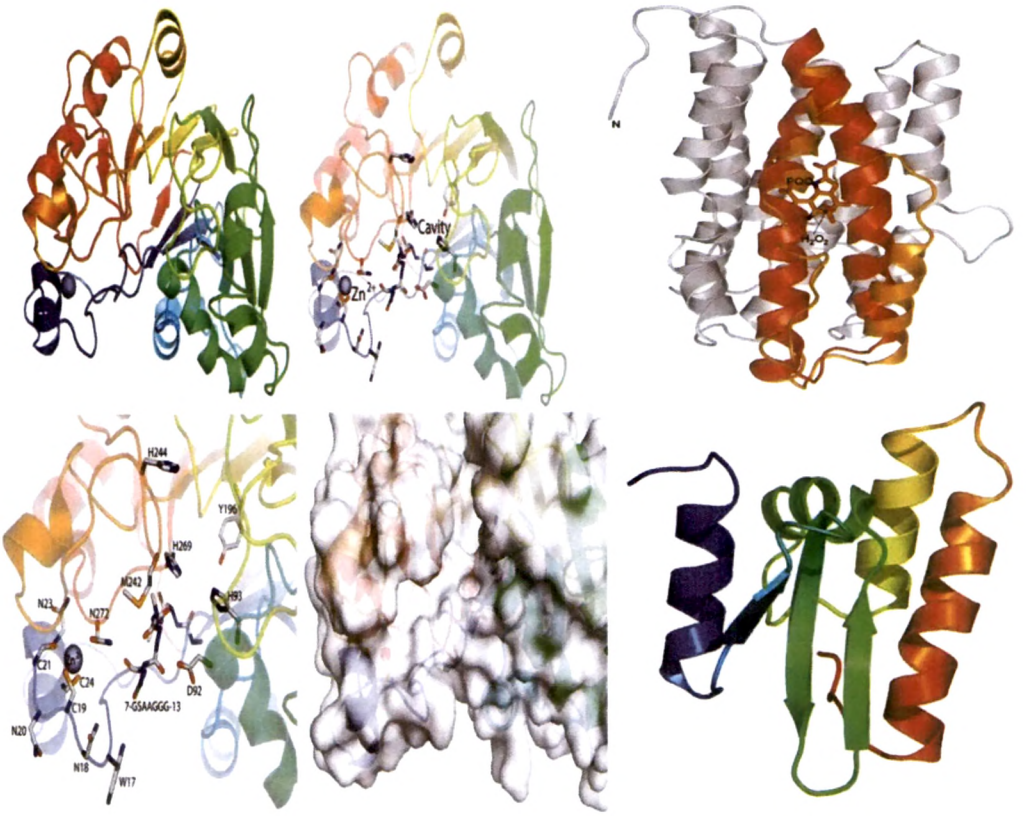
Chapter 4



Monitoring the MPS ability of *H. seropedicae* Z67 harboring *pqq* gene clusters.

4.1: Introduction

H. seropedicae is an endophytic nitrogen-fixing bacterium which colonizes roots of economically important crops such as rice, sugarcane, and wheat (Pedrosa et al., 2001; da Silva et al., 2003; Monteiro et al., 2003). *Herbaspirillum* bacteria have been isolated from the interior of roots, stem, and leaves of many Graminaceous plants without any harmful effect on plants (Baldani et al., 1992; DSbereiner *et al.,* 1994; Olivares *et al.,* 1996). *Herbaspirillum* spp. has the pectinolytic and cellulolytic enzymes that are essential for the cell wall degradation of plant during infection (Elbeltagy et al., 2001; Gyaneshwar et al., 2002). *H. seropedicae* increased biomass and N content of rice plants equivalent to the treatment with 40 kg of N/ha (Pereira and Baldani, 1995).

Nitrogen fixation in field conditions is limited by the availability of P and co-inoculation of nitrogen fixing bacteria with phosphate solubilizing microorganisms (PSMs) have increased growth and N status of plants (Gull et al., 2004; Elkoca et al., 2007; Valverde et al., 2007; Kumar and Chandra, 2008). *Rhizobium* species with high ability to nodulate and fix nitrogen was isolated from common bean *Phaseolus vulgaris* was able to solubilize phosphate (Abril et al. 2003). *Rhizobium leguminosarum* showing P-solubilizing ability has shown to increase the growth of maize and lettuce (Chabot et al., 1996). Phosphate solubilizing *Mesorhizobium meditteraneum* increased growth, N, P, Ca, Mg, and K in chick pea and barley (Peix et al., 2001). Some rhizospheric organisms have the ability to solubilize insoluble phosphate through organic acid production, such as citric and gluconic (Khan et al., 2011; Archana et al., 2012). In Gram negative bacteria, gluconic acid is generated by the direct oxidation of glucose that is mediated by a membrane bound glucose dehydrogenase (GDH) which possesses pyrroloquinoline quinone (PQQ) as cofactor (Goldstein, 1995).

PQQ biosynthesis in bacteria involves varying number of genes present in clusters (Choi et al., 2008). PQQ biosynthesis is not completely understood but a putative biosynthetic pathway has been proposed on the basis of the functions of conserved genes in bacteria (Puehringer et al., 2008). *Klebsiella pneumonia* possesses *pqqABCDEF* genes which are involved in PQQ biosynthesis (Meulenberg et al., 1992). *pqqA* gene encodes for 23-24 amino acid polypeptide which a substrate for a

set of enzymes modifying the glutamate and tyrosine residues leading to PQQ formation (Goosen et al. 1992; Meulenberg et al. 1992; Velterop et al. 1995). PqqB belongs to metallo-β-lactamases and has been suggested to help in the transport of PQQ into periplasm (Velterop et al., 1995). PqqC is a cofactor less oxidase, activates oxygen, and catalyzes the final step of ring closure reaction (Magnusson et al., 2004). PqqD has been shown to interact with PqqE possesses reductively cleavage activity of S-adenosyl methionine to form 5' deoxyadenosine and methionine (Wecksler et al., 2010). PqqE catalyzes the first step of linking glutamate and tyrosine residues of PqqA peptide. Additionally, PqqF, G, H, I, J, K and M are found in some bacteria possessing putative Zn dependent peptidase, non catalytic subunit of peptidase, transcriptional regulator, aminotransferase, cytosolic protein, DNA binding and prolyl oligopeptidase, respectively (Choi et al., 2008).

