionally, increase in acetic acid secretion by *Hs* (pJNK3) could result in increased carbon dioxide levels which in turn form oxaloacetate by pyruvate carboxylase (Papagianni, 2007).

CS activity of *Hs* (pJNK4) is similar to *Hs* (pJNK3) but secreted ~2.79 mM citric acid which is ~2.3 fold more than *Pf* (pAB7). Thus, enhanced citric acid secretion could be attributed the CitC transporter but not to the overexpression of *cs** gene. Both *P. fluorescens* 13525 and *H. seropedicae* Z67 contain H^+-citrate transporter but it is not efficient in efflux of citrate. Although *H. seropedicae* Z67 contains Na^+ dependent citrate transporter, overexpression of *citC* gene has improved citric acid secretion. The active transport system for citrate secretion appears to be the main rate-determining factor in citrate overproduction by yeasts (Anastassiadis and Rehm, 2005). Thus, both *P. fluorescens* 13525 and *H. seropedicae* Z67 appears to require high CS activity coupled with an efficient efflux system for citric acid secretion.

Hs (pAB7), *Hs* (pJNK3) and *Hs* (pJNK4) had no significant change in growth and glucose depletion rates which were similar to that of pseudomonas and *E. coli* (Walsh and Koshland, 1985; Buch et al., 2009). In *Hs* (pAB7), whose biomass yield was unaltered while *gltA* overexpression in *E. coli* increased the maximum cell dry weight by 23% (De Maeseneire et al., 2006; Buch et al., 2009). In contrast, the biomass was decreased in *Hs* (pJNK3) and *Hs* (pJNK4) which correlated with high acetic acid secretion. In *E. coli,* high acetic acid secretion has detrimental effect on growth (Wolfe, 2005). *Hs* transformants also showed similar extent of MPS ability under both aerobic and microaerobic nitrogen fixing conditions suggesting that levels of secreted citric acid was not influenced by the oxygen status. Similar results were observed in *A. brasielense* by overexpression of *pqqE* for gluconic acid secretion which solubilized tricalcium phosphate on N free medium (Vikram et al., 2007).

Significant contribution in plant growth promotion is shown by plant growth-promoting bacteria by producing factors (EPS, Biofilm and IAA) responsible for plant growth promotion in adverse conditions (Vicente et al., 2012). *Herbaspirillum* is known to produce IAA (Radwan et al., 2002). Improved IAA levels, EPS production and biofilm in HRP medium could help in better colonization of *H. seropedicae* Z67 transformants as the bacteria known to form mucilaginous and a fibrillar material in maize and sorghum root surfaces (Gyaneshwar et al. 2002; Roncato-Maccari et al. 2003b). EPS plays an important role during the colonization of the protoxylem vessels of sorghum and sugarcane by *H. seropedicae* and *H. rubrisubalbicans* (James et al. 1997; Olivares et al. 1997; James and Olivares 1998). Biofilm formation is required for the attachment in plant tissues (Baldotto et al., 2011: Santacecilia et al., 2012). Microbial growth and biofilms production is known to be increased by phosphorous (Lehtola et al. 2002; Chu et al. 2005; Fang et al., 2009). The production of EPS is directly correlated with biofilm formation as EPS can protect the cells External addition of phosphorous in water also enhanced biofilm formation (Mohamed et al., 1998). EPS productivity was increased in *Bacillus cereus* GU812900 by increasing nitrogen and phosphate (Bragadeeswaran et al., 2011).

Plant inoculation studies demonstrated the positive effect of *Hs* transformants on growth of rice plants which is cumulative effect of improved N and P status combined with increased IAA and EPS levels. PSB are known to enhanced yield in rice and maize (Tiwari et al., 1989; Pal, 1999). Similar effects are found with wheat plant increased in yield and P uptake when inoculated with *Rhizobium* with phosphate solubilizing bacteria. (Afzal and Bano, 2008). Co-inoculation of N_2 fixing and phosphate solubilizing bacteria showed similar growth improvement in sorghum and wheat (Algawadi and Gaur, 1992; Belimov et al., 1995; Galal, 2003).

Conclusion

This study revealed that overexpression of NADH insensitive citrate synthase (*cs**) and combination of NADH insensitive citrate synthase (*cs**) with Na^+ dependent citrate transporter (*citC*) in *H. seropedicae* Z67 leads to citric acid secretion, enhanced mineral phosphate solubilization and improved growth of rice plant (*Oryza sativa* Gujarat - 17). Thus, genetic manipulations could be useful in the development of better plant growth promoting rhizobacteria.