E. coli, Azospirillum, Rhizobium and *Herbaspirillum* do not encode PQQ biosynthesis genes in their genome but possess glucose dehydrogenase (GDH) apoprotein. Incorporation of *pqq* gene clusters in *E. coli* resulted in PQQ biosynthesis and active GDH enzyme (Goosen et al., 1989; Meulenberg et al., 1990; Khairnar et al., 2003; Yang et al., 2010). Interestingly, expression of *Erwinia herbicola pqqE* gene alone in *E. coli* HB101 and *Azospirillum* resulted in active GDH enzyme and secreted gluconic acid (Liu et al., 1992; Vikram et al, 2007). Similarly, expression of *Rahnella aquatilis pqqED* genes also resulted in gluconic acid secretion in *E. coli* (Kim et al., 1998). The mechanism of PQQ biosynthesis either in *pqqE* or *pqqED* gene transformants of *E. coli* is not clear as *pqqABCDE* genes are necessary for PQQ biosynthesis (Choi et al., 2008; Shen et al., 2012). Thus, the present study investigates the effect of incorporation of *E. herbicola pqqE* and *pqq* gene clusters from *Acinetobacter calcoaceticus* and *Pseudomonas fluorescens* in gluconic acid secretion in *H. serpedicae* Z67 and monitors its effect on mineral phosphate solubilization (MPS) ability.

4.1.1: Rational of study

H. seropedicae Z67 contain apo GDH enzyme but does not possess *pqq* genes for which is cofactor of GDH for gluconic acid production, through direct oxidative pathway. *H. seropedicae* Z67 does not secrete organic acid and thus does not show MPS ability. In present study, we demonstrated the effect of over expression of three different *pqq* genes (*pqqE* of *E. herbicola*, *pqq* gene cluster of *A. calcoaceticus* and *pqq* gene cluster of *pqq* gene cluster of *Pseudomonas fluorescens* B16). Over expression and their effect on MPS ability and PQQ secretion which is known as plant growth promoter.

4.2: EXPERIMENTAL DESIGN

4.2.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
pBBR1MCS-2	Broad-Host-Range vector; Km^r	Kovach et al., 1995
pMCG898	Containing *pqqE* of *Erwinia herbicola*	Goldstein and Liu; 1987
pJNK1	pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	This study
pSS2	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *Acinetobacter calcoaceticus*	Goosen et al., 1989
pOK53	35Kb plasmid contains 13.4-kb *pqq* gene cluster of *Pseudomonas fluorescens* B16	Choi et al., 2008
E. coli DH10B	Used to maintain plasmids for routine use	Invitrogen, USA
Hs	Nitrogen fixing rice endophyte , Nal^r	Baldani et al., 1986a
Hs (pBBR1MCS2 Km^r)	*H. seropedicae* Z 67 with (pBBR1MCS2) Km^r	This study
Hs (pJNK1)	*H. seropedicae* Z 67 with pJNK1 Km^r	This study
Hs (pSS2)	*H. seropedicae* Z 67 with pSS2 Tet^r	This study
Hs (pOK53)	*H. seropedicae* Z 67 with pOK53Tet^r	This study
Enterobacter asburiae PSI3	Amp^r secretes gluconic acid	Gyaneshwar et al., 1999
Pseudomonas aeruginosa P4	Secretes gluconic acid	Buch et al., 2008

Table. 4.1: Bacterial strains used in this study

4.2.2: Bacterial strains, plasmids, media and culture conditions.

E. coli and *H. seropedicae* Z67 strains and plasmids used for this work have been listed in (**Table 1**). DNA manipulation and molecular biology experiments were performed using *E. coli* DH10B as the host strain using standard protocols (Sambrook and Russell, 2001). Plasmid bearing derivatives of *E. coli* DH10B was routinely grown at 37^0C on Luria Bertani (L.B) and maintain on Luria Agar (L.A) plate with 40 μg ml^{-1} streptomycin and *Hs* was grown on JNFb plate at 30 °C with 10 μg ml^{-1} Nalidixic acid and Gentamycin 20 μg ml^{-1}, respectively, *H. seropedicae* Z67 was routinely grown at 30^0C on Luria Bertani (L.B) and maintained on semisolid JNFb medium (Baldani et al., 1992) was used at pH 5.8 at 30°C. *H. seropedicae* Z67 form small moist colonies with blue centers.

4.2.3: DNA manipulation

Plasmids pMCG898 containing *pqqE* was gifted by (Dr. A. H. Goldstein). Appropriate method was used for subcloning of *pqqE* in broad host range vector pBBR1MCS2 Km^r under *lac* promoter in EcoRI/BamHI site, as described by Sambrook et al. (1989), which resulted in pJNK1 Km^r and transformed into *E. coli* DH10B, followed by selection on LB agar medium containing kanamycin, further plasmids pBBR1MCS2 Km^r as vector control, pJNK1 Km^r, pSS2Tet^r and pOK53 Tet^r were transformed by electroporation in *H. seropedicae* Z67 described by Unge et al. (1998).

4.3: Results:

4.3.1: Subcloning of *pqqE* gene of *E. herbicola* in broad host range vector pBBR1MCS2 km^r from pMGC898.

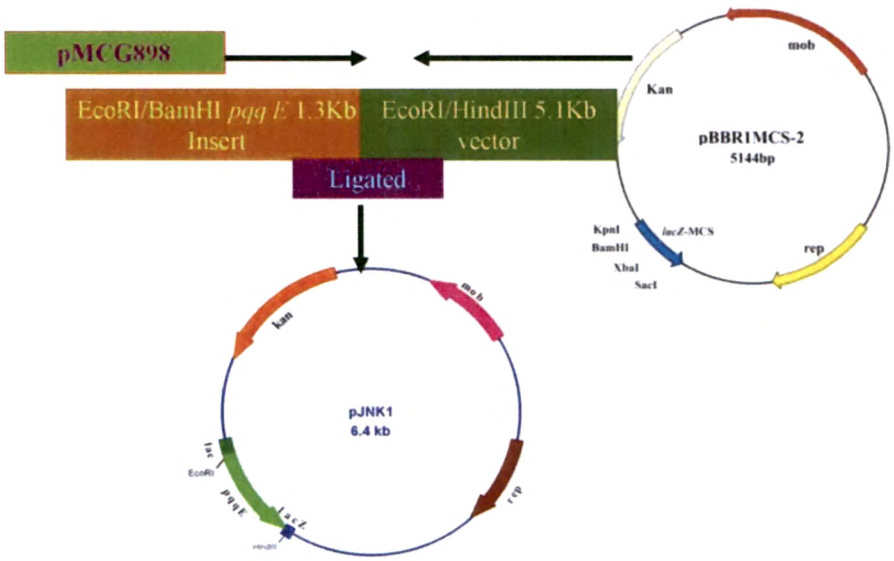


Fig. 4.1: Schematic representation of construction of *H. seropedicae* Z67 stable vectors containing *pqqE* gene under *lac* promoter.

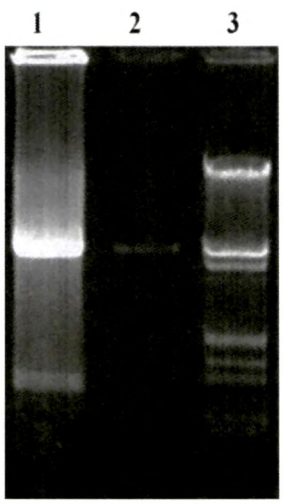


Fig. 4.2: Lane. 1- pJNK1 digested by EcoRI/BamHI, Lane. 2- pBBR1MCS2 digested by BamHI. Lane. 3- Marker EcoRI/HindIII

Fig. 4.2: Restriction digestion pattern for pJNK1.

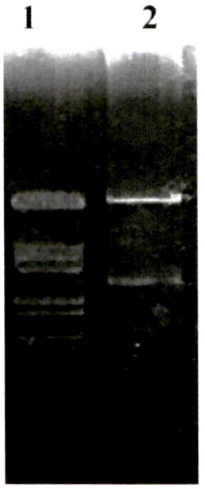


Fig. 4.3: Lane 1- Marker Lambda DNA *BstEII*. Lane 2- pSS2 plasmid digested by EcoRI/BamHI

Fig. 4.3: Restriction digestion pattern for pSS2.

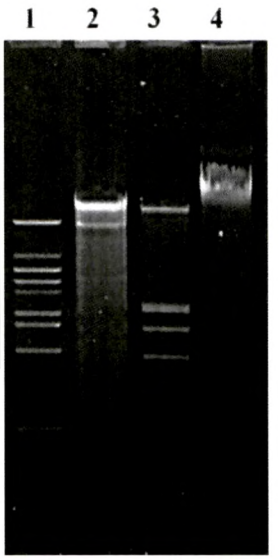


Fig. 4.4: Lane 1- *BstEII* marker, Lane 2- Release of 14 Kb *pqq* cluster digested with BamHI. Lane 3- Marker EcoRI/ Hind III. Lane 4 - Plasmid pOK53

Fig. 4.4: Restriction digestion pattern for pOK53

4.3.2: Growth and MPS ability of *H. seropedicae* Z67 transformant of *pqq* genes.

Hs Z67 transformant containing *E. herbicola pqqE* (pJNK1), *A. calcoaceticus pqq* gene cluster (pSS2) and *P. fluorescens* B16 *pqq* gene cluster (pOK53) plasmid were obtained by electroporation (Eppendorf electroporator). *Hs* transformants containing pJNK1 plasmid showed poor P solubilization on Pikovaskya's agar, HEPES buffer methyl red plates while transformants containing pSS2 and pOK53 plasmids showed very good acidification on 50 mM glucose, 50mM xylose and a mixture of 25mM glucose and 25mM xylose (**Fig. 4.5**).

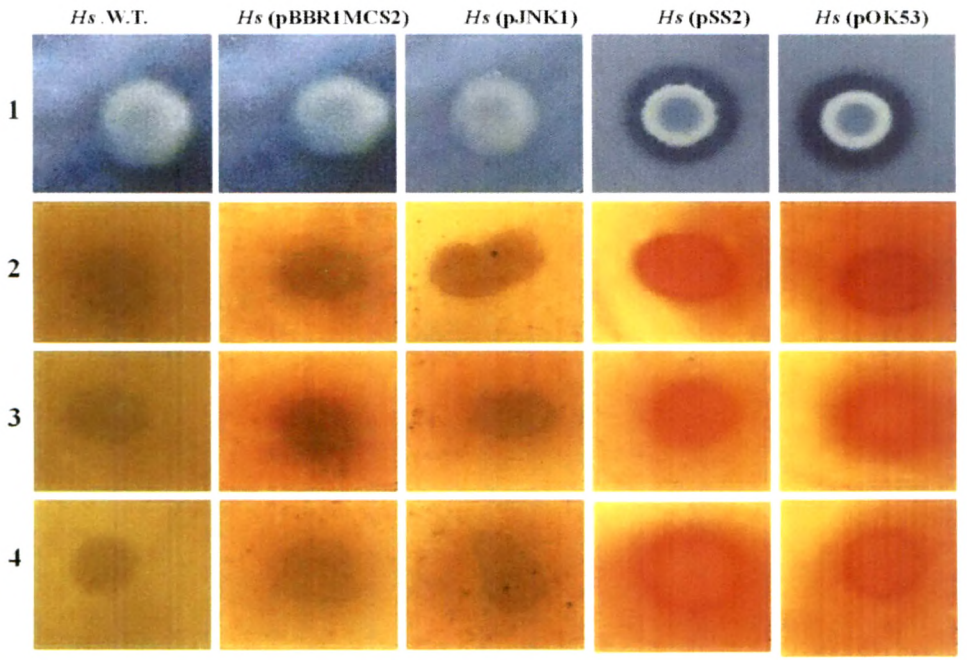


Fig. 4.5: MPS phenotype. 1-Pikovskaya's medium; **2**- 50 mM HEPES 50mM glucose rock phosphate medium; **3**- 50 mM HEPES 50mM xylose rock phosphate medium and **4**- 50 mM HEPES 25mM glucose and 25 mM xylose rock phosphate medium.

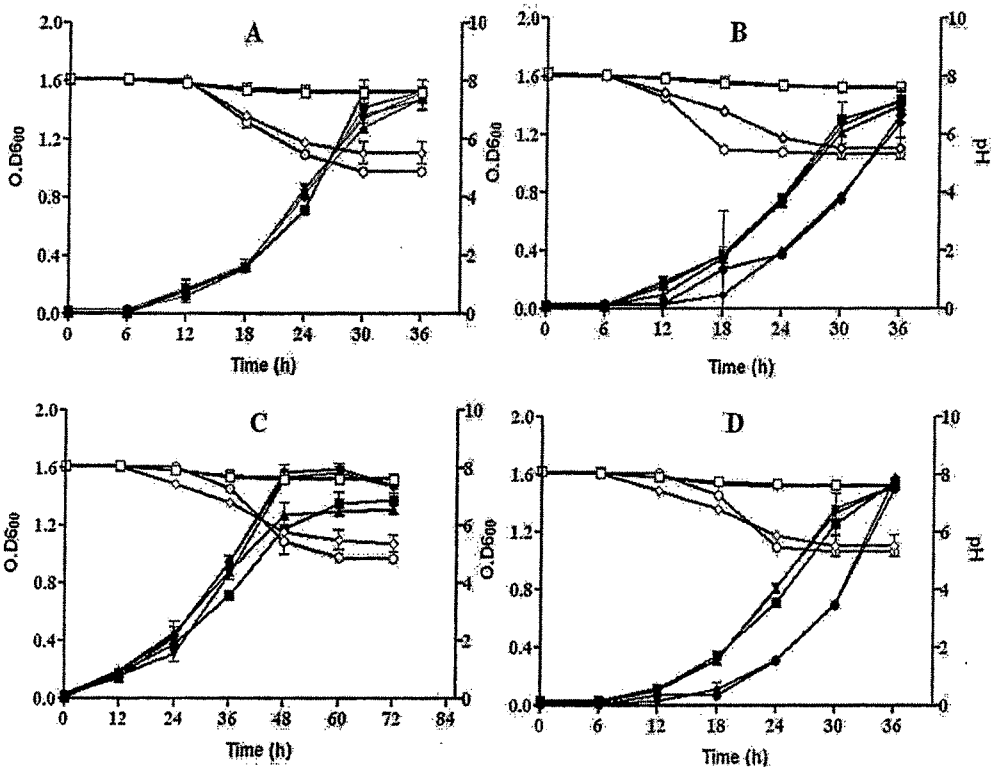


Fig. 4.6: Growth and pH profile. Extracellular pH (□, Δ, ▽, ◊, ○) and growth profile on 50mM HEPES rock phosphate medium (■,▲,▼,◆,●) of *H. seropedicae* Z67 over expressing *pqq* gene clusters, □, ■ *Hs* (wild type); Δ, ▲, *Hs* (pBBR1MCS2); ▽,▼, *Hs* (pJNK1) ; ◊, ◆, *Hs* (pSS2), & ● ,○, *Hs* (pOK53). A,B,D –Aerobic condition on 50 mM glucose and C - Nitrogen free medium 50 mM glucose; B- Glucose and Xylose Mixture of 25 mM each. D- 50 mM Xylose. OD600 and pH values at each time point are represented as the mean ± SD of six independent observations.

Growth rates of *Hs* Z6 and the transformants (pJNK1, pSS2 and pOK53) was similar on HRP medium (**Table 4.2**). Total glucose depletion of *Hs* Z67 transformants (pSS2 and pOK53) was ~4.4 and ~ 2.4 folds higher as against native strain and *Hs* Z6 (pJNK1), respectively, on HRP medium. On the other hand, the specific glucose utilization rate of *Hs* Z6 transformants (pSS2 and pOK53) was ~2.7 and ~ 1.3 folds higher than wild type and *Hs* (pJNK1), respectively, on HRP medium. However, glucose consumption was almost similar in all *Hs* Z67 transformants when compared with wild type. In contrast, biomass yield of *Hs* Z67 transformants (pSS2 and pOK53) was decreased by ~ 2.4 and ~ 2.8 folds on HRP medium when compared

with wild type. *Hs* (pJNK1) showed ~2 fold less biomass yield as compared with native strain.

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] ($g.g\ dcw^{-1}.h^{-1}$)
Hs	0.34 ±0.04	10.200± 0.91	10.200± 0.91	0.068±0.005	0.40 ±0.03
Hs (pBBR1MCS2)	0.20 ±0.003	11.66 ±0.61^{ns}	11.66±0.61 ^{ns}	0.054±0.006	0.51±0.06
Hs (pJNK1)	0.21±0.02 ^{ns}	18.70 ± 1.04*	14.61±1.05 ^{ns}	0.034±0.001*	0.81±0.02*
Hs (pSS2)	0.22±0.008 ^{ns}	44.56 ±0.58***	12.35±0.90 ^{ns}	0.024±0.001**	1.13±0.08***
Hs (pOK53)	0.24±0.008 ^{ns}	45.16 ±0.30***	11.48±0.61 ^{ns}	0.028±0.001**	1.19 ± 0.06***

Table 4.2: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown on HRP minimal medium with 50mM glucose as C source under aerobic condition.

Growth rate and glucose depletion rate were determined using mid-log phase cultures. Parameters compared HRP minimal medium for each transformant. 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 wild type culture controls) ; [a] Determined from mid log phase of each experiment. [b] Determined at the time of pH drop (36 h).

When inoculated in HRP minimal medium, similar pattern of absence of pH change was found in the transformants containing pJNK1 but the transformants containing pSS2 and pOK53 plasmids showed good growth and a drop in pH up to 5.0 within 36 h in the presence of glucose, xylose and mixture of xylose and glucose and solubilized RP (Fig. 4.6). *Hs* native culture and transformant of pJNK1 plasmid did not show acidification and solubilize RP while *Hs* transformants containing pSS2 and pOK53 released P up to ~168.07 µM, ~127.96 µM, and ~155.07µM on 50 mM glucose, 50 mM xylose and, 25 mM glucose and 25 mM xylose, respectively (**Table 4.2**). In micro aerobic condition similar results were obtained (**Table 4.3**).

Table 4.3: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown on N free under microaerobic condition 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] ($g.g\ dcw^{-1}.h^{-1}$)	Nitrogen Free HRP medium [b]
Hs	0.073 ± 0.030	0.011 ± 0.001	0.62 ± 0.11	UD
Hs (pBBR1MCS2)	0.098 ± 0.004*	0.011 ± 0.001ns	0.66 ± 0.08ns	UD
Hs (pJNK1)	0.13±0.017***	0.0072 ± 0.0008***	0.98 ± 0.11*	UD
Hs (pSS2)	0.12 ± 0.022***	0.0042 ± .0003***	1.41 ± 0.09***	130.19 ± 6.2***
Hs (pOK53)	0.13 ± 0.041***	0.0054 ± 0.0005***	1.28 ± 0.14***	172.04 ± 9.1***

Parameters compared HRP minimal medium for each transformant. 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 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* Z67 transformants (pJNK1, pSS2 and pOK53) were higher by ~1.5 fold when compared with wild type culture (**Table 4.3**). On the other hand, the specific glucose utilization rate of *Hs* Z67 transformants (pSS2 and pOK53) was ~2 folds higher than wild type and *Hs* (pJNK1), respectively, on N free HRP medium. In contrast, biomass yields of *Hs* Z67 transformants (pSS2 and pOK53) was decreased by ~ 2 folds on N free HRP medium when compared with wild type. *Hs* (pJNK1) showed ~2.5 fold less biomass yield as compared with native strain.

Bacterial transformants of *H. seropedicae* Z67 when inoculated in N free HRP minimal medium, no change in pH was found in the transformants containing pJNK1

but the transformants containing pSS2 and pOK53 plasmids showed good growth and a drop in pH upto 5.0 within 72 h in the presence of glucose and solubilized RP (**Fig. 4.6**). *Hs* native culture and transformant of pJNK1 plasmid did not show acidification and did not solubilize RP, whereas *Hs* transformants containing pSS2 and pOK53 released P up to ~130.19 μM and ~172.04 μM, respectively, on 50 mM glucose (**Table 4.3**). Extent of P release in microaerobic condition is similar to that of aerobic conditions.

4.3.3: GDH activity and gluconic acid secretion in *H. seropedicae* Z67 transformant of *pqq* genes.

Native culture did not show any GDH activity but *Hs* transformants pJNK1 on M9 medium showed GDH activity 14.98 U with glucose, 12.47 U with xylose and 15.89 U on mixture of glucose xylose. In HRP medium, the activity was 19.14 U with glucose, 14.46 U with xylose and 19.14U with mixture of glucose and xylose (**Table 4.4**). *Hs* pSS2 and *Hs* pOK53 showed ~5.5 to ~6 fold increase in GDH activity with glucose, xylose and mixture of glucose xylose in M9 medium whereas *Hs* pSS2 and *Hs* pOK53 on HRP medium GDH activity was enhanced by ~12 to ~13 fold when compared with pJNK1. Gluconic acid secretion was not found in *Hs* native culture and vector transformant. Gluconic acid production was ~4.26 mM with *Hs* pJNK1 transformants while *Hs* pSS2 and pOK53 transformants secreted 32.0 mM and 33.46 mM gluconic acid, respectively (**Fig. 4.7**). Yield of gluconic acid by *Hs* Z67 transformants (pSS2 and pOK53) was ~ 3 fold more than *Hs* Z67 (pJNK1) on HRP medium.

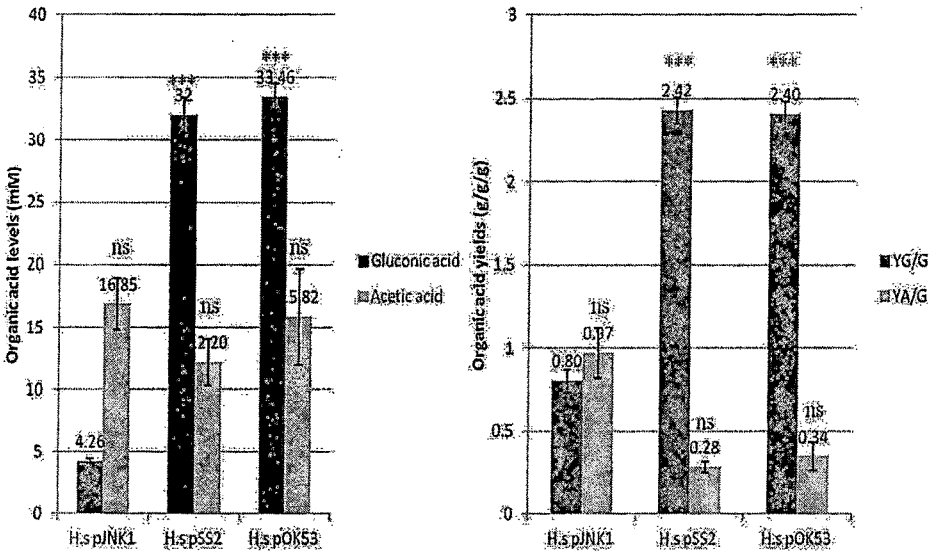


Fig. 4.7: Organic acid levels and yield. Parameters compared HRP minimal medium for each transformant. The results are expressed as Mean ± SEM of readings from 3 independent observations, ***P < 0.001, ** P < 0.01, * P < 0.05 and ns=non-significant (as compared wild type culture controls).

Table 4.4: GDH activity gluconic acid secretion and mps activity of *H. seropedicae* Z67 transformants.

H. seropedicae transformants	GDH activity (U)						Phosphate released. Pi (µM)		
	M9 minimal medium			HEPES rock phosphate medium			HEPES rock phosphate medium		
	Glucose	Xylose	Glucose + Xylose	Glucose	Xylose	Glucose + Xylose	Glucose	Xylose	Glucose + Xylose
Hs	UD	UD	UD	UD	UD	UD	UD	UD	UD
pBBR1MCS2	UD	UD	UD	UD	UD	UD	UD	UD	UD
pJNK1	14.98±2.6	12.47 ± 3.3	15.89 ± 1.5	19.14 ± 2.8	14.46 ± 2.53	19.14 ± 2.2	UD	UD	UD
pSS2	87.50±6.9	80.70±10.4	82.43 ± 5.4	240.93 ± 2.8	215.24 ±15.2	179.20 ± 2.4	125.47 ±15.1	106.64 ± 6.4	119.20 ± 16.4
pOK53	86.34±7.0	79.32 ± 7.1	88.34 ± 18.4	228.86 ± 7.1	224.36 ± 0.9	197.99 ± 3.1	168.07 ±14.5	127.96 ± 17	155.07 ± 7.3

UD – Undetectable

4.3.4: Secretion of PQQ by *H. seropedicae* Z67 transformants.

Hs Z67 wild type did not produce PQQ in M9 minimal medium. *Hs* Z67 (pJNK1) secreted 0.011 μM PQQ while *Hs* Z67 transformants (pSS2) and (pOK53) showed ~ 100 and ~ 200 fold increase in PQQ secretion, respectively. On the other hand, two phosphate solubilizing strains, *E. asburiae* PSI3 and *P. aeruginosa* P4 produced 4.52 μM and 1.4 μM, respectively, in M9 minimal medium.

Table 4.5: Amount of PQQ secretion in native and transformant bacteria.

Host	Nature of the genes incorporated	Amount of PQQ in the medium	Ref.
E. coli K12	*A. calcoaceticus pqq* gene cluster	Very Low	Goosen et al., 1989
E. coli	*Klebsiella pneumoniae* pqq gene cluster	0.18 μM	Meulenberg et al., 1995
E. coli (JM109, BL21)	*G. oxydans* M5 *pqqABCDE* gene cluster	6 μM*	Yang et al., 2010
Methylovorus sp. MP688		45 μM*	Ge et al., 2013
P. aeruginosa ATCC 17933	-	0.455 μM*	Gliese et al., 2010
H. seropedicae Z67	*Erwinia herbicola pqqE*	0.011 ± 0.002 μM	This study
H. seropedicae Z67	*Acinetobacter calcoaceticus pqq* gene cluster	1.10 ± 0.64 μM	This study
H. seropedicae Z67	*Pseudomonas fluorescens* B161 *pqq* gene cluster	2.55 ± 0.10 μM	This study
E. asburiae PSI3	-	4.52 ± 0.84 μM	This study
P. aeruginosa P4	-	1.41 ± 0.12 μM	This study

*Calculated from amount of PQQ given in μg/ml.

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

Hs transformants (pSS2 and pOK53) showed increase in EPS production up to (~28 µg/ml) while wild type culture, vector control and (pJNK1) produced ~15 µg/ml (**Fig. 4.8. A**). Under N fixing conditions, the EPS production was similar to that under aerobic N sufficient condition (**Fig. 4.8. B**). Biofilm formation also showed similar pattern of *Hs* transformants (pSS2 and pOK53) had enhanced biofilm formation by ~2 fold in both N-sufficient aerobic and N-free microaerobic conditions when compared with wild type culture (**Fig. 4.8. C&D**). On the other hand, IAA production was enhanced in *Hs* transformants (pSS2 and pOK53) to ~1.6 fold (~35 µg/ml) in aerobic conditions than that of wild type culture, vector control and pJNK1 transformant (~22 µg/ml) in aerobic condition (**Fig. 4.8. E**). All *Hs* transformants showed 50% decrease in IAA under N free microaerobic conditions as compared to aerobic conditions (**Fig. 4.8. F**).

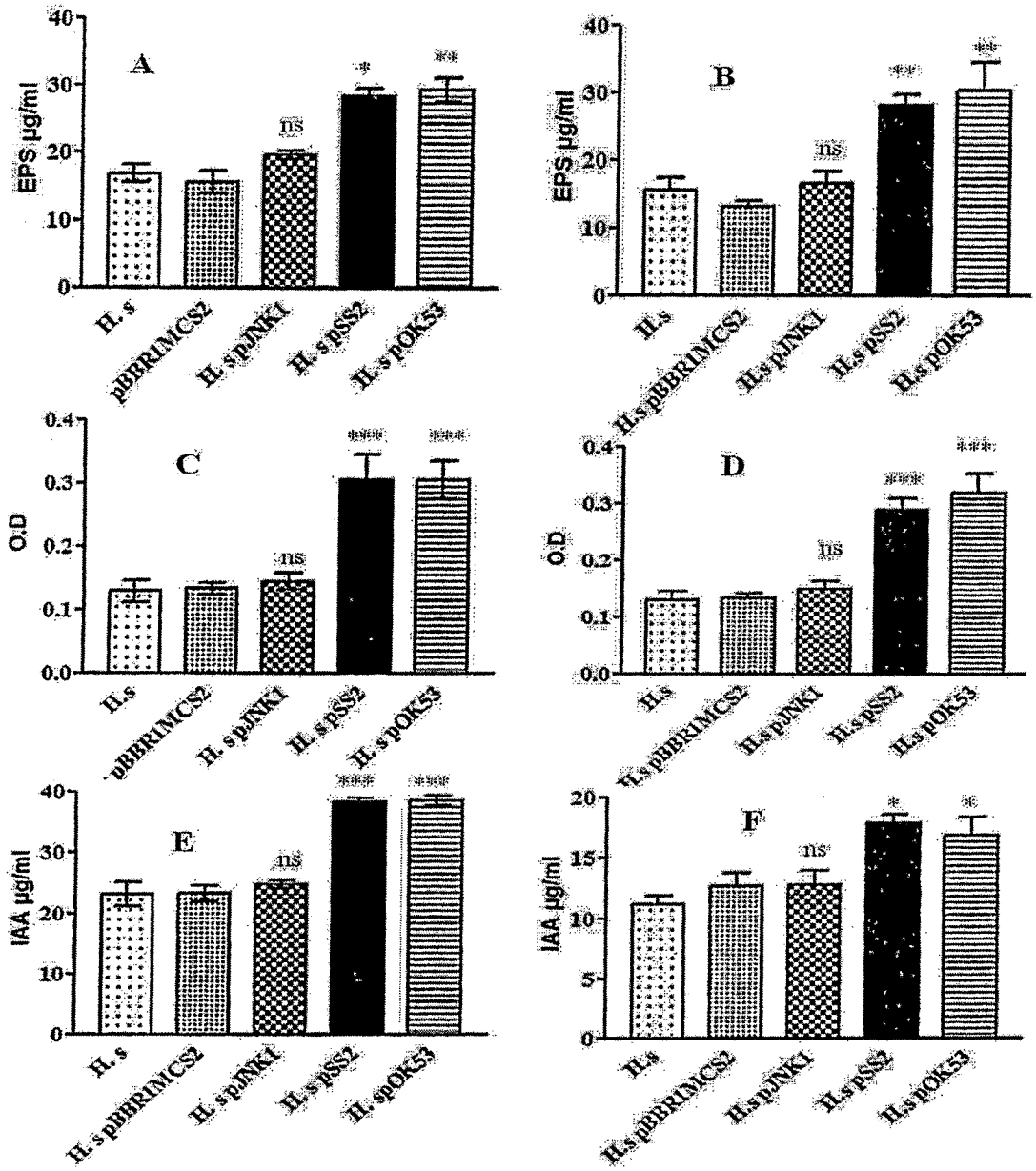


Fig. 4.8: Effect of *Hs* transformants on plant growth promoting factors.

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 wild type culture controls) ; **A, C** and **E** under aerobic , **B, D** and **F** under microaerobic condition.

4.4: Discussion

Phosphate solubilizing rhizobacteria produce chiefly gluconic acid by direct oxidation pathway, gluconic acid production is mediated by periplasmic Glucose dehydrogenase (GDH) which requires PQQ as a redox cofactor for direct oxidation of glucose to gluconic acid (Goldstein, 1995). Although many rhizobacteria *viz* fluorescent pseudomonads, *Klebsiella*, *Gluconobacter*, *Azotobacter* possess PQQ biosynthesis genes, only a few strains secrete high amount of gluconic acid. Exogenous supplementation of PQQ has been shown to secrete gluconic acid (Babu-Khan et al., 1995). Additionally, overexpression of *pqq* biosynthesis gene(s) in *Pseudomonas sp., Burkholderia Azospirilluim brasileinse* and *E. coli* resulted in gluconic acid secretion. Thus, amount of PQQ but not apoprotein limits the gluconic acid secretion.

PQQ biosynthesis in bacteria is not clearly understood but a putative pathway suggests a set of modifications on an oligopeptide involving glutamate and tyrosine residues (Puehringer et al., 2008). Analysis of PQQ biosynthesis genes from a variety of bacteria showed that *pqqABCDE* genes are conserved and presence of additional genes is variable. A single *pqqE* and PQQ transporter gene incorporation in *E. coli, P.cepacia* and *Azospirillum brasiliense* resulted in gluconic acid secretion leading toi MPS ability (Goldstein and Liu, 1987; Liu et al. 1992; Vikram et al., 2007). In contrast, over expression of *pqqE* gene in *Hs* Z67 could not confer MPS phenotype and gluconic acid secretion was limited ~4 mM, Reasons for these variations in *pqqE* overexpression is not clear.

Incorporation entire *pqq* gene cluster of *A. calcoaceticus* in *E. coli* produced very low amount of PQQ but high amount in *A. lwoffi* (Goosen et al., 1992). Additionally, incorporation of *pqq* gene clusters of *Deinococcus radiodurans* and *Serratia marcescens* resulted in gluconic acid secretion and MPS phenotype (Apte et al., 2003; Kim et al., 2006). *H. seropedicae* Z67 transformants of both *pqq* gene clusters of *A. calcoaceticus* and *P. fluorescens* B16 resulted in good amount of PQQ secretion. However, the amount of PQQ level, GDH activity, gluconic secretion and MPS phenotype were better with *Hseropedicae* Z67 containing *P. fluorescens* B16 *pqq* gene cluster as compared to that of *A. calcoaceticus*. *A. calcoaceticus* encodes

pqq genes in 5.1 kb region containing III, II, I, V and IV which are similar to *K. pneumonia pqqE, pqqD, pqqC, pqqB* and *pqqA*. Two additional genes *pqqR* and *pqqL* are found in this gene cluster. On the other hand, *P. fluorescens* B16 *pqq* gene cluster is of 13.4 kb in size and contains *pqqABCDEFHIJKM*. Incorporation of *A. calcoaceticus* gene cluster in *Hs* Z67 has produced 1.10 µM of PQQ while *P. fluorescens* B16 *pqq* gene cluster has produced 2.4 µM which is ~2 folds more (Table 5). This may be due to the differences in the extent of *pqq* gene expression or to the presence of additional proteins. Both pSS2 (25Kb), which is derived from pRK290 and pOK53 plasmid (33Kb), a derivative of pLAFR3, are low copy plasmids (Ditta et al., 1980; Staskawicz et al., 1987) low copy number both the clusters are under their native promoter, low copy number of plasmid might play major role in low production in PQQ production in organism. Interestingly, overexpression of *G. oxydans pqqABCDE* in *E. coli* has produced 6 µM PQQ under pET28a-P_{T7}-pqq expression vector (Yang et al., 2010). Thus expression of minimal set of genes appears to be sufficient for high levels of PQQ biosynthesis. Nevertheless, it will be interesting to understand the functions of *pqqHIJKM* of *P. fluorescens* B16 and *pqqR* and *pqqL* genes of *A. calcoaceticus* in PQQ biosynthesis.

PQQ biosynthesis by overexpression of both gene clusters is less than some of the natural phosphate solubilizing rhizobacteria. *E. asburiae* PSI3 and *P. aeruginosa* P4 have been isolated from the rhizosphere of pigeon pea and mung bean secreted PQQ at 4.5 µM and 1.4 µM, levels, respectively. This suggests that PQQ secretion could be further improved for better MPS ability. It would be interesting to understand the basis for high PQQ secretion in such natural isolates.

The present results demonstrated the overexpression of *pqq* gene clusters of *A. calcoaceticous* and *P. fluorescens* B16 in *H. seropedicae* Z67 resulted in rock phosphate solubilization on glucose, xylose and mixture of both sugars. When both the GDH substrates were supplied 25 mM of each of these sugar sufficient while 50 mM was necessary individually. Similarly, *E. asburiae* PSI3 showed MPS phenotype at 15 mM concentration of a mixture of seven GDH substrates while independently 75 mM amount of sugar was required (Sharma et al., 2005). The amount of the sugars was further reduced to 12 mM when *E. asburiae* PSI3 was

incorporated with *Pseudomonas putida* KT 2440 gluconate dehydrogenase (*gad*) operon secreting 2-ketogluconic acid (Kumar et al., 2013). Earlier, it has been suggested that an organism that is capable of using a variety of C sources for its bioenergetics could have an advantage over competitors that use limited C sources in natural environments (Goodwin and Anthony 1998). In rhizospheric soils, xylose is one of the predominant monosaccharide of plant root exudates (Roy et al., 2002; Kumar et al., 2006).

H. seropedicae Z67 transformants also showed MPS ability under microaerobic nitrogen fixing conditions. Similar results were observed in *A. brasielnse pqqE* transformant which solubilized tricalcium phosphate on N free medium (Vikram et al., 2007). Additionally, incorporation of *pqq* genes improved the biomass and specific glucose utilization of *H. seropedicae* Z67 in both aerobic and microaerobic conditions in HRP medium.

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 kmown to produces IAA (Radwan et al., 2002). PQQ secretion also improved IAA production in *H. seropedicae* Z67. Similarly, exopolysaccharide production and biofilm formation was better in *Hs* Z67 transformants in HRP medium. *Hs* Z67 has been shown to be present in the 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* Z67 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 kmown 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). *Hs* Z67 transformants containing *pqq* gene clusters could also have better colonization. In conclusion, incorporation of *pqq* gene cluster could significantly enhance the plant growth promoting ability of *Hs* Z67 by MPS phenotype, better colonization, and IAA secretion.